ROLES OF *SHIGELLA FLEXNERI* H_2-OXIDIZING HYDROGENASES IN ACID TOLERANCE

BY

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ABSTRACT

NiFe hydrogenases are membrane bound enzymes that catalyze the oxidation or evolution of molecular hydrogen. It has been shown that molecular hydrogen can be used as an energy source. Two uptake (oxidizing) hydrogenases Hya and Hyb are described in *Shigella flexneri*. Hya and Hyb are expressed in acidic and alkaline conditions, respectively. These uptake enzymes are linked to the electron transport chain through a cytochrome *b* carrier. It has been shown that deletions in the genes encoding these hydrogenases within several pathogens resulted in strains that were either defective in uptake of nutrients or unable to colonize the host animal. Thus, for this thesis work mutants in the hydrogenases were constructed in *S.flexneri* to determine their contribution to bacterial physiology, and especially to acid challenge.

S. flexneri is an enteric bacterium that is the causative agent of shigellosis. This disease is characterized by diarrhea stools containing blood and mucus. In order to cause disease the bacteria must first survive in the human gastric environment (~pH 2).

S. flexneri uses 2 acid resistance pathways to survive in a pH of 2. One which is controlled by RpoS and Crp, and the second which requires glutamate.

However, an alternate pathway was described herein whereby *S. flexneri* requires Hya hydrogenase in absence of exogenous glutamate to survive acidic pH values. Hya was shown to provide a membrane potential. The cationic dye JC-1 and the protonophore CCCP was used to access membrane potential in *S.flexneri* wild type and *Hya* mutant strains. Compared to the wild type, the *Hya* mutant had negligible Δψ activity using JC-1 fluorescence. Acid sensitivity comparable to the *Hya* mutant was observed when CCCP was added to the wild type strain. A model whereby Hya provides robust periplasmic proton levels to counteract proton (acid) influx is proposed.

The deletion of Hya has a global impact on protein expression in *S. flexneri*. It was observed that proteins involved in glycolysis and fatty acid metabolism increased in expression in the mutant. Decreased expression of proteins associated with detoxification, gluconeogenesis, serine-derived amino acids, and nitrate reductases was observed.

INDEX WORDS: *Shigella*, acid resistance, hydrogenases, hydrogen oxidation, Enterobacteriaceae, proton motive force, protein expression

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DEDICATION

Thank you to God for bringing me through this graduate education process. Thank you to my wonderful and inspirational Mom, Janice Hughes for her unwavering love, support, and encouragement. To my Grandma words cannot describe your influence in education; I wish you were here to celebrate with me.

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LIST OF ABBREVIATIONS

ΔΨ: membrane potential

AR1: oxidative acid resistance pathway

AR2: see GDAR

AspA: aspartate ammonia-lyase

AspC (aspartate aminotransferase)

CCCP: carbonyl cyanide m-cholorophenylhydrazone

CysK: cysteine synthase A

DapB: dihydrodipicolinate reductase

DMSO: dimethyl sulfoxide

FabB: 3-oxoacyl-[acyl-carrier-protein] synthase I

FabH: 3-oxoacyl-[acyl-carrier-protein] synthase III acetyl CoA ACP transacylase

FabI: enoyl-[acyl-carrier-protein] reductase (NADH)

Fba: Fructose-bisphosphate aldolase

GadA: glutamate decarboxylase

GadB: glutamate decarboxylase

GadC: glutamate: γ- aminobutyric acid

GDAR: glutamate-dependent acid resistance pathway

GlyA: serine hydroxymethyltransferase

GltX: glutamate tRNA synthetase

Gpt: guanine-hypoxanthine phosphoribosyltransferase

Hya: hydrogenase-1, uptake, H_2-oxidizing

Hyb: hydrogenase-2, uptake, H_2-oxidizing

Hyc: hydrogenase-3, H_2-evolving

IlvD: dihydroxyacid dehydratase

LB: Luria-Bertani Broth

LdhA: fermentative D-lactate dehydrogenase

MopB: chaperone

NapA: nitrate reductase 3

NarG: nitrate reductase 1

PckA: phosphoenolpyruvate carboxykinase

PdxJ: pyridoxal phosphate biosynthetic protein

PepD: Aminoacyl-histidine dipeptidase

PfkA: 6-phosphofructokinase I

Pgk: Phosphoglycerate kinase

PLP: pyridoxal 5'-phosphate, vitamin B_6

PMF: proton motive force

PMSF: phenylmethylsulfonyl fluoride

PNP: Pyridoxine-5' phosphates

PPP: pentose phosphate pathway

SDH: L-serine dehydratase

SerC: 3-phosphoserine aminotransferase

SodB: superoxide dismutase

TalB: transaldolase B

WrbA: trp repressor binding protein

WT: wild type *Shigella flexneri* 2a 2457T

CHAPTER 1

LITERATURE REVIEW OF HYDROGENASES AND *SHIGELLA FLEXNERI*

Hydrogenases are ancient proteins most commonly found in bacteria and Archaea. These enzymes catalyze the oxidation of molecular hydrogen into 2 protons and 2 electrons. This reaction can sometime be reversible to evolve hydrogen. Hydrogenases were formally described by Stephenson and Stickland in 1931 (46).

Hydrogen can serve as an alternative energy source to power machines and be a viable energy source for bacterial growth (19). With the dwindling resources available for energy conversion, the scientific community has become increasingly interested in these enzymes for their ability to produce hydrogen (21). The gas can be used by machines (after combustion) in a fuel cell format to drive a process with water as a by-product or it can be used (oxidation) in bacteria as a driving force to produce ATP or energy for transport of biomolecules.

Most hydrogenase research has centered on their roles in bacterial and archaeal organisms. This dissertation research will focus on the role of *Shigella flexneri* 2a 2457T NiFe-hydrogenases in conferring acid survival.

HYDROGENASES

The hydrogenase enzyme was first discovered in mud samples (47). The bacterium containing the protein was hypothesized to be *Escherichia formica*; it was able to reduce methylene blue in the presence of H_2 (47, 52). Stephenson described the enzyme as an “intracellular reducing agent” with properties similar to that of dehydrogenases (52). Hydrogenases are believed to be very ancient enzymes in part because of the accepted theory that before humans, the Earth was a hydrogen rich world (21). Also, in sulfur-rich areas where life may have originated some archaeal hydrogenases couple H_2 oxidation to anaerobic sulfur reduction. It would make sense that early organisms could utilize hydrogen via hydrogenase. The enzymes are membrane bound, and catalyze the reversible reaction $H_2 <--> 2H^+ + 2e^-$ (53). Phylogenetically these enzymes are classified as Fe-, FeFe-, or NiFe- hydrogenases (21, 44, 50). These metal centers contain the catalytic site needed for a functioning enzyme. All three have solved crystal structures (33, 43, 51). They are involved in energy conservation and fermentation in many diverse microorganisms.

Iron (Fe-) hydrogenases do not contain the FeS clusters commonly found in FeFe- and NiFe- hydrogenases. This enzyme was first known as metal free hydrogenase, because of the uncertainty of Fe involvement in the catalysis of hydrogen; this term was later changed to iron-sulfur cluster free hydrogenase (44). The iron is not redox active, this is in contrast to the metal active centers in NiFe- hydrogenases (21, 50). Fe- hydrogenases have only been found in methanogenic bacteria that convert CO_2 to CH_4. Only in nickel limiting conditions are these hydrogenases necessary (50). The specific activity of the Fe hydrogenase was 6 times higher in *Methanobacterium*

thermoautotrophicum grown in a Ni-limited environment compared to non limiting Ni. In conditions where nickel is abundant the methanogen can use F420-reducing hydrogenase for CO_2 utilization (3, 49).

Iron-Iron (FeFe-) containing hydrogenases are monomeric proteins. These are noted as the only type of hydrogenase ever to be found in eukaryotes. These enzymes tend to produce hydrogen. However, a FeFe- uptake enzyme was found in *Desulfovibrio vulgaris* enabling the bacterium to grow without nickel in hydrogen and sulfate media. Other bacteria that respire in anaerobic conditions may contain this type of hydrogenase.

NiFe- hydrogenases are dimeric proteins with a large (α) subunit containing the nickel active site and the small (β) subunit with the Fe-S clusters (4Fe-4S or 3Fe-4S). These were identified in archaea, and in many gram negative H_2-metabolizing bacteria (44). Some enzymes can only oxidize or only evolve hydrogen. Unlike the FeFe- and Fe- hydrogenases, NiFe- enzymes can be further separated into 4 phylogenetic groups.

