POSSIBLE ROLES OF SlyD IN HELICOBACTER PYLORI HYDROGENASE MATURATION

by

ANDREW BURK

(Under the Direction of Robert Maier)

ABSTRACT

SlyD is a two domain protein shown to have many and varied roles in *E. coli* cell physiology. Recently, SlyD has been show to be important for hydrogenase maturation. *H. pylori* has a homolog of the SlyD protein. To investigate the role of the whole protein and two domains of SlyD in hydrogenase maturation, I created two mutant strains, one lacking the full gene (*slyD*) and one lacking only the C-terminal domain (*mbd*). I also purified SlyD to use in crosslinking studies. The *slyD* mutant strain had a 60% decrease in hydrogenase activity and the *mbd* mutant strain had a 30% decrease as compared to the wildtype. Nickel complementation in the growth media was unable to cure the strains. Purified *H. pylori* SlyD was unable to crosslink with HypB suggesting that SlyD probably affects the hydrogenase maturation pathway differently than in *E. coli*.

INDEX WORDS: *Helicobacter pylori, slyD,* hydrogenase maturation

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ANDREW BURK

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ANDREW BURK

Major Professor: Dr. Robert Maier

Committee: Dr. Mark Schell
Dr. William Whitman

Electronic Version Approved:

Maureen Grasso
Dean of the Graduate School
The University of Georgia
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TABLE OF CONTENTS

LIST OF TABLES

Page

LIST OF FIGURES

Page

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Helicobacter pylori

Spiral bacteria in the gastric mucosa of the stomach were reported back in the late 19th century, but they were never isolated and were ultimately forgotten by several generations of medical doctors and scientists. It was not until 1982 when Robin Warren and Barry Marshall used microaerophilic conditions, similar to that used for culture of *Campylobacter*, that *Helicobacter pylori* was first isolated (54). Within two years time from its first isolation, *H. pylori* infection in the gastric mucosa of humans would be strongly correlated to inflammatory gastric disease (8).

Helicobacter pylori is a gram negative, spiral shaped, epsilon proteobacteria well adapted to survive in its biological niche, the human gastric mucosa. No other substantial natural reservoir is known for *H. pylori*. Other than nonhuman primates and cats under particular circumstances, no other living organism can be infected with *H. pylori* (19, 29). Originally thought to be microaerophilic, studies have shown that under high cell density, *H. pylori* can grow at aerobic oxygen levels (around 20 % O_2 partial pressure), though they better mimic typical growth physiology when grown under microaerobic conditions. The only other gas requirement needed is elevated CO_2 tensions of 5-10% partial pressures (11). Nutritional requirements for *H. pylori* are complex and not very well understood. Physically, *H. pylori* are about 2.5 to 5.0 μm long and about 0.5 to 1.0 μm wide (33). Motility, which is essential in *H.*

pylori virulence, is achieved by 4 to 6 unipolar sheathed flagella that are approximately 30 μm long and 2.5 nm thick (32, 34).

To date, three different strains of *H. pylori* have been sequenced and made available through online resources. The first strain sequenced, 26696, was done by The Institute for Genomic Research (TIGR) in 1997. It has a relatively small genome size of 1.67 Mbp with a predicted 1590 annotated genes and a G+C content of 39% (86). The second strain of *H. pylori* sequenced, J99, was performed by Astra and Genome Therapeutics. J99 is slightly smaller in size, 1.64 Mbp and has 1495 predicted genes (2). Even though 26695 and J99 are the same species, there are notable differences between the two strains. It has also been observed that *H. pylori* has a higher rate of mutational frequency and recombination than most other microorganisms (82). This can help explain the amount of diversity observed between the two strains. Most recently a third strain, HPAG1, was sequenced by Washington University (96).

In developing countries, *H. pylori* infect approximately 70-90% of the population with that number being reduced to about 20-50% in developed countries (83). This difference can be attributed to better hygiene practices and easier access to antibiotics in the developed part of the world. The majority of *H. pylori* transmission is conducted via two routes: fecal-oral and oral-oral (56). The fecal-oral route could be a major mode of transmission in less developed countries due to use of a single water source for drinking and for human waste. This cannot be substantiated because *H. pylori* has never been isolated from water. *H. pylori* has been detected, however, in dental plaque, tongue scrapings, and saliva in infected persons which could make oral-oral the major mode of transmission (63).

Since the bacterium was first identified by Warren and Marshall, much research has been put into determining what factors make *H. pylori* pathogenic. Volunteers who ingested *H. pylori*

suffered from acute or chronic gastritis (53, 58). When given antibiotics to clear the bacteria, gastritis was eliminated. The gastritis returned upon recurrence of infection (87). *H. pylori* is specifically associated with gastric mucosal cells and not with either the small intestine or in the intestinal metaplasia of the stomach (16). Evidence has shown that if the gastritis is allowed to persist over decades, it can convert to chronic atrophic gastritis, a precursor of gastric carcinoma and mucosal associated lymphoid tissue (MALT) lymphoma (22, 64, 81, 94, 102). Treatment of patients having MALT lymphoma with antimicrobial therapy often led to regression of the tumor (6). In fact, people infected with *H. pylori* have a 2 to 6 fold greater risk of gastric cancer or MALT lymphoma. In 1994, the International Agency for Cancer Research officially labeled *H. pylori* as a carcinogen. A bacterium that was once disregarded because of our inability to culture it, is now a significant concern of today's medical field.

There has been a vast amount of research put into *H. pylori* colonization and virulence. Motility of the bacterium has been shown to be essential for colonization. Motility in *H. pylori* is achieved by 4 to 6 flagella which are essential for penetration of the viscous mucous layer covering the gastric epithelium (40). Non-flagellated *H. pylori* was shown to be unable to colonize the mucous layer in gnotobiotic piglets (21). Because of the unique and harsh environment that *H. pylori* lives in, it has to have a number of different virulence factors in order to survive.

Virulence factors of *H. pylori* can be grouped under three different pathological effects they elicit: gastric inflammation, disruption of the gastric mucosal barrier, and alteration of gastric physiology. The two major proteins responsible for gastric inflammation are VacA and CagA. Vacuolating cytoxin is encoded by the *vacA* gene and is known to cause acidic vacuoles in gastric epithelial cells which causes tissue damage (66). In patients with peptic ulcer disease,

strains of *H. pylori* with the *vacA* gene are isolated more so than those without (27,84). CagA, the protein produced by cytoxin associated gene, is encoded by genes found on the 40 kbp cag pathogenicity island. This island contains a type IV secretion system that allows cagA to be injected into host cells. CagA is thought to be responsible for disrupting host signaling pathways and inducing IL-8 production causing inflammation and recruiting neutrophils (9,13,44,73). Once neutrophils are recruited, another protein, NapA (neutrophil activating protein), activates the neutrophils and causes them to aggregate (25).

Neutrophils and macrophages produce a number reactive oxygen species (ROS) such as superoxide, hydrogen peroxide, and hydroxyl radicals (15). These ROS cause damage to both host and bacterial biological molecules (10, 17, 26, 30, 45, 52). In order to combat oxidative stress, *H. pylori* contains an impressive number of defense mechanisms to combat damage. These include numerous ROS detoxifying enzymes, oxidative DNA damage repair enzymes, and oxidative protein damage repair enzymes. Two major detoxifying enzymes important for *H. pylori* virulence are superoxide dismutase (SodB) and catalase (KatA). SodB uses two superoxide molecules with two protons to convert superoxide radicals into oxygen and the less damaging ROS, hydrogen peroxide. *H. pylori sodB* mutants have been shown to have increased sensitivity to high oxygen stress (60). The enzyme catalase is able to convert hydrogen peroxide into water and oxygen. *H. pylori* catalase is encoded by the gene *katA*. *katA* mutants demonstrate an increased sensitivity to hydrogen peroxide and are deficient in mouse colonization (5,37,38,70). KapA, catalase accessory protein, is a protein that has been uniquely associated with the *H. pylori* catalase. Disruption of *kapA* in *H. pylori* results in a strain that does not cause a decrease in overall catalase activity but does display an increased sensitivity to hydrogen peroxide stress (37). In KapA mutants, there is a 5.5 fold decrease in catalase activity

in the periplasm, yet the strain still has similar to wild-type levels of activity in a cell free extract. Decreased catalase activity in the periplasm can be attributed to the inability of catalase to be properly translocated to the periplasm. KapA has been shown to possess the twin-arginine motif used in the TAT (Twin Arginine Translocation) system in *H. pylori.* This provides a potential mechanism for the translocation of catalase (36).

H. pylori has numerous proteins that play roles in repair of DNA oxidative stress damage. Nth, MutsS, MutY, and MutT are all DNA repair proteins with homologs in *E. coli*. Nth removes oxidized pyrimidines, such as thymine glycol, and mutants in *H. pylori* are shown to be deficient in mouse colonization (62). MutS functions as a sensor of DNA structure anomalies, such as that caused by 8-oxo-G (7,8-dihydro-8-oxoguanine), in its role of combating DNA oxidative stress damage (78). *mutS* mutants display an increased mutation rate when compared to wild-type, with a predominance of G/C to A/T transversions, typical of 8-oxo-G damage. In fact, *mutS* mutants had 3 times the level of 8-oxo-G when compared to the wild-type (97). MutY is the most recently characterized DNA repair protein in *H. pylori*. MutY is an adenine glycosylase that repairs A/8-oxo-G mismatches caused by oxidative stress damage. The spontaneous mutation rate of a *mutY* mutant can be increased through exposure to atmospheric oxygen. An *H. pylori mutY* strain also lacks the ability to repair A/G mismatches, demonstrating its specificity in oxidative stress repair (24). MutT has not yet been studied in *H. pylori*.

The only protein-specific oxidative damage repair system studied in *H. pylori* is Msr, methionine sulfoxide reductase. It is able to convert reduced oxidized methionine back to its natural state. Methionine is one of the most easily oxidized amino acids and causes enzymes to lose functionality when the amino acid is in the oxidized state. Catalase activities in *H. pylori*

were reduced to one-half that of the parent strain in *msr* mutants, showing that repair of oxidized proteins by Msr has a more global effect on oxidative stress repair (1, 91).