Biological relatedness: group I and II are more closely associated than group III and IV. The two sets have diverged before association into the NiFe-hydrogenase type group.

Table 1.1 NiFe- hydrogenase classification

Group	Description	Found in
I	the membrane bound uptake hydrogenases	bacteria and archaea
II	uptake hydrogenases	cyanobacteria
	hydrogen sensors	bacteria

III	F_{420}-reducing	archaea
	NADP reducing	bacteria and archaea
	F_{420}-non-reducing	archaea
	bidirectional NADP/NAD reducing	bacteria
IV	Hydrogen evolving membrane associated	bacteria and archaea

(21, 50)

Shigella flexneri has hydrogenases that fall into the groups I (Hya, Hyb) and IV (Hyc). The hydrogenases are not essential enzymes for *S. flexneri* even though they do provide energy for optimal growth (22, 31). Group I is associated with membrane bound uptake hydrogenases. When H_2 is oxidized an anaerobic electron accepter such as CO_2, NO_3^-, fumarate, or SO_4^{2-} or an aerobic acceptor O_2 are reduced (50). Group I enzymes also have the benefit of being physically connected to the respiratory chain of the organism, thus making the enzymes a source of energy generation. The electrons produced can directly be shuttled to carriers in the respiratory chain and protons can be translocated to produce a membrane potential (9). Cytochrome *b* is connected to the C-terminus of the small subunit of the hydrogenase, which also anchors the enzyme to the membrane. One example of a well-studied NiFe- hydrogenase is found in *Wolinella succinogenes* (50).

Table 1.2 NiFe- uptake hydrogenases of enteric bacteria

Shigella	***Escherichia***	***Salmonella***	***Helicobacter***
Hya	**Hya**	**Hya**	**Hya**
Hyb	**Hyb**	**Hyb**	
	Hyd		

Of these enteric bacteria listed only *Helicobacter* lacks an evolving (Hyc) hydrogenase (Table 1.2).

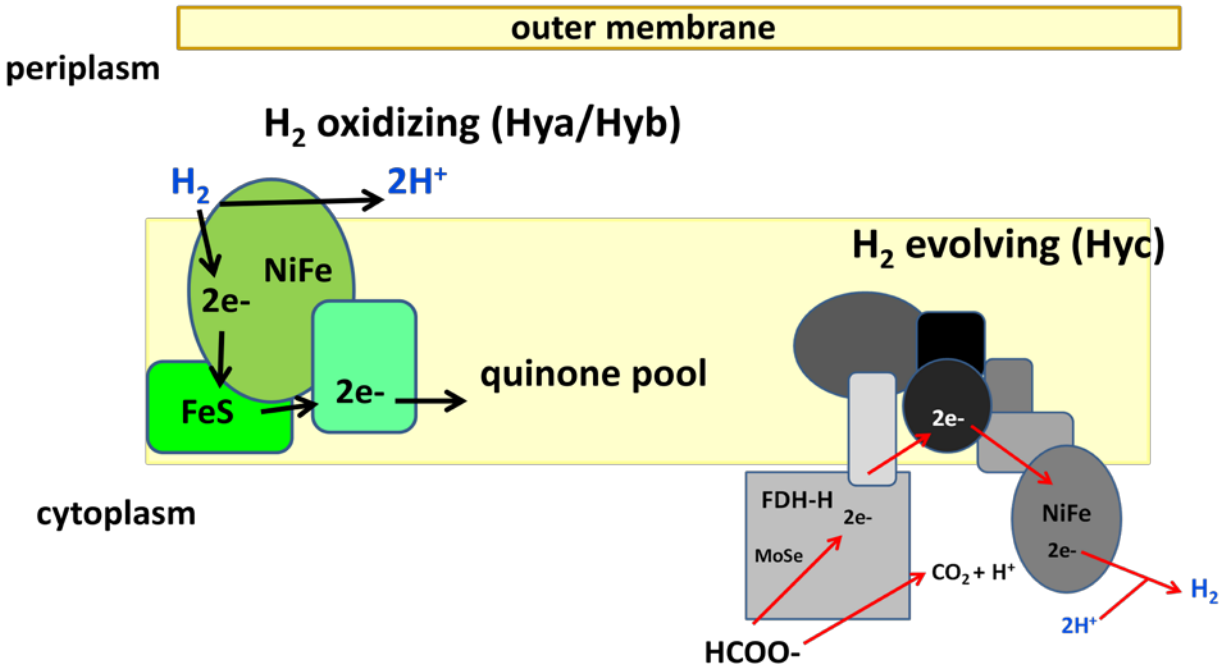

Figure 1.1 Schematic of NiFe-hydrogenases. Abbreviated schematic adapted from Sawers *et al*., (41).

Escherichia coli hyc is the type hydrogenase for group IV H_2-evolving enzymes. An organism can use this reaction to get rid of excess protons in the cytoplasm. The evolving enzymes work in concert with other complexes to rid the cell of excess reducing products. In *E.coli* this complex is called formate hydrogenlyase (FLH-1), and the complex converts formate to H_2 and CO_2 (50). These types of proteins have been discovered disproportionally in archaea compared to bacteria.

Characterization of Hydrogenases found in *Shigella flexneri*

Group I NiFe Hydrogenase

Hya, an uptake NiFe hydrogenase is encoded by 6 genes loci 1040-1045 (*hyaA-hyaF*). The genes encoding the small and large subunit are designated *hyaA* and *hyaB*

respectively. Biologically this enzyme is proposed to recycle H_2 produced from the formate hydrogenlyase (including Hyc) complex (25, 54). Metabolically this enzyme oxidizes H_2 in anaerobic and fermenting conditions (22). Transcriptional fusions to the *hya* promoter site in *E. coli* revealed that anaerobic induction was 20 fold higher in anaerobic conditions compared to aerobic (9). AppY and ArcA regulators help control expression of the *hya* promoter (22). Hya has been described as being more resistant to oxygen than Hyb (22, 25).

Gene expression of *hya* is repressed by addition of nitrate but increased in glucose and formate supplemented media. Also, formate induces expression of *fdhF* which encodes a component of the formate hydrogenlyase complex (9); thus connecting the biological role of Hya to recycling hydrogen produced by Hyc.

Further characterization of Hya has revealed that its pH optimum range is between 6-8 (22). This range agrees with activation of the *hya* operon in fermentative conditions with glucose, which lowers the pH of the media. Also it can be inferred that this enzyme may be of importance when *Shigella* invades the host. It must survive the acidic mileu of the stomach in order to invade the epithelial layer of the intestines. Furthermore Hya's biological role has also been linked to stress conditions. The expression of *hya* can be modified according to external pH. When expression is monitored in unbuffered Luria Bertani broth (LB) with glucose, the highest expression of *hya* was seen in late stationary phase where the pH was 4.7 (22). Regulators AppY and RpoS are activated in stress conditions which can occur when the bacteria are in acid. These regulators have been shown to aid in *hya* expression. An RpoS mutant (grown in unbuffered LB with glucose) showed an increased expression of *hya* as the pH was

lowered (22). However this expression was at a reduced level compared to the wild type strain.

Hyb shares the same metabolic activity as Hya, it oxidizes H_2. Both are similar in that they optimally oxidize hydrogen in anaerobic conditions. The Hyb operon encodes 7 genes (*hybA-hybG;* S3244- S3238), with *hybA* and *hybC* encoding the small and large subunits. Biologically these proteins have different roles in the life of a bacterium. The major difference is that the pH optimum of Hyb is pH 8 while Hya can be optimally expressed at pH 6. Expression of *hyb* is increased in alkaline media supplemented with glycerol and fumarate (22).

Hyb is regulated by NarX/L and NarQ/P two-component regulation systems. Deletion of *narP* results in no *hyb* expression. Expression of *hyb* is influenced by ArcA. Catabolite repression (cAMP/CRP) plays a role in *hyb* expression while there is no evidence of such repression for *hya* (39).

Group IV NiFe hydrogenases

Hyc is an H_2 evolving protein complex. The operon (*hycA-hycF*; S2934-S2929) comprises of 6 genes. *hycB* and *hycE* encode the small and large subunits. Hyc is a part of the formate hydrogenlyase complex (FHL). This complex consists of formate dehydrogenase (*fdhF*), electron carriers, and *hyc*. The dehydrogenase oxidizes formate via pyruvate formate lyase to CO_2. Hyc uses the protons from formate oxidation and electrons from electron carriers to evolve H_2 (9). The complex is active in the presence of formate. In the presence of alternative electron acceptors such as nitrate, trimethylamine N-oxide (TMAO), or oxygen there is little gene expression (41).

Alternative formate consuming dehydrogenases FDH-O and FDH-N oxidize formate, preventing accumulation of the substrate inside the cell.

Bacterium: *Shigella flexneri*

History

The *Shigella* genus was discovered over 100 years ago by Kiyoshi Shiga. He isolated the bacterium (*Bacillus dysenteriae, later* renamed *Shigella dysenteriae* type 1) during an endemic episode of dysentery in Japan in the 1890s. In 1919 the genus was formally referred to as *Shigella* after Dr. Shiga by Castellani and Chalmers (35, 48). This genus is mostly found in fecal samples from patients suffering from bacillary dysentery (mucoid bloody stool), though *Shigella* has also been isolated from various parts and liquids in the body including the ear, bones or joins, blood, and urine.

Classification

Before 1919 *Shigella flexneri* was first referred to as *Bacillus dysenteriae. S. dysenteriae* was selected by Castellani and Chalmers as the type species. *Shigella* are G*ammaproteobacteria* grouped in the order *Enterobacteriales*. They are placed in the family *Enterobacteriaceae,* which simply means that these bacteria are intestinal (8). *Shigella* are facultative anaerobic bacilli sized 1-3 µm in length. Unlike other enteric bacteria like *Escherichia coli* and *Salmonella enterica*, they are non-motile.

Table 1.3 *Shigella* species

Species	Serotypes	Isolated/described	Named after bacteriologist
dysenteriae	**15**	**1898, Kiyoshi Shiga**	**Kiyoshi Shiga**
flexneri	**6**	**1919, Simon Flexner**	**Simon Flexner**
boydii	**20**	**1949, William Ewing**	**Sir. John Boyd**
sonni	**none**	**1920, Max Levine**	**Carl Sonne**

(16, 48)

Disease

Shigella is the causative agent of shigellosis, which infects around 140,000,000 people per year worldwide (5). Before the onset of scant stools the patient may experience watery diarrhea (15, 34). This disease is characterized as watery, bloody, mucoid (dysentery) diarrhea. Shigellosis is also referred to as bacillary dysentery. This is a disease of the large intestine, which is characterized by scant stools, while an infection of the small intestine would be characterized by watery diarrhea (2). Accompanying the dysenteric symptoms are painful bowel movements (with little to no excretion), fever, tenesmus (painful stools with little excretion), and stomach cramps (1). These clinical signs of shigellosis can appear in as little as 24 hours. Studies have shown that shigellosis can develop from as few as 10-100 bacterial cells (1).

Treatment

Treatment for shigellosis can be as simple as oral rehydration for those who are dehydrated due to multiple episodes of diarrhea. Though shigellosis is more commonly associated with mucoid and scant bowel movements, some patients can benefit from this method if they experience dehydration (2, 35). Antibiotics are not typically given to

every victim of shigellosis. There are at least two reasons for this: for a mild case the disease is self-limiting; and secondly the rise of antibiotic resistance among *Shigella* spp. is of concern. When oral treatment is properly given this can limit the duration of the disease by a few days, and also decrease the titer of *Shigella* in feces (35).

Epidemiology

Shigella is classified in the *Enterobacteriaceae* family along with its notorious colleagues including *Escherichia*, *Salmonella*, and *Yersinia*. Unlike *Salmonella* and *Escherichia* which can be spread from contaminated food to person and person to person transmission, *Shigella* can only spread from person to person contact (fecal-oral route) (1). This bacterium's natural reservoirs are humans and higher primates (16).

S. sonnei is the primary species that contributes to the incidence of shigellosis in the US while *S. flexneri* is commonly associated with endemic cases of shigellosis internationally (2, 11, 16, 35).

S. flexneri is of global relevance, with most cases of shigellosis found in children less than 5 years of age, and the majority of patients residing in underdeveloped nations. It is quite difficult to know the true incidence of shigellosis because this is a self limiting disease (for those who are relatively healthy) so temporarily ill individuals may not seek treatment. In these individuals the time of clinical disease onset is anywhere from 4 to 7 days (6).

Pathogenesis

Shigella are enteric bacteria that have adapted to not only survive the host stomach at a pH of 2 for hours but also are able to use the hosts' own machinery to infect the

intestinal epithelia to gain access to the submucosa. Once *Shigella* are ingested the bacteria uses a suite of acid resistance pathways to combat the host acidic environment. Then they are emptied into the small intestine where the pH is significantly more neutral or less acidic than the stomach. The large intestine (colon) is the preferential invasion spot for the bacteria because of the presence of sampling cells called micro fold or M cells. These cells provide a portal to allow *Shigella* to cross the membrane. *Shigella* are intracellular pathogens that enter the host epithelial cell encased in a phagosome. *Shigella* use a type III secretion to deliver proteins that lyse the phagosome in as little as 15 min. *Shigella* are then released into the cytoplasm to replicate (42). The bacteria can then use the host machinery to polymerize an actin tail to invade the next cell.

Acid Survival

Enteric pathogens ability to survive the low acidity of the stomach is attributed to acid resistance pathways. The *in vitro* test of bacterial acid resistance measures bacterial survival after 2 hours of acid exposure at pH 2.5. If greater than or equal to 10% of the initial inocoulum survives after this time it is deemed acid resistant (18). *Escherichia coli* has 4 active acid-combating pathways: glucose-repressible oxidative pathway (AR1), glutamate-dependent (AR2, GDAR), arginine-dependent (AR3) and lysine-dependent (AR4). AR1 and AR2 have been identified in *Shigella flexneri* but only recently have these pathways been fully described (17).

The mechanism of action of AR1 in both *E. coli* and *S. flexneri* is quite similar. AR1 protects cells above pH 3.0 (4). To induce acid resistance cells must first be grown

in mildly acidic (pH 5.0) complex media. If cells are grown in slightly basic medium AR1 was not activated (10, 17). Oxygen is required in the adaptation medium; for a *S. flexneri* wild type strain acid challenged at pH 2.5, a 100 fold difference in survival was seen with oxygen versus without oxygen.

AR2 or the glutamate-dependent pathway requires extracellular glutamate, isoenzymes *gadA* and *gadB*, and the antiporter *xasA* (also referred to as *gadC*) for proper action (4, 17, 40). Protons are consumed during the decarboxylation of glutamate to γ–aminobutyric acid (GABA), XasA transports glutamate in and GABA out. Studies in *E. coli* have found that decarboxylases *gadA* and *gadB* are both low pH inducible and that *gadB* is more strongly induced in stationary growth phase than is *gadA* (10). The pH optima of both GadA and GadB is pH 4.0 (40). The presence of both enzymes allows for greater acid survival at pH 2.0. XasA is a glutamate:GABA antiporter, and it is not ATP-dependent. It is a member of the amino acid-polyamine-organocation (APC) superfamily (tcdb.org).