To survive in the harsh environment of the human stomach, *H. pylori* has to have the ability to combat more than just the oxidative stress response mounted by the human immune system. One of the first barriers to survival in the human stomach is to survive the low pH environment of the stomach. *H. pylori* accomplishes this transit of the stomach through the use of the metalloenzyme urease. A catalytically active urease is essential for *H. pylori* virulence (20). Urease catalyzes the hydrolysis of urea into ammonia:

$$H_2N - CO - NH_2 + 2H_2O \rightarrow 2NH_3 + H_2CO_3$$

The end effect of this reaction is the raising of the pH in the microenvironment around the bacterium allowing it to survive in the low pH until it can burrow down into the higher pH gastric mucosa (pH 7.0-8.0). As a further indication of its importance, *H. pylori* urease makes up approximately 10% of the total soluble protein (39). The urease enzyme has a very low Km for urea (app. 0.3 ± 0.1 mM) so it is efficient even when there is very little urea present. The gastric mucosa has a urea concentration of about 4 mM (20, 43). All the genes required for urease maturation are clustered in one area of the *H. pylori* genome. All in all, there are 2 genes that encode for the structural subunits of urease, *ureA* and *ureB*, and 7 genes that encode for accessory proteins, *ureC, ureD, ureE, ureF, ureG, ureH,* and *ureI*, and all but 2, *ureC* and *ureD*, are known to play an essential role in the maturation of a catalytically active urease enzyme (14,42). Of the five accessory proteins, only one, UreI, does not play a role in nickel delivery. Each UreB monomer stoichiometrically binds 2 Ni^{2+} ions and is essential for urease activity (20).

Hydrogenase

H. pylori possesses a second metalloenzyme essential for colonization: a hydrogen uptake hydrogenase. Hydrogenase is responsible for the catalytic conversion of molecular hydrogen to protons and electrons:

$H_2 \leftrightarrow 2H^+ + 2e^-$

In *E. coli,* this oxidation of hydrogen is often coupled to the reduction of a number of different electron acceptors such as O_2, NO_3^-, SO_4^-, CO_2, and fumarate. These electrons can then be used in respiratory chains to generate energy through oxidative phosphorylation.

There are three major classes of hydrogenase enzymes, characterized by their metallocenters: [Ni-Fe], [Fe], and metal free hydrogenases (65, 89, 92, 106). *H. pylori* possesses a hydrogenase of the first grouping, [Ni-Fe]. Much of what is known about hydrogenase structure and maturation has been learned from model systems, such as those of *Escherichia coli*, *Bradyrhizobium japonicum*, and *Rhizobium leguminosarum.* The full maturation of hydrogenase requires three structural genes and a multitude of accessory genes, most of which are responsible for the proper insertion of the nickel and iron ions and carbon-containing ligands into the metallocenter. The genes for these are all located together within a single operon in these model systems.

The small subunit of hydrogenase, HydA, contains three [Fe-S] clusters, (two [4Fe-4S] and one [3Fe-4S]) and is responsible for directing the flow of electrons from the hydrogen-activating site in the large subunit. The large subunit of hydrogenase, HydB, contains the [Ni-Fe] metallocenter which is the active site for hydrogen "activation" or splitting of the molecule. The third structural protein, cytochrome b, is responsible for anchoring the heteroenzyme, HydA

and HydB, to the cellular membrane, and it probably receives the electrons from hydrogenase (68)

The collection of accessory genes, commonly known as the *hyp* genes, is necessary for the full maturation of hydrogenase. These genes are *hypA, hypB, hypC, hypD, hypE,* and *hypF* (12). The following proposed pathway was determined from mutational studies done in *E. coli*. The first major step in the formation of a fully mature hydrogenase is the insertion of the iron into the large subunit, HydB. HypC and HypB are required to bind free iron for metallocenter assembly. HypC is a chaperone like protein that forms a complex with HypD, an [Fe-S] protein, to provide a scaffold for the liganding of the iron ion. The liganding of the iron ion requires two electrons, possibly provided by the HypD protein (18, 101). Once free iron has been bound by the HypC/HypD complex, a complex of HypE and HypF is needed to generate the 2 CN and CO ligands needed to incorporate the iron into HydB. HypF is responsible for the cleavage of carbamoyl phosphate (CP) which is thought to be the source of the CN and CO ligands. No definite role has been assigned for the HypE protein but it does possess ATPase activity. The cleavage of ATP by HypE could be the source of the energy that is absolutely required for the conversion of CP (25, 69). After this step, the liganded iron bound to the HypC/HypD complex is ready to be inserted into HydB. After the liganded iron is transferred to HydB, the HypD protein disassociates from the complex leaving the HypC still bound to HydB. Bound HypC is thought to keep the large subunit in the correct conformation for the next essential step of hydrogenase maturation, nickel insertion. HypC may also play a possible role in linking the two metal ions once both have been inserted into the large subunit (49).

Nickel insertion is one of the better studied steps in hydrogenase maturation. The two major proteins that play an essential role in the insertion of nickel are HypA and HypB. HypB is

a GTPase that is thought to play an important role in nickel sequestering. This is shown by the fact that *hypB* mutants can be cured to wildtype phenotype by the addition of excess nickel to the growth media (98). In *R. leguminosarum* and *B. japonicum*, HypB contains a stretch of histidine residues at the N-terminus that is shown to bind multiple nickel ions (31, 71). The nickel bound to this histidine rich domain could be the source of nickel that eventually gets incorporated into the final nickel-sink, namely hydrogenase. The *E. coli* and *H. pylori* HypB protein lack this stretch of histidines. It has been hypothesized that the lack of the N-terminal histidine stretch is compensated for by efficient nickel uptake systems (23). HypA has also been shown to bind up to 2 nickel ions per monomer in *H. pylori*. The ability of HypA to bind nickel is essential for hydrogenase activity (61). It is unknown how HypA gets its nickel. It could be hypothesized that in *H. pylori* that HypB sequesters nickel and transfers it to HypA which is an essential part of the sequential Ni-delivery pathway to the large subunit for its metallocenter. Whether or not this is the case, the source of nickel for donation to HypB is unknown.

Once nickel has been inserted into the large subunit of hydrogenase, HypC dissociates and is free to complex with HypD. The fully developed HydB and HydA form a heterodimer and the catalytically functional hydrogenase. The last step in hydrogenase maturation is the translocation of the enzyme to the cell membrane. The *H. pylori* HypA protein contains the signal sequence for the TAT system. Hydrogenase maturation is a very complex process with many different parts in order to make a functional nickel-enzyme. Recently another protein, SlyD, has been identified as playing a role in hydrogenase maturation in *E. coli*.

SlyD

The *slyD* gene was first identified by screening for survival of *E. coli* after induction of the cloned lysis gene *E* of bacteriophage ΦX174. Spontaneous mutants were isolated and tested

for the locus of the mutation using P1 transduction experiments. When a wild-type copy of the gene was placed under control by the *lacZ* promoter, cell lysis by E was restored. It was further discovered that mutations in the gene caused the accumulation of complete phages within the cell without lysis. This gene was termed *slyD* (Sensitivity to LYsis) due to sensitivity in the mutants (51). The SlyD protein was later rediscovered as a persistent contaminant of recombinant proteins that had been purified by immobilized metal affinity chromatography (IMAC) (3, 67, 103). At this time the protein was named WHP (Wondrous Histidine-rich Protein) due to the histidine stretch in the protein. Though the protein now is referred to simply as SlyD, the fact that the protein had two names can be directly attributed to the different and distinct nature of its 2 domains.

Based on its amino acid sequence (*E. coli*), SlyD consists of two conserved regions. The N-terminal region is a stretch of 146 amino and has significant sequence homology to the FKBP (FK Binding Protein) family of rotamases. FKBP proteins are members of the immunophilin group known to have affinities for FK506 antibiotics in eukaryotic systems. They also play a role as a chaperone protein and are involved in protein folding, specifically catalyzing the *cis/trans* conversion in proline containing peptide (4, 77). The *cis/trans* conversion is an enzymatically slow reaction and requires rotamases in order to make the switch efficiently. Because of this ability, this class of proteins is commonly referred to as peptidyl-prolyl isomerases or PPIases. FKBPs also play a role in signal transduction pathways, and in the modulation of cell activities (85). The C-terminal end contains a stretch of histidine and cysteine residues with the ability to bind divalent metal ions. Similar stretches of histidines are found commonly in HypB proteins and are implicated in the nickel binding or nickel delivery into

hydrogenases. However, this nickel-binding, His-rich stretch is absent in the HypB of *E. coli* (50).

Since the discovery of SlyD, there have been many attempts to elucidate its function. The net conclusion is that it may have numerous functions within the cell. All studies on the effects of SlyD on cell physiology or intracellular function have been done in *E. coli*. The first studies of SlyD involved the role of SlyD in resistance to lysis by the E protein. In spontaneous mutants that were resistant to E lysis, genome analysis all showed that there were mutations in *slyD*. Lysis was restored upon complementation of the *slyD*$^-$ strain with a wild-type *slyD* introduced via transposon mutagenesis (72). In experiments where other proteins other than SlyD are shown to play a role in E mediated lysis, none were able to demonstrate that E was expressed properly (48, 93, 99). In a study done with strains that had 2 point mutations in *slyD* and were resistant to E lysis, SlyD was shown not to have any effect on the transcriptional or translational regulation of E. Pulse chase labeling with [^{35}S]-Met of E protein showed that in the absence of SlyD, E protein was almost completely degraded within 10 minutes. Experiments done with the detection of the levels of c-*myc* tagged E protein in a *slyD* mutant strain showed that E was rapidly degraded. Overexpression of the E protein was able to restore cell lysis but it was still unstable and unable to accumulate as strains that did not overexpress the E protein (7). This suggests that SlyD is absolutely essential for E-mediated lysis and acts as a chaperone for the E protein, allowing it to remain stable in the cell. The role of SlyD as a PPIase for E was never tested even though E has 5 proline residues, with one residue in particular (Pro21) having to be properly positioned in order for E to be fully functional (100). A loss of SlyD function could hypothetically cause E to be in an incorrect conformation. This could explain the raid degradation of the protein.

Analysis of SlyD mutants in *E. coli* demonstrate the overall effect that SlyD plays on cell physiology. E resistant strains of *E. coli* grew as smaller colonies than that of their wild-type counterparts. Different spontaneous SlyD mutants were tested and showed that there was no specificity of a particular site mutation to growth limitation. Growth curves of SlyD mutants in complex media had an approximately 20% decrease in growth rate compared to the parent strain. This shows that SlyD plays an important but non-essential role for *E. coli*, at least in the complex media. In strains where SlyD is under the control of *lacZ* promoter¸ induction with 1mM IPTG yielded cells up to 10 times their normal length. Overall cell mass did not decrease when compared to wild type. Somehow SlyD overexpression caused a defect in cell division (72).

The 146 amino acid stretch at the N-terminus of *E. coli* SlyD has a 28.1% similarity to human FKBP12. This similarity is what first gave SlyD its designation as a PPIase. It was not until 12 years after its identification that the PPIase activity was actually assayed. PPIase activity is commonly assessed with a protease-coupled enzymatic assay. Linear peptides with the generic formula Suc-Yaa-Xaa-Pro-Zaa-4NA (Suc, succinyl; 4NA, 4-nitroaniline) can be generated with the Xaa-Pro bond in the *cis* conformation through preparation in dimethyl sulfoxide. This is important because cleavage of the 4NA group can only occur if the bond is in the *trans* conformation. The *trans* conformation of the Xaa-Pro bond is the favored one *in vivo* (47). For the substrates Suc-Ala-Phe-Pro-Arg-4NA, Suc-Ala-Ala-Pro-Arg-4NA, and Suc-Ala-Leu-Pro-Arg-4NA, specificity constants k_{cat}/K_m of 29,600, 6,200, and 5,600 $M^{-1}s^{-1}$ were measured in a trypsin-coupled enzymatic assay respectively. Trypsin was used rather than the more common chymotryspin due to SlyD instability in its presence (41). These activities are low when compared to that of other more well characterized PPIase enzymes such as trigger factor (for Suc-Ala-Ala-Pro-Arg-4NA, trigger factor of *E. coli* is 499,000 $M^{-1}s^{-1}$) (80). The catalytic

efficiency of SlyD was approximately 1000 fold higher with a protein substrate when compared to proline containing peptides (104).