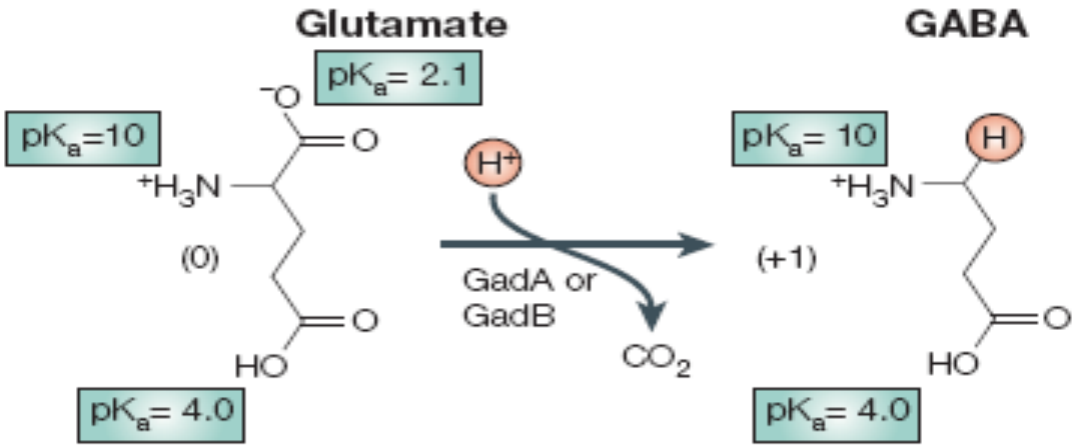

Figure 1.2 Mechanism of AR2 or GDAR acid resistance pathway. Figure source Foster, (13)

Rationale for study of *Shigella* hydrogenases

Shigella are acid resistant bacteria and the causative agents of bacillary dysentery (shigellosis). The incidence of shigellosis is highest among children under the age of 4 (24, 35) but it can commonly cause clinical symptoms in those who are immunocompromised, like HIV patients (20). In the United States almost 10,000 cases are reported each year in comparison to the 163.2 million cases reported in developing nations (11, 18). Such disparity of cases is due to poor sanitation practices in underdeveloped areas, as well as the overall poor health of the population.

Shigella infection can also lead to serious complications: vomiting, toxic megacolon, painful bowel movements, reactive arthritis, and hemolytic uremic syndrome (HUS). Toxic megacolon occurs when inflammation destroys the integrity of the intestine. This condition is medically defined as segmental or total colonic distention of greater than 6 cm in the presence of acute colitis and signs of systemic toxicity (12). People with this condition have little smooth muscle contraction due to electrolyte imbalance caused by diarrhea (14, 23, 27). This is often a fatal disease (12, 16).

HUS is a severe kidney complication that can lead to acute renal failure in those under 3 years of age. It is most commonly associated with *S. dysenteriae* Type 1 that contains the shiga toxin (stx) (16). The condition can also be associated with non-diarrheal symptoms. Glomerular endothelial cells aggregate in the kidneys which cause clots (1). The cells that do happen to pass the clot are disfigured and fragmented (schistocytes). This leads to the inability of filtering by the kidneys, so that the patient must have dialysis treatment (7, 38).

Only 10-100 viable bacteria are necessary for disease symptoms. Shigellosis manifests itself only in humans and higher primates. The small infection niche of this disease has made it difficult to find suitable animal models. The stomach on average empties every 2 hours, during this time bacteria activate acid resistance pathways that allow them to persist in minimal concentrations in the stomach. The bacterium possesses complex mechanisms to survive the gastric mucosa. While *Shigella* normally grow in a pH range of 5-9, they can survive for a prolonged period in a pH as low as 2 (45).

Environmental factors such as pH of growth media, and nutrients added to said media will also impact acid resistance (17, 45); as well as growth state of the bacteria. Sigma factor 38 encoded by *rpoS* is believed to have a role in acid resistance, however this factor may only be effective in aerobic conditions (26). In fact the 2a strain contains a truncated *rpoS* transcribed protein. This protein does not seem to have a large impact on acid resistance for this particular strain (17).

Hydrogenases have been thought to take part in survival of bacteria in acidic conditions (36). The Hyc protein can help regulate excess proton pools in the cytoplasm and therefore to control the internal pH by catalyzing the reduction of H^+ to H_2. In that way excess reducing equivalents can be used to regenerate molecular hydrogen.

The Centers for Disease Control and Prevention and the World Health Organization consider the *Shigella* species a prevalent human pathogen. Many of the world's inhabitants live in conditions that enable *Shigella* to thrive. In addition to

deficiency of health resources and lack of education, proper ways to combat the bacterium needs to be addressed.

Dissertation focus

Hydrogenases can either oxidize molecular hydrogen or they can produce H_2. The two reactions can have a major impact on how *Shigella* survives in various environments. It is important to focus on the roles of these enzymes in the gastric and colonic environments of the human body. A variety of gases are available for bacterial use in the intestinal tract, and their levels undoubtedly vary as the dynamics of the colonic community varies. The colonic flora ferment food products into short chain fatty acids (i.e. acetate, butyrate) and the by-products can be beneficial to bacterial survival (28). The gaseous by-products (H_2 and CO_2) produced by bacteria are unable to be utilized by the host, so instead the host eliminates these products via the feces, flatus, or human breath exhalant (29). How special are bacteria to use the trash for its treasure?

Of the total H_2 generated from the colonic bacteria upwards to 20% has been identified in the bloodstream. In a paper by Olson and Maier, it was determined that there is a strong correlation between H_2 use by *Helicobacter pylori* and its ability to colonize the stomach (37). This was quite an amazing find; in effect the bacteria that are supposed to aid host digestion and outcompete invaders are providing ammunition (energy in the form of hydrogen) to be utilized by pathogenic organisms. The average concentration of hydrogen in the mouse tissues was upwards of 43µM which is more than what is required to saturate the uptake hydrogenases. Half saturation affinity for H_2

was reported as 2.1µM for *S. enteric*a and 1.8µM for *H. pylori (30)*. This saturation refers to whole cell measurements and not pure protein determinations.

For a related pathogen (*Helicobacter hepaticus)*, a hydrogenase (uptake) mutant, colonized the mouse liver at a reduced level compared to the parent strain, these mutants were also markedly attenuated in H_2-supported amino acid uptake (32). Oxidation of hydrogen can be coupled to energy generation that can then facilitate transport of nutrient molecules across membranes. This finding helped to further strengthen the connection between hydrogenase and optimal bacterial survival.

A bacterium more related to *Shigella*, *Salmonella enterica* showed dependence on hydrogenase for its pathogenesis. It was found that when only one hydrogenase was present, the strain was able to colonize the mouse host. However a triple mutant strain (lacking all uptake hydrogenases), was unable to cause mouse mortality nor colonize the mouse tissue (30). In addition *S. enterica* showed differences in their hydrogenase expression depending on the tissue colonized (55).

My research highlights the pathogenic nature of *Shigella flexneri* by focusing on the connections between the bacterium's hydrogenase and its acid tolerance. The outcome from this study can give insight into how research may identify factors that could be targeted to combat infections from *Shigella* and perhaps other *Enterobacteriaceae*.

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CHAPTER 2

ROLES OF H_2 UPTAKE HYDROGENASES IN *SHIGELLA FLEXNERI* ACID TOLERANCE

ABSTRACT

[1]Hydrogenases play many roles in bacterial physiology, and use of H_2 by the uptake type enzymes of animal pathogens is of particular interest. Hydrogenases have never been studied in the pathogen *Shigella*, so targeted mutant strains were individually generated in the two *Shigella flexneri* H_2-uptake enzymes (Hya and Hyb) and in the H_2 evolving enzyme (Hyc) to address their roles. Under anaerobic fermentative conditions, a Hya mutant strain (*hya*) was unable to oxidize H_2, while a Hyb mutant strain oxidized H_2 like wild-type. A *hyc* strain oxidized more exogenously-added hydrogen than the parent. Fluorescence ratio imaging with dye JC-1 showed that the parent strain generated a membrane potential 15 times greater than *hya*. The *hya* was also by far the most acid sensitive strain, being even more acid sensitive than a mutant strain in the known acid combating glutamate dependent acid resistance pathway (GDAR). In severe acid-challenge experiments, the addition of glutamate to *hya* restored survivability, and this ability was attributed in part to the GDAR system

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(removes intracellular protons) by mutant strain (e.g. *hya/gadBC* double mutant) analyses. However, mutant strain phenotypes indicated a larger portion of the glutamate rescued acid tolerance was independent of GadBC. The *hya* strains acid tolerance was aided by adding chloride ions to the growth media. The whole cell Hya enzyme was more active upon (20 min) acid exposure, based on assays of *hyc*. Indeed, the very high rates of *Shigella* H_2 oxidation by Hya in acid can supply each cell with 2.4 x 10^8 protons per min. Electrons generated from Hya-mediated hydrogen oxidation at the inner membrane likely counteract cytoplasmic positive charge stress, while abundant proton pools deposited periplasmically likely repel proton influx during severe acid stress.

INTRODUCTION

Four predicted unidirectional hydrogenases are identified in the genome of *Shigella flexneri*: two uptake (*hya, hyb*) and two evolving (*hyc, hyf*) types. No physiological studies on *S. flexneri* hydrogenases have been published, but the *S. flexneri* hydrogenases are expected to have similar function and mechanism to the *E.coli*-described enzymes. Still, physiological and pathogenesis differences between *S. flexneri* and other *Enterobacteriaceae* justify studies on the comparative roles of the *Shigella* enzymes. Similarly, variations in enterobacterial acid-stress responses warrants further studies on *Shigella*.

Upon H_2-oxidation by uptake hydrogenases, electrons are commonly donated to various redox potential electron carriers at the inner membrane (31). Due to the membrane orientation of the H_2-oxidation reactions, the model for hydrogenase-

mediated proton motive force (pmf) generation involves the deposition of protons at the periplasm so that ATP production or other work can be accomplished by the cell (1) and (31). In *Enterobacteriaceae*, it is believed the Hya hydrogenase is the primary enzyme to recycle protons and electrons originally produced anaerobically by the formate hydrogenlyase complex (25, 32). One component of FHL is HycE, a hydrogen-evolving enzyme (25, 28).

Shigella flexneri causes diarrheal disease symptoms in humans upon ingestion of less than 100 bacilli (24) and (12). *Shigella* cells enter the acidic environment of the stomach (pH 2) where they subsist (~2hr) in stationary phase but typically grow very little (12). *Shigella* eventually enters the colon (pH 5.5-7.0) and invades the colonic epithelium via exterior sampling immune structures called M-cells (10). Again they encounter extreme acid conditions after their ingestion by macrophages. (6).

S. flexneri's ability to express stationary phase acid resistance (AR) mechanisms in the stomach contributes to the bacterium's unusual pathogenicity (17). Two AR pathways have been identified in *S. flexneri*: AR1 requires oxygenation of cultures and AR2 is glutamic acid-dependent and glucose repressed (2, 12). However, cells grown anaerobically were the most acid-resistant. It is clear the AR2 provides the most effective acid resistance, allowing cells to persist in the human stomach at pH levels below 3 (4). The genes involved in AR2 include decarboxylases (*gadA* and/or *gadB*) and an antiporter (*gadC*). Glutamate is taken into the cell via GadC, and decarboxylated via GadA/B which consumes an intracellular proton; the product, gamma aminobutyric acid (GABA) is exported out of the cell through the action of GadC (26). The net result is a neutralization of the cytoplasm, aiding acid tolerance.

Another acid resistance mechanism used by enteric bacteria involves chloride ion channels to prevent proton buildup in the cytoplasm (8). The exchange of a chloride ion for a proton in the cell decreases the intracellular pH, but also counterbalances the excess intracellular positive charges accumulated due to the decarboxylation products (7); the exchange allows the cell to eventually recover an internally negative membrane potential.

Deletion of all uptake-type hydrogenases in *Salmonella enterica* yields an avirulent strain with respect to mouse mortality (19) and H_2 has been postulated to be an important energy source for many enteric bacteria *in vivo* (18). The large intestine is a rich source of H_2, but the gas is found (dissolved in the bloodstream) at μM levels in many tissues colonized by pathogens and no known mechanism for animal hosts utilizing the gas have been described. Therefore, H_2 uniquely represents (to pathogens) an energy source for which competition with host cells is not a factor. Activity studies on *E. coli* Hya revealed that the *hya* operon is anaerobically induced under acid conditions (14, 30). Our study focuses primarily on the Hya hydrogenase in *Shigella* acid tolerance.

METHODS

Bacterial Strains and Growth conditions

S. flexneri 2a 2457T strain ATCC 700930 was used. *Salmonella enterica Typhimurium* JSG210 containing the recombinase plasmid pKD46, and *Escherichia coli* pCP20 and *E.coli* pKD4 were used for lambda red recombination. Strains with temperature sensitive plasmids (pKD46 and pCP20) were grown aerobically at 30°C on ampicillin (100ug/ml). pKD4 containing the kanamycin resistance cassette was

maintained at 37°C. Growth was performed in Luria-Bertani Broth (LB, pH 7.0, NaCl 10g/L) supplemented with 0.4% vol/vol glucose in 165 ml serum stopper-sealed vials. The liquid volume was 20ml and the atmosphere contained anaerobic mix (10% H_2, 5% CO_2, 85% N_2). Single mutant deletions are annotated as ALZ44 (Δ*hya*), ALZ47 (Δ*hyb*), and MMM01 (Δ*hyc*). The double mutants are designated as MMM02 (Δ*gadBC*) and MMM03 (Δ*hya*Δ*gadBC*). The strains are shown in Table 2.1.

Lambda red mutant constructs

Genes for both large and small subunits of the hydrogenase proteins were deleted. The procedure was adapted from (5). The temperature sensitive recombination plasmid pKD46 was electroporated into background strain 2457T. pKD46 was induced with 0.1M L-arabinose at 30°C. PCR constructs containing the large and small subunit deletions along with the flip-recombinase recognition sites (FRT) were transformed into pKD46 induced *Shigella* cells. After 1h at 37°C, cells were plated onto LB + kanamycin (25ug/ml) at 37°C overnight. pCP20 containing the flip-recombinase enzyme (3, 5) was electroporated into the Kan cassette containing mutants and plated (LB agar). Colonies were then plated on LB at 37°C to expel pCP20. The final mutant constructs are kanamycin and ampicillin sensitive and each contain a 'scar' site from the Kan cassette removal. Table 2.2 denotes primers used for deletion mutants

Hydrogenase assays

S. flexneri wild-type and mutant constructs were grown overnight (shaking at 200 rpm) in 165 ml vials (20ml LB) to stationary phase (16-18 hr). H_2 concentrations were determined using a microelectrode probe (19). A Unisense H_2-50 (Unisense, Aarhus,

Denmark) probe was used. The cell samples (7 ml) were removed anaerobically via syringe for addition to the (anaerobic) amperometric assay chamber, or for acid treatment prior to assay (see below). A 10 ml sample was placed into a beaker and swirled for 10-15 sec in air and then (7ml) evaluated aerobically for amperometric hydrogen consumption/evolution (20, 32). Exposure to air inactivates the H_2 evolution enzymes (32). For studying acid affects, stationary phase cells were added to vials (previously sparged with anaerobic mix) containing acid challenge media, which was composed of buffered citric acid phosphate (Mcilvaine) pH 2.5. Oxyrase for broth (Oxyrase© Inc., Mansfield, OH) was added to the vials, to maintain anoxic conditions, and the amperometric assay chamber was sealed throughout the procedure. Initial H_2 concentrations were noted after electrode stabilization (20-30 sec), and then H_2 levels were recorded for one min. The For H_2 assays upon acid challenge, samples were loaded directly from the acid-challenge milieu into the amperometric chamber (i.e. without washing) so the H_2 levels (i.e. activities) were measured while cells were suspended in the acid medium. After calculating nmoles H_2 consumption or evolution in one min the cell number was determined from a standard curve of cell number versus OD_{600} for specific activity calculations. Oxyrase-only controls oxidized a negligible amount of H_2.

Acid survival

The acid challenge procedure was adapted from one described previously (12). Wt and mutant constructs (*hya, hyb, hyc, gadBC,* and *hya gadBC*) were grown to stationary phase (16-18hr) in LB (pH 7.0). The challenge media atmosphere was adjusted by sparging with anaerobic mix. Stationary phase cells (10^7cfu/ml) were

added to the acid challenge media, which was composed of buffered citric acid phosphate (Mcilvaine) pH 2.5. After the indicated incubation period, serial dilutions were plated (LB) and colonies were counted after overnight growth.

For assessment of AR2 influence, 1.5 mM L-glutamic acid (Sigma) was added to the challenge media upon cell inoculation.

For NaCl acid survival, 1% NaCl w/vol was either supplemented to Luria-Bertani Broth and/or added to challenge media (pH 2.5) where indicated.

^{14}C-glutamate uptake and amino acid pool analysis

^{14}C-glutamate uptake was done as described in (15, 21)**.** Uniformly labeled glutamate (Sigma) was added to either exponential or stationary phase *Shigella* cultures and glutamate uptake was determined at numerous points. Amino acid pools were determined as follows: stationary phase cells were subjected to French Press disruption and then sonication. The extract (containing phenylmethylsulfonyl fluoride and always maintained at 0-4°C) was subjected to ultracentrifugation to remove the membranes. The supernatant was removed and concentrated by lyophilization. The physiological samples containing the amino acid pools were sent to the Molecular Structure Facility at Univ. of CA at Davis. There the samples were analyzed for full amino acid content by use of a Hitachi L-8800 amino acid analyzer.