A recombinant protein was created by expressing only the N-terminal portion of SlyD. It was tested for PPIase activity to assess the role of the N-terminal PPIase domain versus the C-terminal metal binding domain (MBD). SlyD PPIase activity was shown to be reversibly inhibited by the binding of Ni^{2+} to the MBD (41). The MBD has been shown to bind a number of different divalent cations: nickel, zinc, copper, and cobalt, with the highest affinity shown for zinc and nickel (103). Atomic emission spectroscopy was used to determine that SlyD MBD has 3 different metal binding sites with binding constants of $9.5x10^5$ M^{-1}, $4.9x10^5$ M^{-1}, and $4.4x10^5$ M^{-1}. Incubation of 1μM of SlyD protein with 50 μM $NiCl_2$ decreased the PPIase activity by 90%. The recombinant protein only expressing the N-terminal domain displayed comparable levels of PPIase activity to the full length version, showing that the MBD is not required for PPIase function and probably plays some regulatory role (on PPIase activity). CD (circular dichroism) spectra of the SlyD protein bound to nickel show an increased amount of beta-turns when compared to SlyD MBD without bound nickel. This suggests that if the MBD is playing some regulatory role, it might do so through structural changes of the N-terminal domain (41).

Aside from its isomerase activity, SlyD also displays function as a general chaperone protein. One of the major hypotheses on SlyD's role in E-mediated lysis is that it acts as a chaperone protein allowing E to maintain stability. SlyD has been shown in numerous bacterial species to display a high affinity for unfolded proteins, regardless of their proline content. In *E.* coli, SlyD has been shown to be an efficient PPIase for the reduced and carboxymethylated RNase T1 (RCM-T1) protein. The process of RCM-T1's folding is known to be limited by a prolyl *trans-cis* isomerization (55,59,74). The refolding proceeds at a rate with a specificity

constant k_{cat}/K_m of $6.8x10^6$ $M^{-1}s^{-1}$. It was also shown that SlyD showed specificity for unfolded RCM-T1 and was able to bind the unfolded protein substrate with a K_M of 1.65 μM. Because similar values were obtained for various recombinant forms of SlyD that excluded the MBD, it was determined that the MBD does not play a role in the ability of SlyD to act as a chaperone (75). This is highly debatable because the full length protein used was stripped of metals before use and the ability to bind unfolded proteins was never assayed with nickel supplementation of SlyD.

SlyD was shown to irreversibly bind to permanently unfolded protein substrates. To investigate this inhibition of activity, reduced and carboxymethylated α-lactalbumin (RCM-α-La) was used as a competitor to RCM-T1. In the presence of 1.0 μM RCM-α-La, PPIase activity was reduced 30% due to the binding of the unfolded protein. The inhibition of the PPIase activity was observed even when the competing unfolded protein lacked proline residues. The α-amylase inhibitor Tendamistat contains 3 prolines and its form is permanently destabilized when subjected to reduction and carboxymethylation (88, 90). A variant of this protein in which all 3 proline residues are changed to alanine was able to inhibit SlyD PPIase activity approximately 40%. A similarly created variant of RNase T1 inhibits the catalytic refolding of RCM-T1 approximately 30% (75). The ability of SlyD to bind unfolded proteins, regardless of proline content, shows that it plays a more general role in cell physiology. This property of SlyD allows it to be used in solubilizing aggregation-prone viral envelope proteins, such as gp120 and gp41, important membrane proteins in HIV infection (76)

A more recent discovery for SlyD function is a possible role in TAT translocation. The TAT system in bacteria is responsible for transporting folded proteins with N-terminal twin-arginine signal peptides to and across the cytoplasmic membrane. It is important to note that

SlyD itself does not possess this signal peptide. The best studied TAT signal sequence is that of the high potential iron-sulfur protein $(HiPIP)^2$ from *Allochromatium vinosum*. The TAT signal on $(HiPIP)^2$ is in an unfolded state and therefore susceptible to proteolytic attacks (46). For this reason, signal sequence binding proteins are necessary for preservation of the signal sequence until it is recognized by the TAT system.

Because HiPIP is recognized and translocated by *E. coli* TAT machinery, it was used as a tool for the identification of TAT signal sequence binding proteins. This study used a DnaK-deficient (DnaK is a known TAT signal sequence binding chaperone) strain of *E. coli* as the base for determining these proteins. Homogeneous HiPIP precursor was coupled to a binding column and membrane free extract was passed through. Proteins that bound to HiPIP were eluted and identified by mass spectrometric analysis. Two major proteins were identified: SlyD and GroEL (35). GroEL is a major and important chaperone protein in *E.* coli (95). This experiment was repeated with CueP and AmiA, both *E. coli* native proteins known to be translocated by the TAT system. Again, the dominant protein that was associated with the TAT translocated proteins was SlyD. SlyD displays *in vivo* interaction with the signal sequences of the TAT substrates YcdB, CueO, and HiPIP. Even though SlyD bound to multiple TAT signal sequences, a *slyD* deletion mutant had no apparent effect on the targeting of TAT substrates. Furthermore, in a double deletion of *slyD* and *dnaK,* SlyD seemed to have only minor effects on translocation (35).

The ability of SlyD to bind TAT signaling sequences shows that it has some role in translocation, but that role remains unidentified. It is interesting to note that an overexpression of SlyD causes *E. coli* cells to become elongated and filamentous (72). Deletion mutants in *tatA, tatC,* and *tatB* all cause long chains of cells to form (79). These defects can be accounted for by the inability to translocate a protein normally exported by the TAT pathway that is required for

cell separation and division. The question then becomes how a TAT deletion and SlyD overexpression yield the same phenotype. One answer could be that the overexpression of SlyD can cause a rapid binding of all the TAT signal sequences so that the TAT system never gets a chance to recognize the signal and translocation is blocked.

SlyD has also been shown to be a nucleotide binding protein. Nucleotide binding was assessed by covalent labeling with 3 different ATP analogues and by fluorescence enhancement using 2',3'-*O*-2,4,6-trinitrophenyl (TNP)_ATP. It was shown that binding of (TNP)-ATP in SlyD was only possible when a divalent cation was bound to the MBD. Zinc was the metal that allowed for the highest amount of nucleotide binding. Nickel was not one of the tested divalent cations. Metal-bound SlyD was also able to bind other nucleotides such as ADP, GTP, and UTP. This was shown by incubating SlyD with $^{8}N_3$-[α-^{32}P]ATP in the presence of the different nucleotides and measuring the radioactivity of the protein by autoradiography. All of the nucleotides were able to inhibit the radioactive ATP binding to SlyD. Though SlyD is able to bind ATP and GTP when Zn^{2+} is bound to the MBD, no discernable GTPase or ATPase activity was ever measured (57).

The previous study is very interesting because of its possible implications on the hydrogenase maturation pathway. Homology searches on the MBD of SlyD yielded matches to the N-terminal histidine-rich portions of HypB of both *B. japonicum* and *R. leguminosarum.* HypB is a known GTPase. The HypB of *E. coli* has measured GTPase activity but lacks the metal binding domain. Even though HypB has been well established to play a role in the *E. coli* hydrogenase maturation pathway, SlyD could also be playing a role in this process.

Recently, it was shown that a *slyD* deletion mutant led to decreased hydrogenase expression in *E. coli*. In trying to identify new hydrogenase pathway accessory proteins, the

sequential peptide affinity purification was used to purify protein complexes with HypB. SlyD was identified and shown to physically interact with HypB through a covalent crosslinking reaction. Deletions in *slyD* caused a hydrogenase deficient phenotype for both hydrogenase 1 and 2 that was able to be rescued to wild-type levels upon addition of 500 μM Ni^{2+} to the growth media. This could be the result of a requirement for SlyD in transferring nickel for the metallocenters of the mature hydrogenase enzymes, especially in nickel-deficient conditions. Experiments with ^{63}Ni showed that intracellular nickel levels were increased 2 fold in *slyD* knockouts. This could indicate a role for SlyD in nickel storage for the bacterium (105).

H. pylori and *SlyD*

H. pylori possesses a hydrogen uptake hydrogenase shown to be important for stomach colonization in mouse models. *H. pylori* possesses the full array of *hyd* and *hyp* accessory genes necessary for the complete production of a mature hydrogenase complex. HypB, like in *E. coli*, lacks the N-terminal histidine rich metal binding domain. *H. pylori* has a homolog to SlyD of *E. coli*, with 35% identity. They both share an N-terminal PPIase domain but differ in the MBD, most specifically with regard to histidine content. *E. coli* contains 15 histidine residues while *H. pylori* has only 5, 3 of which are in positions conserved in *E. coli* SlyD. To date, no experimental data has been gathered on SlyD in *H. pylori*. The purpose of this study was to clarify the role that SlyD plays in *H. pylori* hydrogenase maturation. This was approached via mutagenesis. The role of the metal binding domain was also of importance because of the differences of the MBD between *E. coli* and *H. pylori*. Through my studies on whole protein and on domain specific mutants, some possible roles of SlyD in hydrogenase maturation can be suggested. (see Chapter 2)

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CHAPTER 2

POSSIBLE ROLES OF SlyD IN HELICOBACTER PYLORI HYDROGENASE MATURATION

Abstract:

Helicobacter pylori possesses a hydrogen uptake, [Fe-Ni] hydrogenase that is essential for virulence. SlyD was recently discovered as an important accessory protein in the hydrogenase maturation pathway in *E. coli. H. pylori* possesses a homolog to the SlyD protein. SlyD is a two domain protein with a N-terminal petidyl-prolyl isomerase domain shown to play a role as a chaperone, and a metal binding domain with homology to the metal binding domains of some HypB proteins in other organisms. This study uses mutational analysis and pure protein studies to help assess the role of SlyD and that of its two domains in the *H. pylori* hydrogenase maturation pathway. In this study, two mutants were constructed in *slyD*: one mutant lacking the entire *slyD* gene (*slyD* strain) and another lacking only the C-terminal metal binding domain (*mbd* strain). Hydrogenase activity was determined amperometically in the two mutants. Hydrogenase activity was diminished approximately 25% in the *mbd* strain and about 50% in the *slyD* strain (compared to the parent strain) suggesting different roles for the two domains in the hydrogenase maturation pathway. Unlike in *E. coli*, nickel supplementation was unable to cure either of the two mutant *H. pylori* strains. Purified SlyD was unable to crosslink with purified HypB. This demonstrates that the role that SlyD is playing in *E. coli* is likely different than the role in *H. pylori.*

Introduction:

SlyD is a two domain protein with largely unknown or unspecified roles. It was first discovered by screening for survival of *E. coli* after induction of the cloned lysis gene *E* of bacteriophage ΦX174 (7). This is how *slyD* obtained its designation (Sensitivity to lysis). SlyD is essential for maintaining E stability while within the cell (3). This discovery was based on properties of the first domain of SlyD. The N-terminal domain (app. 150 amino acids) shows homology with the FK506 binding proteins and SlyD was therefore classified as a rotamase (15). The N-terminal domain of SlyD has been shown to be a peptidyl-prolyl isomerase capable of catalyzing the *cis/trans* conversion in peptides, a slow step in protein folding (5). The N-terminal domain is also proposed to be a chaperone known to bind and stabilize peptides and proteins regardless of proline content (11). This is the hypothesized method through which SlyD confers E stability *in vivo*. It has also been shown to bind ATP and GTP but without any known nucleotide hydrolyzing catalytic activity suggesting another role of SlyD that is not fully characterized or understood (8). The most recent function that SlyD has been found to have is as a binding protein to protect twin arginine translocation pathway (TAT) signal peptides from potential degradation (4). The N-terminal domain, to this date, has been characterized to a greater extent than has the C-terminal metal binding domain, though both domains are likely to play important roles.