Membrane potential (Δψ) measurement

The fluorescent cationic dye JC-1 (5, 5', 6, 6'-tetrachloro-1, 1', 3, 3'-tetraethylbenzimidazolylcarbocyanine iodide) was used to stain viable *Shigella* cells for

subsequent visualization via confocal microscopy. The fluorescence shift was used to approximate the electrical membrane potential (Δψ) of cells (15). JC-1 dissociation shifts the red emission to green and indicates a decrease in Δψ (13, 15). Cultures were grown (with H_2) to stationary (16-18 h) phase, and samples were prepared as described in (15). For the negative control a chemical protonophore, carbonyl cyanide m-chlorophenylhydrazone (CCCP), was used (dissipates membrane potential). The negative control samples were incubated with JC-1 (10 µg/ml final concentration) for 28 minutes. Then 40 µM of CCCP (stock solution was dissolved in DMSO, and used at 1:2000 dilution) was added for an additional two minutes (JC-1 still present). Samples were centrifuged at 18,000 x g for 2 min and the pellet was resupended in 250ml of fresh permeabilization buffer (10 mM Tris, pH 7.5, 1 mM EDTA, 10mM glucose). Ratios were taken from 5 fields with an average of 100 cells per field. Four independent experiments were conducted with similar results. Ratios are expressed in green/red; a lower ratio indicates a higher Δψ (13)

Statistical measurements and precision

One way analysis of variance (ANOVA), Tukey HSD test for independent samples or student T-test were used to compare data significance. Asterisks above bars in graphs indicate significant difference compared to wt except for fig. 2.5 where the difference was in comparison to (untreated for the same time point) *hya strain*: $P < 0.01$ (*) and $P < 0.05$ (**) or for fig. 2.6 $P < 0.02$ (*). All experiments were performed at least one additional time, with results similar to those presented herein.

RESULTS

Hydrogenase activities associated with gene-targeted mutants grown in fermentative conditions

The strains were grown in fermentative conditions and then assayed in conditions favoring H_2 oxidation and not H_2 evolution (see methods). In many separate experiments we found the *hya* mutant *(hya)* was unable to oxidize H_2 (see Fig. 2.3). Indeed *hya* showed slight hydrogen evolution, undoubtedly due to residual activity of the Hyc (H_2-evolving) system. In contrast, the *hyb* mutant strain (*hyb*) had almost wild-type levels of H_2 oxidation. The hydrogenase assays confirm that Hya in anaerobic fermentative conditions is responsible for hydrogen oxidation while Hyb contributes little and cannot replace Hya in H_2 oxidation. As expected, the strain lacking the evolving enzyme Hyc showed high levels of H_2 oxidation. Only in this strain can the full H_2-oxidation activity be measured, as H_2 produced by Hyc is eliminated as a factor.

Acid challenge

The Hya hydrogenase was implicated as playing a role in *S. typhimurium* acid resistance (32). The *Salmonella* strain lacking Hya was both acid sensitive and poorly able to survive in RAW 264.7 macrophages compared to the wild-type (wt) (33). After fermentative growth with glucose in LB to stationary phase, individual deletion mutation strains of *Shigella* were subsequently severely acid-challenged in a buffered medium (pH of 2.5). The results are shown in fig. 2.3. The viable cell counts decreased over the 6 h period for the wt and the three hydrogenase mutants. Compared to initial cell counts, the wt had 65 and 38% survival at 4 and 6 h respectively. In contrast, a dramatic

drop (almost 100 fold) in survival after just 2 h was observed for the *hya* mutant. At 4 h ~0.01% of the *hya* cells remained. No *hya* cells were recovered at 6 h post acid-challenge; its survival ability was statistically significantly less than all the other strains at all sampling periods (2, 4, 6 hr). The *hyb* strain was slightly more acid sensitive than wt; it was statistically significantly different than wt only at the 2 hr time point.

Glutamate-dependent acid tolerance mediated by GadBC is known to confer acid resistance to *Shigella* (12). Therefore a *gadBC* mutant (*gadBC*) was generated to compare its properties to *hya*; the strain lacks both the *gadB* decarboxylase and the *gadC* antiporter necessary for AR2 activity. This strain unexpectedly showed more acid resistance than the *hya* mutant, but (expectedly) was more acid sensitive than the other strains (see 2.3 at 4 and 6 hr points). Significant acid sensitivity (more than the other four strains in the table 2.1) was also observed in pH 3 challenge medium by *hya* (data not shown).

Hydrogenases are active in acid conditions

The roles/activities of the hydrogenases specifically during acid challenge (as opposed to in LB medium) was of interest. For example, it was possible that H_2 metabolism during pre-challenge growth had the major effect in conferring subsequent acid tolerance, and that H_2 metabolism enzyme activities in acid were negligible due to the acid shock. However, we found the whole cell activities in acid were even greater than in LB (see Table 2.3). In pH 2.5 conditions the wt, *hyb* and *hyc* strains oxidized considerable hydrogen, while the *hya* strain evolved some hydrogen in the anaerobic and acid stress conditions. In line with the hydrogen oxidation results (Fig. 2.2) *hyb* has

a similar phenotype as the wt strain. As expected, the *hyc* strain showed the highest H_2 consumption because only in that strain could the full H_2 disappearance (i.e. oxidation) be monitored. After 5 or 20 min in acid the *hyc* strain took up more than 200 nmoles of H_2 per minute per 10^9 cells. This means more than 2.4 x 10^8 H^+ are produced per cell per min.

The Hya enzyme (uptake activity) was stimulated by acid. In one experiment the *hyc* strain had an activity of 74 ± 10 nmoles of H_2/min/10^9 cells for cells taken directly from LB (n=5) and 228 ± 33 nmoles of H_2/min/10^9 cells (n=9) for the same cell culture placed into the acid shock media. Hydrogen evolution in *hya* undoubtedly originates via the Hyc enzyme. H_2-uptake enzymes normally consume all Hyc-evolved H_2 so that *Enterobacteriaceae* H_2 metabolism is interconnected (28, 32). For *E.coli* it was noted that Hya is transcriptionally activated in acid conditions and Hyb more so in alkaline conditions (14, 23). Hydrogenase activity comparable to wt *E.coli* was observed in a *hybC* deletion at pH 5.5, while no hydrogenase activity was observed in a *hyaB* mutant (29). The uptake activity we observe in the *hyc* strain is due to both Hya and Hyb enzymes. However, Hya must play the major uptake role, as the *hyb* mutant (contains Hyc and Hya) oxidized all Hyc-produced H_2 and some exogenous H_2. The *hya* mutant could not even oxidize all the H_2 produced by the formate hydrogenlyase system. (Table 2.3). Therefore the *hya* mutant evolved H_2.

Influence of glutamic acid-dependent acid resistance pathway

The AR2 pathway is the most efficient *Shigella flexneri* acid-combating pathway at pH below 2.5 (26). It is dependent upon efficient glutamate uptake. Therefore we

thought Hya might play a role in providing membrane PMF to facilitate amino acid uptake, a role attributed to one of the *Salmonella* hydrogenases (15)**.** If glutamate transport was facilitated by Hya, causing high internal glutamate pools, one might also expect that adding high levels of glutamate may diminish the strong acid sensitivity of the *hya* strain. To address this, 1.5mM L-glutamic acid was added to the challenge medium vials along with the various strains (see Fig. 2.4).

In fig. 2.4, the wt was mildly acid sensitive, with 63% of the cells surviving at 4 h and 31% of the cells surviving 6 hr of acid challenge. From an initial cell level of 10^7cells/ml, less than 0.1% of the *hya* strain cells were recoverable after 4 hr of acid challenge. As before (Fig. 2.3), at the 6 hr point no cells were recovered. However, glutamate supplementation aided in the recovery of the *hya* mutant; it achieved wild-type (no-glutamate added) survival levels (2 and 4 hr) or nearly so (6hr). Glutamate supplementation had little effect on (aiding) acid survival of wild type (data not shown). The double mutant *hya gadBC* was also acid challenged with and without glutamic acid. Although glutamate aided recovery of this strain, viable cell recovery by glutamate addition was nearly 67-fold less (see 4 hr time point) than for the *hya* strain; therefore most, but not all the glutamate-dependent rescue of *hya* is due to the GDAR system.