Around the same time as the E studies, SlyD was "discovered" by others based on the unusual properties of the C-terminal metal binding domain. SlyD was found to be a consistent contaminant of recombinant proteins that had been purified by immobilized metal affinity chromatography (IMAC) (1,10,15). This protein was called named WHP (Wonderous Histidine-rich Protein) because of a large stretch of histidine proteins found in the C-terminal domain. The

C-terminal metal binding domain of *E. coli* has three separate metal binding pockets and has been shown to be able to bind numerous divalent cations including nickel (5). Nickel binding is noteworthy because the metal binding domain of SlyD has some homology to the N-terminal metal binding domains of nickel-storage HypB proteins in *Bradyrhizobium japonicum* and *Rhizobium leguminosarum.* The HypB protein in these species is known to bind nickel necessary for hydrogenase maturation. It is also interesting that while *E. coli* possesses a HypB protein; it does not have this N-terminal metal binding domain but does possess a SlyD. Interestingly, *B. japonicum* and *R. leguminosarum*, organisms that have HypB with the N-terminal metal binding domain, they lack the gene for *slyD*. The C-terminal domain of SlyD mainly has been studied with respect to its effect on the various properties associated with the N-terminal domain, namely PPIase and chaperone abilities. Binding of nickel to the C-terminal portion effectively inhibits the peptidyl-prolyl isomerase activity possibly through causing conformational changes at the N-terminal domain (5). Metal binding to the C-terminal domain also increases the affinity of the N-terminal domain to nucleotides (8).

Because of the similarity between the C-terminal domain of SlyD and the N-terminal His-rich domain of some HypB proteins, it was hypothesized that SlyD played some role in hydrogenase maturation (5,16). A recent study discovered that *E. coli* SlyD could complex with the *E. coli* HypB protein. A *slyD* deletion mutant caused a decrease in hydrogenase activity for hydrogenase 1 and 2 that could be fully restored to wildtype levels upon the addition of nickel. It was also shown that without SlyD, there is an increase in intracellular nickel concentrations. Because of this data, it was hypothesized that SlyD plays a role in nickel storage and transport into the hydrogenase maturation pathway through a direct interaction with HypB (16).

Helicobacter pylori contains a homolog to *slyD* that has never been studied. *H. pylori* is a common gastric pathogen that is known to infect over half of the world's population (13). It has numerous defense mechanism and adaptations to survive in its unique biological niche: the human gastric mucosa. One of these adaptations is the presence of a hydrogen uptake hydrogenase that is important for virulence and colonization (9). The *H. pylori* homolog of SlyD has 35% identity to the *E. coli* protein with the most significant differences in the C-terminal metal binding domain. The *E. coli* SlyD has 15 histidine residues while the *H. pylori* SlyD has only 5, though 3 of which are conserved. Many of the accessory genes for hydrogenase maturation have been well studied and characterized. This study uses a mutational analysis and biochemical approach to address the possible role of SlyD and its two separate domains in hydrogenase maturation.

To examine the roles each of the domains play in hydrogenase maturation, two different mutations were made in *slyD*. The first construct is a full length mutation of the gene (*slyD* strain) with the second mutant strain only having the N-terminal peptidyl prolyl isomerase domain (*mbd* strain). Here we show that there are differential levels of hydrogenase expression between the two mutant constructs. This is the first indication that the metal binding domain plays an important role in hydrogenase activity in *H. pylori*. It was also determined that the *H. pylori* SlyD plays a different role in nickel physiology than its *E. coli* counterpart, as neither mutant strain's hydrogenase activity was able to be restored to any extent by supplementation of nickel to the growth media. Biochemically, the proteins behave differently as well; from protein-protein crosslinking studies, no evidence for an intimate interaction between purified *H. pylori* SlyD or HypB could be demonstrated.

Materials and Methods:

Bacterial strains and construction of mutants

H. pylori ATCC strain 43504 was used as the parent strain for all mutants. Strains were grown on Brucella agar (BA) (Difco) supplemented with 10% defibrinated sheep blood. Kanamycin (30 μg/ml) was added to the media as required. Cells were incubated in jars with CampyPak Plus (Becton Dickinson) providing atmospheric gas concentrations, or in a CO_2/O_2 incubator 93% N_2, 5% CO_2, 2% O_2, and 95% relative humidity at 37°C. *E. coli* Top10 was used for all cloning experiments. *E. coli* strains were grown in Luria-Bertani (LB) plates supplemented with Ampicillin (100 μg/ml), Kanamycin (30 μg/ml), 1, 10, or 50 μM $NiCl_2$ as required.

Strains, primers, and plasmids are listed in Table 2.1. All primers were manufactured at Integrated DNA Technologies. *slyD* strain was created through PCR amplification of the *slyD* gene using primers slyDF-Nde and slyDR-Xho with *H. pylori* 43504 genomic prep as a template (560 bp). *slyD* gene was then ligated into the pGEM-T cloning vector (Promega). *slyD* gene was cut with HindIII at a unique restriction site in the PPIase domain and the *aphA3* cassette, encoding for kanamycin resistance, was ligated into the vector. The *slyD* mutant construct was introduced into 1 day old *H. pylori* via natural transformation by and integrated into the genome via allelic exchange. Successful mutations were selected for by growth on BA + KAN plates. Mutant verification was made using PCR with primers slyDF-Nde and slyDR-Xho and mutant genome as template. *mbd* mutant was created through use of a 2 step PCR method. First a BamHI site was introduced at the junction of the PPIase and *mbd* domains through the creation of 2 PCR products. The first half was created using the slyDF-Nde and smF-Bam primers. The second half was created using the slyDR-Xho and smR-Bam primers. These two products were

then purified and used in a second PCR with the slyDF-Nde and slyDR-Xho primers to generate *slyD* with a unique BamHI site. This product was then ligated into the pGEM-T vector and final steps were done the same for *mbd* as described previously for *slyD*.

Hydrogenase assays

Hydrogen-uptake activity was determined amperometrically for whole cells with O_2 as the final electron acceptor as previously described (6). Briefly, *H. pylori* strains used in the assay were grown in CampyPak Plus for stimulation of hydrogenase activity. Cells were harvested after exactly 24 hours of growth to assure cell viability. Cell growth was measured spectrometrically by measuring $O.D._{600}$. Cells were washed twice with phosphate buffered saline, pH 7.5 (PBS) and centrifuged at 8,000 rpm to pellet cells. Cells were then resuspended in 10 ml of PBS and assayed for hydrogen-uptake activity. Final activity was expressed as the number of nmol of H_2 oxidized per minute per 10^9 cells.

Protein expression and purification

PCR amplified *slyD* was digested with NdeI and XhoI and ligated into the similarly digested overexpression vector pTYB12 (New England Biolabs) resulting in pTYSly. This placed an intein tag in frame at the N-terminus of *slyD*. pTYSly was then transformed into BL21 Rosetta heat shock competent *E. coli* cells for overexpression. Cells were then grown up in 10 separate 50mL sterile tubes containing 25mL of LB broth supplemented with chloramphenicol (30 μg/mL) and ampicillin (80 μg/mL) to a final optical density of 0.6. Smaller batches were used because overexpression was unable to be achieved in large batches. Cells were then induced with 0.5mM IPTG and shaken overnight at room temperature. Cells were pooled after induction, washed twice and resuspended in 15mL of 20mM HEPES with 500mM NaCl, pH 8.0 (HEPES) and lysed via 3 passes through a French press. Broken cells were spun at 8,000 rpm to

remove unbroken cells. Cell lysate was then passed through a column of chitin beads previously washed with HEPES. Next, the fusion protein bound to the chitin column was allowed to incubate at 4° C in HEPES + 500 mM DTT for 48 hours. Cleaved protein was eluted using HEPES. Analysis of final protein product was analyzed via SDS-PAGE.

Purification of HypB was performed as outlined in (9). Briefly *hypB* was inserted into pET21b and transformed into BL21 Rosetta heat shock competent cells. Cells were induced with 1 mM IPTG for 3 hours at 37°C. Next cells were lysed via French press and whole cells were separated by a spin at 8,000 rpm. HypB was then isolated through use of anion exchange and gel filtration.

Crosslinking assay

A 1:1 molar mixture of SlyD and HypB (2.5 μM) wild-type proteins was incubated for 45 minutes at 37°C in the presence of 5mM dimethyl suberimidate (DMS, Pierce). The reaction was stopped through addition of 1M Tris-Cl, pH 7.5 followed by incubation at room temperature for 15 minutes. Following quenching, the samples were run on SDS-PAGE and electrophoretically transferred to a nitrocellulose membrane. This membrane was slightly washed with PBS and then blocked overnight in the presence of 5% gelatin. This was followed by a 1 hour incubation with a 1:1000 dilution of anti-HypB antibody raised in rabbits against purified *H. pylori* HypB proteins (Cocalico Biologicals) supplemented with 1% gelatin. The purified HypB for antibody generation was prepared by Dr. Nalini Metha. The membrane was then washed 5 times with PBS buffer and then incubated with a 1:1000 dilution of the secondary antibody, goat anti-rabbit immunoglobulin G complexed with alkaline phosphatase. The membrane was then rewashed 5 times in PBS. Bound antibody was detected by the addition of Nitroblue tetrazolium (0.25 mg/ml) and 5'-bromo-4-chlor-3-indolyl phosphate (0.125 mg/ml).

Results:

Creation of mutants

To study the roles of the 2 domains in SlyD, mutant strains were created in which SlyD was fully knocked out (*slyD*) or only the metal binding domain was missing (*mbd*) (Figure 2.1). The *slyD* strain was created through digestion of a unique HindIII site located in the area encoding for the PPIase domain of *slyD*, disrupting the gene. Disruption of the gene was verified via PCR amplification of genomic DNA purified from mutants. In order to make the *mbd* strain, a unique BamHI site was created at the junction of the areas encoding the PPIase domain and *mbd* domain through a 2-step PCR reaction. The new gene construct was then cut at the BamHI site and the *ahpA3* kanamycin cassette was inserted to generate the *mbd* construct. This plasmid was then transformed and the mutation verified in the same manner as for *slyD*. An agarose gel with PCR products corresponding to wildtype and both mutants is shown in Figure 2.2.

Hydrogenase assays and nickel supplementation

Hydrogenase activity in both the *mbd* and *slyD* strains showed reduced activity when compared to the wild-type with a more exaggerated defect in *slyD* (Figure 2.3). Hydrogenase activity for the *mbd* strain and *slyD* strain was reduced approximately 25% and 50% respectively when compared to the parent strain. It is important to note that hydrogenase activity is not completely abolished as seen with some other hydrogenase accessory protein mutants (9). This decrease in activity is not likely due to cell death. All of the strains grew well on plates and when observed underneath 1000X magnification, a vast majority of the cells for all 3 strains were still rod shaped and had not proceeded to the coccoid form. The latter would have indicated the cells were unhealthy.

It was proposed that the metal-binding domain of SlyD might be playing a role in the insertion of nickel into the [Fe-Ni] center of HydB; either by transferring nickel to HypB or by storing nickel until needed for the hydrogenase maturation pathway. This hypothesis was supported in *E. coli* because a *slyD* deletion mutant was able to be phenotypically cured for hydrogenase activity by addition of excess nickel. By attempting to phenotypically cure both *slyD* and *mbd H. pylori* strains, I could address whether the metal binding domain is playing this role in *H. pylori.* However, when nickel was added back to the media, hydrogenase activity was unable to be restored for any of the mutant strains. This was tested at levels up to 50μM $NiCl_2$ added. At high concentrations of nickel (50 μM) the decrease in hydrogenase activity can be attributed to cell death. Indeed, cultures grown with 50 μM nickel added to the growth media failed to reach as high of an $O.D._{600}$ as other growth conditions and when observed under a microscope, many had gone into the coccoid phase. Cells used in this assay were grown in an atmosphere with excess hydrogen. When grown in incubators without added hydrogen, hydrogenase activities for all strains were reduced by more than 50% (data not shown). Hydrogenase activities measured when cells grown without the added hydrogen to the atmosphere were erratic for all three strains. Overall, it was not determined if mutations affected hydrogenase protein levels (i.e. apoenzyme) or just activity.

Purification of SlyD and HypB

One method of understanding how SlyD works in the hydrogenase maturation pathway is to see if purified protein has interactions or forms complexes with other known maturation proteins. *E. coli* and *H. pylori* SlyD have virtually identical isometric points and molecular weight values; thus in order to assure purification of only *H. pylori* SlyD, anion exchange and gel filtration could not be used. To solve this problem, an affinity purification approach was

used using a cleavable intein tag fused to the N-terminus of SlyD. Through the use of a chitin column and cleaving with DTT, *H. pylori* SlyD was purified to near homogeneity (Figure 2.4). The observed molecular weight of SlyD based solely on migration was found to be about 27kDa but the actual size is 20kDa. This phenomenon has been reported previously for *E. coli* SlyD, and that the 27kDa band is SlyD was verified via N-terminal sequencing (15). Purification of HypB was done exactly as outlined in (9). A final observed molecular weight of approximately 30kDa for HypB, corresponds with previous findings (data not shown).

Crosslinking of SlyD and HypB

Another possible hypothesis as to how SlyD affects hydrogenase activity is that it acts as a chaperone protein or uses its peptidyl-prolyl isomerase activity to make sure that at least one of the essential proteins in the hydrogenase maturation pathway is folded correctly. If this were happening, then direct contact with one of these proteins would be necessary. In *E. coli* it was shown that SlyD and HypB form a physical interaction (16). In this study, a crosslinking experiment with *H. pylori* SlyD and HypB was performed to determine whether a similar interaction can be found. A 1:1 molar mixture of SlyD and HypB was crosslinked using DMS as the crosslinker. Many conditions were used (with or without nickel addition, altering protein concentrations, altering DMS concentrations and crosslinking times, changing incubation temperatures) to attempt to establish the physical interaction between HypB and SlyD. No set of conditions attempted were able to reproduce the interaction reported for *E. coli* (16).

Discussion:

My study used two mutant strains to ascertain the importance of domains of SlyD on hydrogenase maturation. The mutants were generated by the insertion of the KAN cassette at

specific points in *slyD* to generate a protein that either is nonfunctional or only partly (the PPIase domain) expressed. The rationale behind this approach is that the PPIase domain will be fully translated but termination will happen early and the protein will lack the metal binding domain. In similar mutants made in *E. coli*, the PPIase domain was shown to be expressed and maintained full peptidyl-prolyl isomerase and chaperone activities (5). A polar effect from the insertion of the *aphA3* cassette is not expected because similarly created mutants in *H. pylori* were shown not to produced polar effects (14).

The results obtained in this study show that both domains in SlyD are important for full hydrogenase activity. This conclusion is based on the differential levels of hydrogenase activity between the *slyD* and *mbd* mutant strains. A more pronounced decrease in hydrogenase activity was observed when the whole gene was knocked out as opposed to only just the metal binding domain. This indicates that both domains, the peptidyl-prolyl isomerase domain and the metal binding domain, play separate but important roles in hydrogenase activity. Nickel supplementation, even at 10 μM, was unable to restore any hydrogenase activity. HypB mutations in *H. pylori* are able to be fully complemented with the addition of 5 μM of nickel to the growth media (9). I was unable to show that SlyD and HypB of *H. pylori* physically interact with each other *in vitro*. It was this specific interaction that first implicated SlyD in the hydrogenase pathway in *E. coli*. This all raises some interesting questions and possibilities about how SlyD could play a role in hydrogenase maturation.

The role that SlyD and its two domains play in determining hydrogenase activity is still unclear. All previous work on SlyD's role in hydrogenase activity was done using the *E. coli* system. It is obvious that the hypothesized roles brought up in the *E. coli* studies, are not the same as what occurs in *H. pylori* according to the results from my study. It is still possible that

the PPIase domain of SlyD is responsible for catalyzing the *cis/trans* conversion of a proline bond in one of the other hydrogenase pathway accessory proteins other than HypB. HypA, as well as most other *hyp* accessory genes, are all essential in the pathway (2). If only a small percentage of this protein were fully functional, it can be hypothesized that the expression of fully mature hydrogenase would be decreased and consequently activity would be decreased. Another strong possibility is that *H. pylori* SlyD is required for proper translocation of the mature hydrogenase complex. HydA in *E. coli* and *H. pylori* possesses the TAT signal peptide. In *E. coli,* SlyD has been shown to play an important role in targeting of CueO, a known target for the TAT system. It is shown that SlyD is able to bind to the TAT signal peptides and could play a role in protection of them from degradation (4). In *H. pylori*, HydA, the small structural subunit of hydrogenase, is a known carrier of the TAT signal peptide and SlyD could be needed to target the full hydrogenase enzyme for translocation. A mislocalized hydrogenase represents a problem for a bacterium that depends on a respiratory hydrogenase. To function in a respiratory manner, hydrogenase needs to be membrane-integrated with access to respiratory heme proteins. The hypothesis that SlyD plays a role in the TAT signaling pathway is further supported by the fact that both deletions of *tatC*, essential for the TAT pathway, and an overexpression of SlyD causes a defect in cell division that results in the formation of filamentous cells (12). It would be interesting to test a SlyD overexpression strain to see if it is just as deficient in hydrogenase activity. An attempt was made to assay *H. pylori* membranes from WT, *slyD*, and *mbd*, for activity using methylene blue as the terminal electron acceptor to test this hypothesis, but no hydrogenase activity could be measured in any membrane-containing extracts.

It does not, however, seem plausible that SlyD in *H. pylori* functions as a nickel storage protein, or deliver nickel to the accessory system HypB/HypA. This is evidenced by the fact that

excess nickel in the media could not complement either the *slyD* or the *mbd* mutant strain. By comparing sequencing data, we see that the metal binding domain of *H. pylori* is drastically different than that of *E. coli.* It contains a third of the histidine residues, bringing the nickel binding abilities of this domain into question.

My study brings up some interesting questions on the hydrogenase maturation pathway and the role SlyD plays. It also has helped narrow the focus on different steps in which it could play a role.

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Table 2.1: Strains, Plasmids, and Primers used in SlyD studies

Strains:	Description:
43504	Parent strain/WT *H. pylori*
slyD	*H. pylori slyD:KAN aphA3* inserted in PPIase domain
mbd	*H. pylori slyD:KAN aphA3* inserted between PPIase domain and metal binding domain
Top10	*E. coli* cloning strain
BL21 Rosetta	*E. coli* overexpression strain
Plasmids:	
pGEM-T	Cloning vector
pSlyD	*slyD* inserted in pGEM-T
pSlyD-M	*slyD* w/ BamHI site inserted in pGEM-T
p*SLYD*	*slyD* w/ *aphA3* in PPIase inserted in pGEM-T
p*mbd*	*slyD* w/ *aphA3* between PPIase domain and metal binding domain inserted in pGEM-T
pET21b	Overexpression vector
pETHypB	*hypB* inserted in between NdeI and XhoI sites in pET21b
pTYB12	Overexpression vector w/ intein tag at N-terminus
pTYSly	*slyD* inserted in between NdeI and XhoI sites in pTYB12
Primers (5'→3'):	
slyDF-Nde	CCCCCCATATGCAAAACCATGATTTAGAG
slyDR-Xho	AAAACTCGAGCTACCCATGCGAACATGAG
smF-Bam	CTTTAGCGTTTCGGATCCGTTTCAAGG
smR-Bam	CCTTGAAACGGATCCGAAACGCTAAAG

Figure 2.1 Diagram of mutational approach: Figure shows location of the *aphA3* cassette insertion in both mutant strains. Insertion into the PPIase domain is used to knock out the entire gene. Insertion near the start of the metal binding domain allows expression of the N-terminal domain without the metal binding domain.