Additional experiments (data not shown) included ^{14}C labeled glutamic acid uptake assays and comparative intracellular amino acid pool analysis to try to explain the *hya* phenotype. Increased glutamate accumulation during growth in the pre-shock media would aid AR2 mediated acid resistance. ^{14}C glutamate was added to various growth phase *Shigella* (wt and *hya*) in an H_2-containing atmosphere, and the rate of glutamate uptake over minutes and hours were measured using liquid scintillation

spectrometry. The results (data not shown) were not supportive of the hypothesis that Hya plays a direct role in transporting glutamate.

Amino acid pools of both the wild-type and *hya* mutant were also analyzed to determine if the reason behind the phenotype difference in survival was due to more robust amino acid accumulations into the cytoplasm by the parent. However, the pool analysis showed little differences between the strains; no evidence could be gleaned to conclude that Hya aided *Shigella* in glutamate or other amino acid accumulations. The glutamate-dependent rescue of *hya* strains phenotype may be due to the GDAR system permitting extra positively-charged gamma aminobutyric acid export out of the cell.

NaCl facilitated acid survival of *hya* strain

The reversal of Δψ comes from both proton influx and accumulating positively charged decarboxylation products associated with function of AR2 (7). It is proposed that this acid-combating mechanism (positive internal charge) protects cells from acid by repulsion of proton movement into the cell. However, maintaining a positive electrical potential within the cell can also detrimental, as hyperpolarization can cause additional severe ion stress, resulting in compromising PMF-driven functions (7). Another acid-combating system involving Cl^- influx (via chloride channels) is proposed to prevent hyperpolarization due to excess positive charge stress in *E.coli* (11). The influx of the chloride (in exchange for H^+) is thought to reverse the internal potential from positive to less positive (26). Conceivably *hya*, due to lower ability to generate cytoplasmic electrons could be phenotypically rescued by addition of Cl^-. To address a possible role of Cl^- influx on the *hya* phenotype we thus challenged the wild-type and *hya Shigella*

strains in pH 2.5 buffer with added NaCl. Different challenge conditions were used to address NaCl affects in acid resistance (Fig. 2.5). These were NaCl added to buffer (treatment 1), NaCl added to LB growth medium (treatment 2), NaCl added to both challenge and growth media (treatment 3) and as a control no NaCl added to either LB or buffer (untreated). *Hya* cells grown in LB with NaCl (treatment 2) were acid sensitive, but survived better than the untreated sample or than the other conditions; from an initial level (all numbers as cfu/ml) of 3.5×10^7 (zero time point), ~ 1.0×10^4 cells were recoverable at 6 h post inoculation. Still, this recovery was significant, in that no cells were recovered 6 h post-acid challenge in the untreated *hya* sample. In contrast, adding NaCl to LB greatly inhibited subsequent acid-challenge survival of the wild type; 6 hr of acid-challenge caused its viable cell number to decrease from ~4×10^7 (no NaCl in LB) to about 10^4 cells (by treatment 2). Considering the inhibition of wt, NaCl may aid *hya* greatly, but the inhibition of wt acid tolerance makes interpretations difficult.

The *hya* mutant was also challenged in pH 2.5 with the addition of 2% MgCl in the same manner as NaCl addition. MgCl added to the overnight media provided a similar phenotype to treatment 2. Additional NaCl in the growth media could affect many components. For example, it was shown that NaCl was important not only in the exchange of H^+ for Cl^- ions across the membrane but also in the activation of key enzymes in the *E. coli* AR2 system (27). Still, the salt could have affected many factors not directly related to the acid combating systems.

Membrane potential (Δψ) in wild-type and *hya* strains

Based on the results from the hydrogenase assays, and the predicted roles of membrane-bound hydrogenases, the electrical component (Δψ) of proton motive force (PMF) was assayed in wt and *hya* strains. The fluorescent dye JC-1 was used to determine membrane potential, a critical component of PMF. Green fluorescence indicates the cells contain a lower membrane potential. Wild-type *S. flexneri* had a green to red ratio of 0.064 (see Figure 2.6). The *hya* strain had a lower Δψ, with green/red ratio of 0.876. Four independent experiments were performed and they all yielded a significantly higher membrane potential associated with the wild-type *Shigella* strain.

DISCUSSION

Hydrogenases have been previously implicated as playing roles in acid resistance in *E. coli* and in *Salmonella*. The evolution of hydrogen by Hyd-3 (*hyc*) uses excess protons to neutralize the cytoplasm in *E. coli* (22), and a *Salmonella hya* mutant was acid and macrophage sensitive (32). It was observed (14) that the *E.coli hyb* gene expression was increased in alkaline conditions and an increase in *hya* expression was correlated with a decreased external pH. In our study, based on gene directed mutants, we conclude Hyc and Hyb can confer some *S. flexneri* acid resistance, but not nearly to the degree of the Hya hydrogenase (Fig. 2.3). In our study, the strain lacking Hya was even more acid sensitive than a strain lacking the described primary acid resistance mechanism. The glutamate supplementation and uptake assays indicate a major role of Hya is not in facilitating energy-driven GDAR function or by increasing cell pools of

glutamate. Still, recovery of *hya* cells by glutamate addition could be attributed in part to an active AR2 pathway and likely to glutamate transit via other glutamate-specific or even promiscuous transporters.

Enterobacteriaceae acid resistance is highly complex and dependent on many factors (7, 26). At least two acid resistance (AR) stationary phase pathways have been described in *Shigella flexneri*. Anaerobic conditions, low pH (2.5), and addition of glucose (conditions we used) are expected to prevent the action of the previously described AR1 pathway. The glutamic acid dependent system is expected to function in our conditions, but another proposed *Shigella* system (12) independent of either glucose or glutamate may also play a role in the phenotypes described here. In our experiments, addition of NaCl to Luria broth (LB) provided significant acid protection for *hya* when cells were subsequently acid-challenged. While there was a large reduction in *hya* viability even with NaCl, without salt addition there were no recoverable *hya* cells 6 h post acid challenge. The addition of NaCl was reported to aid acid tolerance of *E.coli* tetracycline-resistant mutants (9).

Gram negative bacterial hydrogenases are oriented across the cytoplasmic membrane to produce protons at the periplasm and electrons at the inner membrane thus enabling generation of a PMF; the system is used to facilitate work in many physiological processes. In addition, from our studies we conclude the Hya hydrogenase confers unexpectedly strong acid resistance to *Shigella flexneri*, and the reason must be related to electron and/or proton production. It is generally perceived that when cells are under extreme acid stress, protons illicitly enter the cell directly thru the cell membrane or via protein channels. One role of hydrogen-utilizing

hydrogenases may be to counteract positive charge stress that occurs cytoplasmically due to positively charged decarboxylation products and other positively charged molecules and ions that accumulate under acid conditions (7). Electron deposition at the cytoplasm due to Hya mediated H_2 oxidation could fill this role. Such negative ion accumulation would therefore play the same role as Cl^- ions do (11). The chloride experiments indicate that the role for Cl^- falls into the adaptation growth phase, rather than having a direct effect on counteracting protons during survival in the acid environment. Influx of the chloride (in exchange for H^+) in *Enterobacteriaceae* is thought to allow negative charges back into the cell, to counterbalance excess positive charge. It is not clear if the negative ion production hypothesis role for Hya in aiding acid tolerance is supported by the glutamate addition experiments. Still, operation of GDAR is known to combat external proton stress, so the *hya* strains sensitivity to acid may be related to loss of cytoplasmic proton management.

Amplifying operation of a system that is known to reduce positive ion stress, namely the GDAR system (by adding glutamate) greatly aided survival of the *hya* strain. A positive Δψ, measured in *E. coli* subjected to acid conditions, comes from both proton influx and accumulating positively charged decarboxylation products that can be associated (i.e. increased) via the function of AR2. It is proposed that this acid-combating mechanism (positive internal charge) whether due to AR2 or other metabolites, protects cells from acid by repulsion of proton movement into the cytoplasm (7). Such positively charged metabolites localize to the cytoplasm, but are acting to repel proton influx from an acid environment that exists outside the cell wall. A hydrogenase that “splits” H_2 such that protons are deposited into the periplasm would

seem to be a better system to capitalize on combating acid via a proton repelling mechanism. Thus, two possible mechanisms for combating acid via H_2 oxidation, one involving electron accumulation internally, and the other involving proton deposition periplasmically are proposed for further investigation.