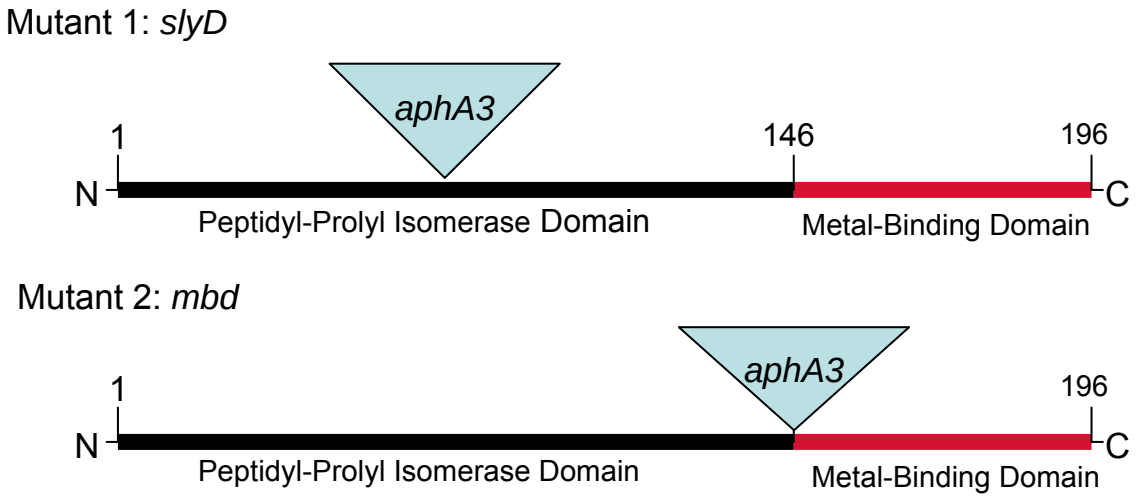


Figure 2.2 Verification of mutation of *slyD* in *H. pylori* by PCR. Lane 1: WT *slyD*, 558bp; Lane 2 + 3: *slyD* strain 1.95kbp; Lane 4: Marker (Fisher MidRange); Lane 5: WT *slyD*, 558bp; Lane 6: *mbd slyD:KAN,* 1.95kbp. *slyD* mutant represents 1.4kbp *aphA3* cassette inserted into native, unique HindIII site in *slyD*. *mbd* mutant represents 1.4kbp *aphA3* cassette inserted into created BamHI site between the 2 domains. Both mutants were successfully constructed as signified by the appearance of the 1.95kbp band (558 bp *slyD* + 1.4 kbp *aphA3*)

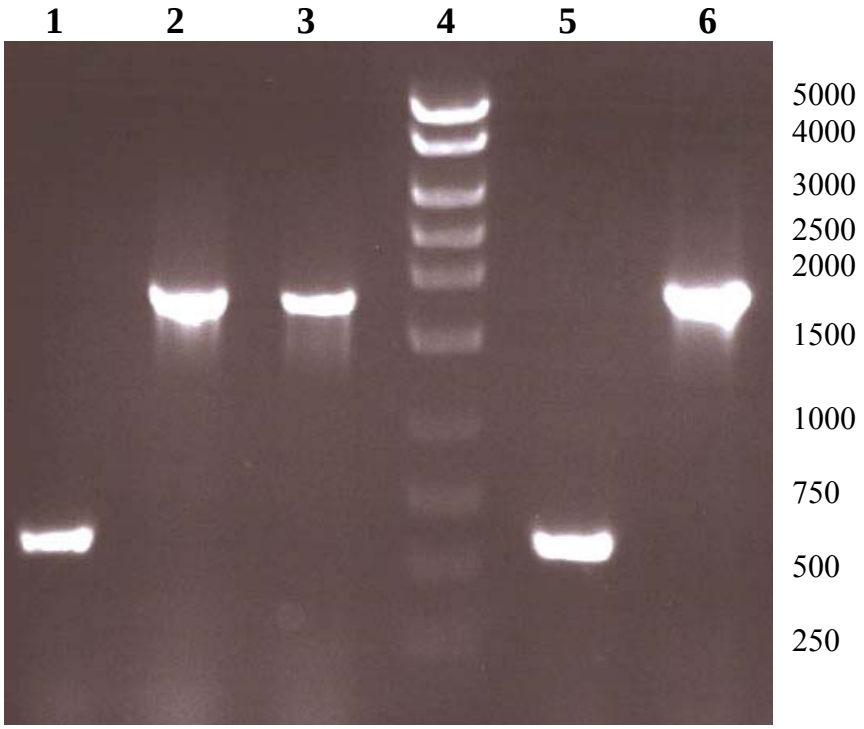


Figure 2.3 Hydrogenase activity of wild-type, *slyD*, and *mbd* mutant strains grown in the absence and presence of added nickel: This data is a compilation of 6 individual trials with standard deviations. Hydrogenase activity of the two mutants strains were decreased when compared to the wild-type. The *mbd* mutant hydrogenase activity was decreased approximately 30% while the *slyD* strain decreased 60%. These were significant differences according to students T-test analysis. Addition of nickel did not show the ability to cure either mutant strain to wildtype hydrogenase levels. Loss of activity at 50µM is attributed to nickel toxicity.

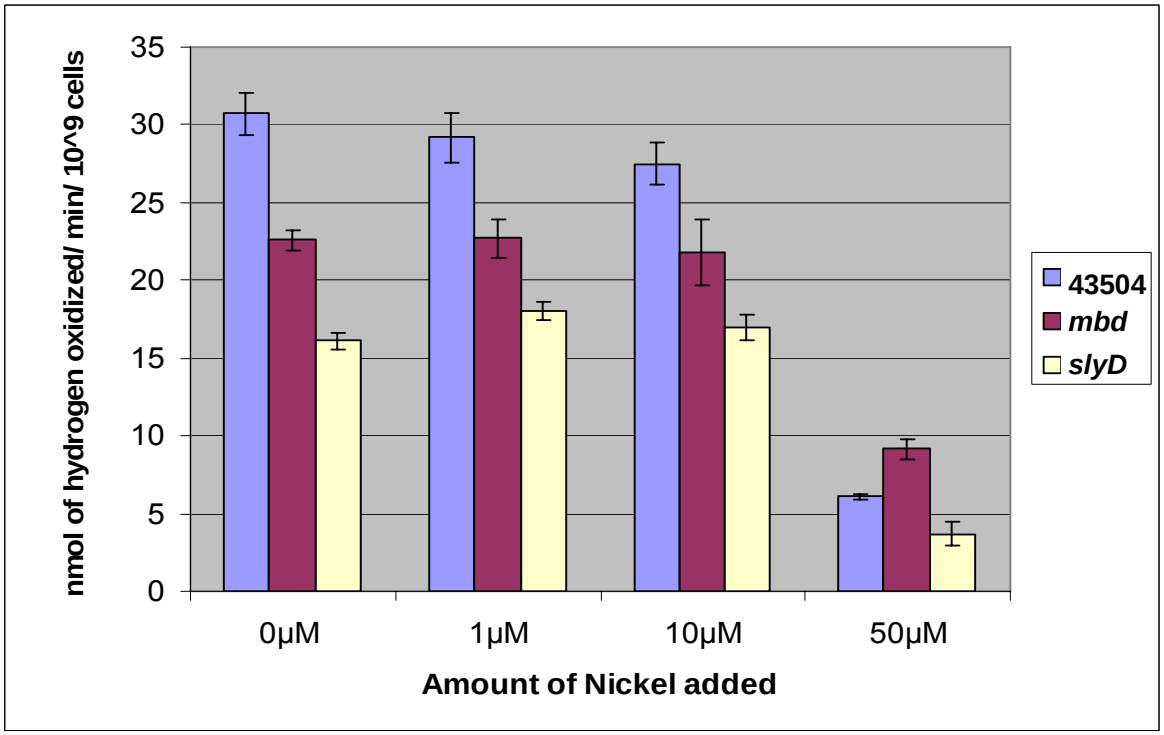


Figure 2.4 Purification of SlyD using intein based affinity purification: Lane M: Protein standard (Biorad); Lane IN: Induced fraction; Lane FT1: Fraction from *slyD* flowthrough after first addition of cell extract; Lane FT2**:** Fraction after second addition of cell extract; Lane QFT: Quick wash before cleavage of fusion SlyD; Lanes F1, 2, 3: Fractions after cleavage of SlyD fusion protein; Lane C: Chitin beads after removal of SlyD protein. Presence of SlyD detected in lanes F1, F2, and F3 (~25 kDa) unbound from intein (~55 kDa). Fractions F1, F2, and F3 were pooled and concentrated to a final protein concentration of 0.3 mg/mL.

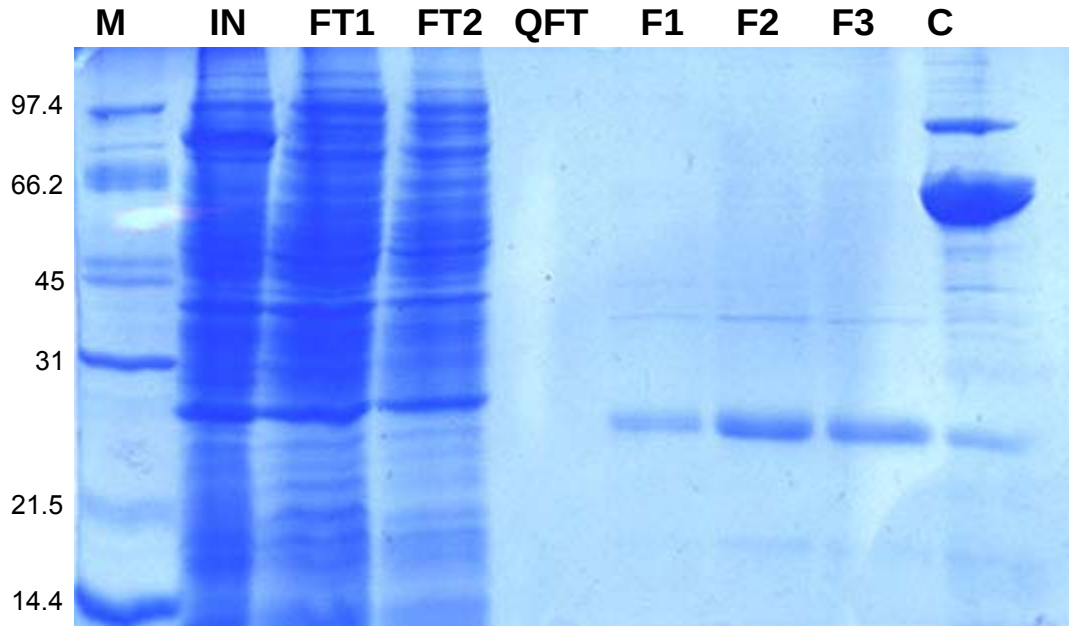
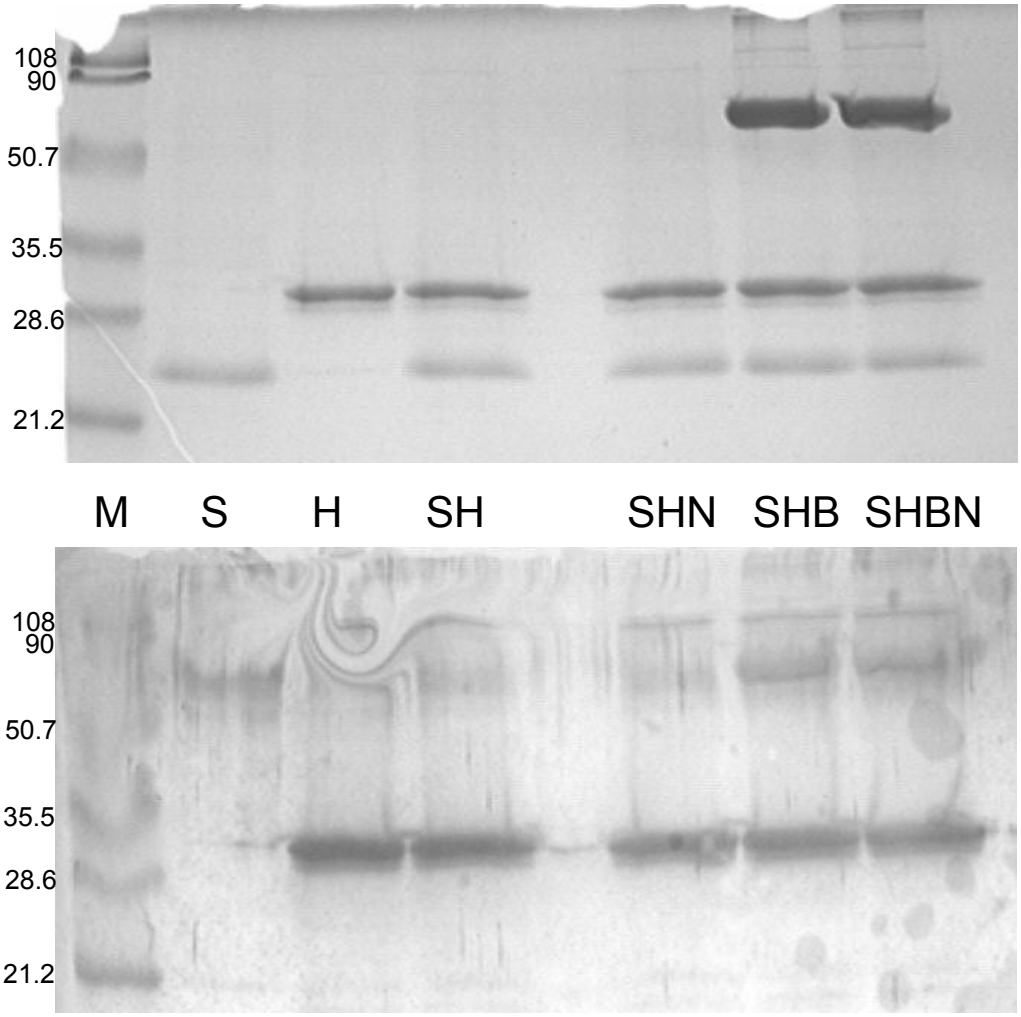


Figure 2.5 SlyD/HypB crosslinking results: Lanes (M = Marker (Biorad), S = SlyD, H = HypB, B = BSA, N = 5μM Ni^{2+}). All lanes represent separate reactions. A final concentration of 2.5μM of SlyD or HypB was added to each reaction as required. A final concentration of 5mM of BSA was added to required reactions. No indication of SlyD bound to HypB in any lane regardless of the presence of nickel was found. The reaction was repeated two additional times with similar results.



CHAPTER 3

ATTEMPTS TO MAKE VIABLE MUTANT STRAINS IN THE IRON SULFUR CLUSTER SCAFFOLDING PROTEINS NifU AND Nfu

Abstract:

Iron-sulfur clusters ([Fe-S]) play important roles in enzymes responsible to many essential functions of the cell: electron transfer, substrate binding/activation, iron/sulfur storage, regulation of gene expression and overall activity of the cell. There are 3 major systems that have been identified to be able to synthesize [Fe-S] clusters: Isc, Nif, and Suf. The Isc is the most well studied of the three systems. *Helicobacter pylori* genome encodes only for the Nif system. The Nif pathway is a two component system involving NifS (cysteine desulfurase) and NifU (scaffolding protein) and is needed for the maturation of the nitrogenase Fe and MoFe components under nitrogen fixing conditions. *H. pylori* does not have a nitrogenase enzyme which means that the NifS/NifU system is likely responsible for the generation of [Fe-S] clusters for many enzymes. NifU in *H. pylori* was previously characterized as an essential protein, as a *nifU* mutant strain could not be recovered; but since that time, new technologies have become available that can possibly allow for such mutant strain recovery. This study attempted to create mutants via cassette insertion in *H. pylori* strains SS1, 26695, and 43504 in NifU and also in a possible third [Fe-S] cluster maturation protein in the system, Nfu. This study shows that even in a 1% O_2 atmosphere and media supplemented with various iron sources, NifU and Nfu are both essential for *H. pylori* viability.

Introduction:

Iron sulfur clusters ([Fe-S]) are ubiquitous prosthetic groups that are essential to sustain various fundamental life processes across all strata of life. These clusters are contained in proteins that have a wide range of characteristics and play many important and essential functions in cell physiology. [Fe-S] clusters have the ability to delocalize electron density over both Fe and S atoms thereby allowing for many different structures and oxidation states (5). Therefore such clusters in proteins can accommodate a variety of redox potentials for reduction and oxidation.

There are 3 major systems that have been characterized that are able to synthesize [Fe-S] clusters: Isc, Nif, and Suf (5). The IscS/IscU system in *Azotobacter vinelandii* and *Pseudomonas putida* have been shown to perform a general housekeeping function in terms of generation of [Fe-S] clusters and maturation of [Fe-S] proteins (1,7). The Suf system has many overlapping roles with the Isc system in *E. coli* but plays a very specialized role during iron-starvation conditions in the maturation of oxidative stress related [Fe-S] proteins (11). The Nif pathway is composed of NifS (cysteine desulfurase) and NifU (3 domain [Fe-S] scaffolding protein) and is responsible for generation of [Fe-S] clusters needed for maturation of the nitrogenase Fe and MoFe components under nitrogen fixing conditions (4). Since the availability of whole genome sequences, it is clear that many organisms have multiple [Fe-S] cluster formation proteins. *Helicobacter pylori* is different in that it does not contain the [Fe-S] cluster formation systems known to play general housekeeping roles, Isc and Suf, but it does have the NifS/NifU proteins. What makes this particularly peculiar is that *H. pylori* does not encode for the nitrogenase protein. This suggests that *H. pylori* uses the Nif proteins for general [Fe-S] cluster formation

purposes. This is surprising because NifS/NifU has been shown to be unable to cure the loss of function of IscS/IscU in *A. vinelandii* (6).

NifU functions in conjunction with NifS to assemble transient [2Fe-2S] and [4Fe-4S] clusters for transfer into apoproteins (3,12). NifU has 3 distinct domains, all with functions important in [Fe-S] cluster synthesis. The N-terminal region has 3 conserved cysteines and is highly homologous to the N-terminal region of many IscU proteins. It is thought to play a role in assembling transient [Fe-S] clusters for the transfer to aponitrogenase (12). The central domain consists of 4 conserved cysteines that hold a permanent [2Fe-2S] cluster thought to be important in maintaining the redox balance of the protein (5). The C-terminal domain has 2 conserved cysteines in the thioredoxin-like motif, -Cys-Xaa-Xaa-Cys-. This domain has recently been implicated specifically in the formation of [4Fe-4S] clusters (10). An interesting aspect in *H. pylori* is the presence of a small protein with high homology to the C-terminal region of NifU, HP1492. This protein has a homolog in *A. vinelandii* that belongs to the Nfu family of iron-sulfur cluster scaffolding proteins and is capable of acting as a scaffold on its own. Though able to act independently, it was still shown to be nitrogenase specific (2).

Previous experiments showed that the *nifU* is an essential gene to *H. pylori* viability under certain conditions (8). The purpose of this project was to attempt to study *H. pylori* through the use of site directed mutagenesis to change the conserved cysteine residues in the 3 domains of NifU to alanine. Then through assays of the various [Fe-S] enzymes, the goal was to determine the specificity of each domain to either manufacturing of a specific form of [Fe-S] clusters or to a specific subset of proteins. The study of Nfu would be conducted through cassette insertion mutagenesis of the gene. The mutated *H. pylori* (i.e. transformants) would be grown in less stressful conditions than in previous attempts to isolate NifU mutants and

additional supplemental forms of iron were added to the growth media to attempt to alleviate the strict need for NifU. The idea is that all mutants would be "sick," so nutrient/survival conditions could perhaps be optimized for strain recovery. This study shows that under even less stressful conditions to the cell, NifU and Nfu are both independently essential for *H. pylori* viability.

Materials and Methods:

Bacterial strains and growth conditions

Mutations were attempted to be created by using *H. pylori* ATCC strains 43504, SS1, and 26695 as parent strains. All strains were grown on *Brucella* agar (BA) supplemented with 10% defibrinated sheep's blood. Kanamycin (20 μg/mL), FeSO4 (15 μg/mL), FeCl3 (15 μg/mL), or Fe-citrate (15 μg/mL) was added to the growth media as required. Cells were incubated in anaerobic gas + 1% O_2 atmosphere in anaerobic jars and placed in a 37°C incubator. DNA manipulations were done in *E. coli* Top10 competent cells grown on LB agar supplemented with Ampicillin (100 μg/mL) or Kanamycin (30 μg/mL) as needed.

Construction of clones designed for nifU and nfu mutagenesis

nifU was PCR amplified using primers NifUF2 and NifUR2 to yield a product of 1.2 kbp. This product was ligated into the cloning vector pGEM-T (Promega) to yield the plasmid pGEMNifU. BclI was used to cut *nifU* at a unique restriction site located in between the middle and C-terminal domain. KSF cassette obtained from Dr. Nalini Metha was then ligated into the BclI cut plasmid to yield pGEMNifUKSF. *nfu* was amplified using the primers NLF-Eco and NLR-Bam to yield a 669 bp product. The product was cut with EcoRI and BamHI and ligated

into a similarly cut pKS plasmid to yield pKSNfu. *nfu* was cut with HindIII at a unique restriction site and an *ahpA3* cassette was ligated to yield pKSNfuKan.

Mutagenesis of H. pylori wildtype strains 43504, SS1, and 26695

H. pylori wildtype strains were naturally transformed using plasmid DNA from mutant plasmid constructs. Briefly, *H. pylori* wildtype was grown up for 24 hours in a 5% O_2/CO_2 incubator. A small dimmer size circle of this bacteria was plated on a new plate and allowed to grow for 5 hours in the same incubator. These plates contained the ferric, ferrous, and organic iron in the concentrations listed before. Approximately 20 ng of the mutant construct plasmid was placed on the circle and mixed and incubated in the 1% O_2 jars for 24 hours. After this the entire circle of bacteria was replated on fresh iron supplemented plates and allowed to incubate in the 1% O_2 jar for 5 days. Colonies that appeared were picked and restreaked for mutant verification. Mutant verification was done using PCR amplification.

Results:

Creation of plasmids for H. pylori mutagenesis

To study the roles of the various domains in *H. pylori* NifU, site directed mutagenesis of the conserved cysteines within each domain were to be changed to alanine. In order to verify cells that underwent the amino acid switch, a selectable marker would have to be used. KSF (*kan-sacB-flaA*) has been used previously in similar experiments in *H. pylori* with sucrose resistance being the selective marker (9). The KSF cassette is used almost extensively for selection of site-directed mutagenesis, which was one of the original goals of this project. A unique BclI restriction site was used as the area of insertion for the KSF cassette. The BclI site is

located in the C-terminal region of *nifU*. This is the safest domain in which to insert the cassette. This is due to the possible redundancy effect of Nfu (based on sequence homology to the C-terminal domain) and that *A. vinelandii* IscU is a fully functional scaffolding protein that lacks the C-terminal domain contained in NifU (1). KSF insertion into the plasmid was successful as determined by PCR amplification (Figure 3.2B).

For *nfu*, site directed mutagenesis is not required because it only has one domain. To study the role of Nfu, a mutant construct was made in which the kanamycin resistance cassette, *aphA3*, was inserted into *nfu* at a unique HindIII site. The insertion of the cassette into the plasmid was successful as determined by PCR amplification (Figure 3.3B).

H. pylori mutagenesis

The plasmid constructs for the mutation of *nifU* and *nfu* were naturally transformed into 3 wild-type strains of *H. pylori*. Since it was previously shown that a mutant in *nifU* was non-viable, the cells were grown in the least stressful environment possible; 1% partial pressure O_2 atmosphere was used to limit oxidative stress. Iron in 3 different forms, ferric, ferrous, and organic, was added to the growth media to help overcome possible iron deficiencies caused by lack of NifU. Numerous attempts were made to obtain the mutant but no colonies were ever observed. Failure to obtain the mutant is not likely due to iron toxicity because attempts to grow cells with no added iron did not produce colonies either.

Attempts to isolate *nfu* mutant colonies using the same procedure as for *nifU* were similarly unsuccessful. After the 5 days of growth at 1% partial pressure O_2, a few colonies appeared for each of the three strains in which mutagenesis was attempted. Colonies were restreaked and their genome was PCR amplified using *nfu* specific primers to check for the

presence of the mutated gene. Results for all 3 of the parent strains showed the presence of the mutated gene (*nfu*) but also for the wildtype copy. The presence of both bands could signify a single crossover event, in which the whole plasmid gets incorporated into the genome and there are 2 copies of the gene, only one of which is mutated. It seems clean mutant strains cannot be isolated. The mutational data shows that *nfu* is likely essential for cell viability under the conditions I attempted.

Discussion:

[Fe-S] clusters are important and essential components of many biological processes and enzymes. Apparently, *H. pylori* uses the NifS/NifU system to generate all the [Fe-S] clusters it needs. This is interesting because the NifS/NifU system had been shown to be specific for nitrogenase maturation; the latter is an enzyme that *H. pylori* does not have. NifU is a 3 domain scaffolding protein that acts as the foundation on which [Fe-S] clusters are made. The analysis of the genome of *H. pylori* showed another putative scaffolding protein with homology to the Nfu proteins in *A. vinelandii*. In order to understand the role that both of these proteins play in the manufacturing of [Fe-S] clusters, a mutational analysis approach was attempted. Even though NifU was shown to be essential for *H. pylori* viability, new approaches were used to try to obtain these mutant constructs. Under the least stressful conditions, neither gene, *nifU* nor *nfu*, was able to be stably mutated. *nifU* mutants failed to produce any colonies and the only colonies produced by *nfu* mutants were ones that still maintained a wildtype copy of the gene.

An attempt was also made to place control of *nifU* under the control of an inducible system to see if we could study the effects of a controlled amount of NifU on the various [Fe-S] cluster proteins. We attempted to adapt the tetracylince resistance (TetAPO) system used for

eukaryotic gene induction in *H. pylori*. This approach is ultimately unviable because it requires a knockout of the gene to be studied chromosomally. In this study, it is concluded that both NifU and Nfu are likely essential proteins and their loss cannot be compensated for by growth in 1% O_2 and/or supplementation with different iron sources.

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Table 3.1: Strains, plasmids, and primers used in NifU/Nfu studies

Strains:	Description
43504	Parent strain
SS1	Parent strain
26695	Parent strain
Plasmids:	
pKS	Cloning vector
pKSNfu	pKS vector with *nfu*
pKSNfuKan	pKS vector with *nfu:aphA3*
pGEM-T	Cloning vector
pGEMNifU	pGEM-T with *nifU*
pGEMNifU-KSF	pGEM-T with *nifU:KSF*
Primers (5' →3'):	
NifUF2	TCTAATATCGCCGCTTCCAC
NifUR2	CGTCTGCTTTTGGTTTGCGT
NLF-Eco	CCCCCCGAATTCTTGGAAGCTTTTTCAAACTC
NLR-Bam	GTTGGATCCGGCTTCTTTAGGGAAATCCTTAG

Figure 3.1 Creation of vectors used for attempted mutagenesis of *H. pylori*: A. PCR amplification of *nifU* and *nfu*. Expected band sizes of 1.2kbp (*nifU*) and 700bp (*nfu*) were amplified via PCR. B. pGEMNifU-KSF cut with BamHI and EcoRI to yield 2 products: 4.5 kbp (1.2 kbp *nifU* + 3.3 kbp *KSF*) and 3 kbp pGEMT. C. pKSNfuKan cut with BamHI and EcoRI to yield 2 products: 3.2 kbp (PKS) and 2.1kbp (700 bp *nfu* + 1.4 kbp *aphA3*)

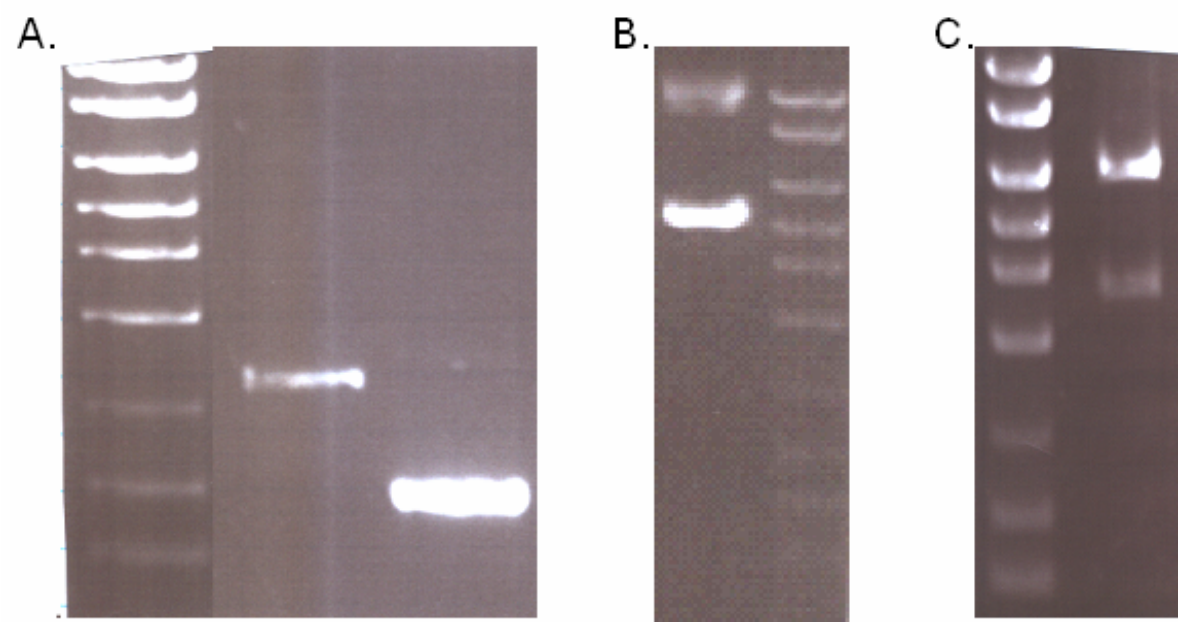

Figure 3.2 PCR products from possible *nfu*- strains: All strains were grown in 1% O2 on BA plates supplemented with Kan, ferric, ferrous, and organic Fe. Colonies that appeared were grown up and DNA was extracted from them. PCR amplification yielded results that displayed both wild-type and mutant bands.

CHAPTER 4

CONCLUSIONS

SlyD

In *E. coli*, SlyD is shown play an important role in hydrogenase maturation (4). The process of hydrogenase maturation is a very complex process that requires the correct insertion of nickel and iron into the large subunit and proper translocation to the cell membrane (1). I have studied the role of the SlyD homolog in the gastric pathogen *Helicobacter pylori*. SlyD is a two domain protein shown to have multiple roles in *E. coli* physiology. In order to study the roles of the two domains in *H. pylori* hydrogenase maturation, 2 different mutant strains were constructed using cassette insertion mutagensis: *slyD* strain (full gene knockout) and *mbd* strain (N-terminal domain only). I have shown that the *mbd* strain and *slyD* strain have a 25% and 50% decrease in hydrogenase activity when compared to the parent strain respectively. Neither of the two mutant strain's activities were able to be cured with nickel supplemented to the media. Pure *H. pylori* SlyD was unable to complex with pure *H. pylori* HypB *in vitro* with DMS used as a crosslinker. My experiments have shown that *H. pylori* SlyD is important for full hydrogenase maturation. The role that it plays is still unclear but it seems likely that its role in *H. pylori* is different than in *E. coli*. A number of different possibilities were discussed in Chapter 2.

NifU

NifU is a chaperone-like [Fe-S] cluster scaffolding protein, that in conjunction with NifS, is normally responsible for manufacturing of [Fe-S] clusters needed for nitrogenase maturation in

nitrogen fixing bacteria. General "house keeping" [Fe-S] clusters are normally produced via the Isc or Suf pathway (2). The gastric pathogen, *Helicobacter pylori* contains the Nif pathway but has neither the Isc or Suf pathway. What makes this particularly unique is that *H. pylori* does not have a nitrogenase enzyme. It was previously shown that a mutation in the NifU gene created a nonviable strain of *H. pylori* (3). Since then, new technologies have been developed that increase the chance of mutant recovery. I made constructs for mutagenesis of *nifU* and *nfu*, a potential NifU-like scaffolding protein, and transformed them into 3 different strains of *H. pylori.* Many conditions were attempted (See Chapter 3), but none were able to yield a clean mutant phenotype. Thus, this study has concluded that the NifU and Nfu proteins are essential to *H. pylori* viability, even under conditions optimized for mutant strain recovery.

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