It has been suggested that proton concentrations inside the cell increase 1,000 fold in response to a shift in external pH to ~2.5 (internal pH shift of 7.5 to 4.5) (26). It is likely that the *hya* strain dies in acid because it cannot attain a negatively charged cytoplasm when needed, because it cannot repel sufficient protons. Due to the sidedness of the H_2 oxidation reactions, protons would deposit in the periplasm, thus not contribute to further cytoplasmic proton overload (16). The proton deposition at the *Shigella* periplasm could be very robust: according to our H_2 uptake activities (from the mutant strain unable to produce H_2, but fully capable of using H_2) upon exogenous H_2 addition the number of protons produced from H_2 activation could achieve more than 2.4×10^8 protons per minute per cell. For perspective, to change the pH within a single *E. coli* cell from pH 4.5 to 7.5 requires a net loss of 10,000 protons from the cytoplasm (26). In the absence of a terminal acceptor, the Hya enzyme would provide a build-up of electrons and reduced carriers on the cytoplasmic side; we propose this is beneficial to combat proton stress and aid in a reversal of the transmembrane potential. At the same time, H_2-oxidation via Hya causing abundant proton accumulation in the periplasm would be expected to repel proton influx across the outer membrane.

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Table 2.1 Strain constructions

Strains	Description	Reference
***Shigella flexneri* 2a**	**2457T wild-type**	**ATCC 700930**
Mutant constructs		
Δ*hya*::FRT ALZ44	*hya* deletion with FRT site (S1040-S1041)	This study
Δ*hyb*::FRT ALZ47	*hyb* deletion with FRT site (S3242-S3244)	This study
Δ*hyc*::FRT MMM01	*hyc* deletion with FRT site (S2930-S2931)	This study
Δ*gadBC*::FRT MMM02	Deletion of AR2 glu/gaba antiporter and glu decarboxylase with FRT site (S1867-S1868)	This study
Δ*hya*::FRT Δ*gadBC*::FRT MMM03	Deletion of *hya*, glu/gaba antiporter, and glu decarboxylase with FRT sites	This study
Plasmids		
pKD46	λ-red recombinase, Amp^r	(5)
pKD4	kanamycin resistance cassette	(5)
pCP20	Flp recombinase , Amp^r	(3, 5)

Table 2.2 Description of primers used in study.

Strain		Primers 5'→3'
		Deletion
ALZ44	F	CCGGTGTTGCTAGGAGAAGAGACGTGCGAT**TGTGTAGGCTGGAG CTGCTTC**
hya	R	TTTCTGTTGCATGATGATTCTCCTTCGCTG**CATATGAATATCCTCC TTA**
		Checking
	F	TTTCTGGCGACGTGTGCCAGTG
	R	ATGCATAACACCGTCAGCCAG
		Deletion
ALZ47	F	AGATAACGCTGACTCACGGGGAGAATAACC**TGTGTAGGCTGGAG CTGCTTC**
hyb	R	TATTGCCGACCCCTAAGACTAAAATACGCA**CATATGAATATCCTCC TTA**
		Checking
	F	AACGTCTCTGCAGGCGCTAATG
	R	AGGACTTCAACATAATCCGGCAG
		Deletion
MMM0 1	F	GGTGATTATTGTTTCACGAGGAGCCTGAGA**TGTGTAGGCTGGAGC TGCTTC**
hyc	R	GACTTTTTTGATAAAGGTAAACATGGCGATTCC**CATATGAATATCC TCCTTA**
		Checking
	F	GCCATGCCGGTTAAACAG
	R	CAACGCAGTGTACTGCACC
		Deletion
MMM0 2	F	TTTTAATGCGATCCAATCATT TTAAGGAGT**TGTGTA GGCTGGAGCTGCTTC**
gadBC	R	CCT TGTCTTATAACCATT CAG ACATGG**CATATGAAT ATCCTCCTTA**
		Checking
	F	CCACCCAAGAATTCTCTATTAC
	R	TCCGGTAAACAGATGTGC

MMM03 (*hya gadBC*) was engineered from strain MMM02 *gadBC* using deletion and checking primers from ALZ44. Deletion primers contain kanamycin resistance cassette

F= forward primer R= reverse primer

Table 2.3 Hydrogenase activity in acid (pH 2.5)

Strains	Specific activity after indicated time period in acid (pH 2.5)	
	nmoles of H_2/min/10^9 cells	
	5 min	**20 min**
WT	150 ± 37 H_2 uptake	180 ± 23 H_2 uptake
hya	51 ± 19 H_2 evolution	44 ± 5 H_2 evolution
hyb	113± 31 H_2 uptake	151± 54 H_2 uptake
hyc	276 ± 66 H_2 uptake	228± 23H_2 uptake

Amperometric hydrogen measurements of strains subjected to acidic (pH 2.5) conditions. H_2 metabolism activity (uptake or evolution) of each strain was measured after their subjection to acid challenge under strictly anaerobic conditions. Standard deviation was determined from 4-6 replicate recordings of H_2 level changes in a one minute period.

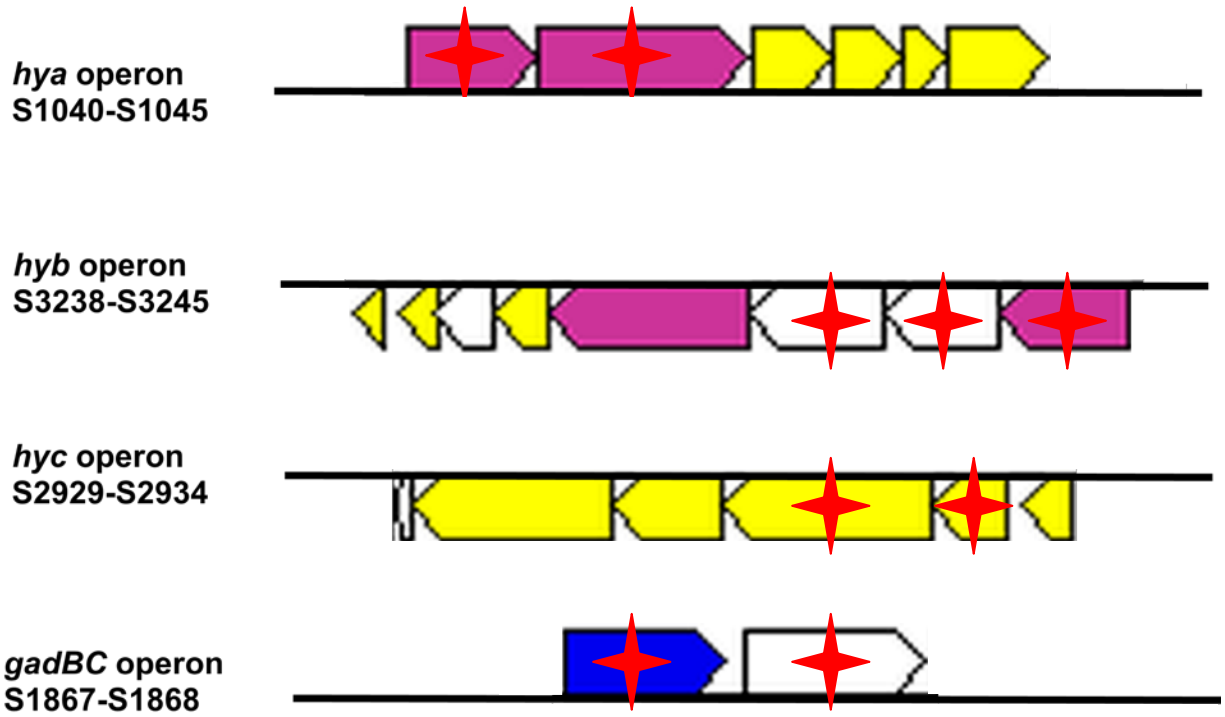

Figure 2.1 *S. flexneri* hydrogenase and glutamate-dependent acid resistance pathway (GDAR) operons. Schematic adapted from KEGG genome map (http://www.genome.jp). Lambda red deletions (red star) were made in S1040-S1041 in *hya*, S3242-S3244 in *hyb*, S2930-S2931 in *hyc*, and S1867-S1868 in *gadBC*.

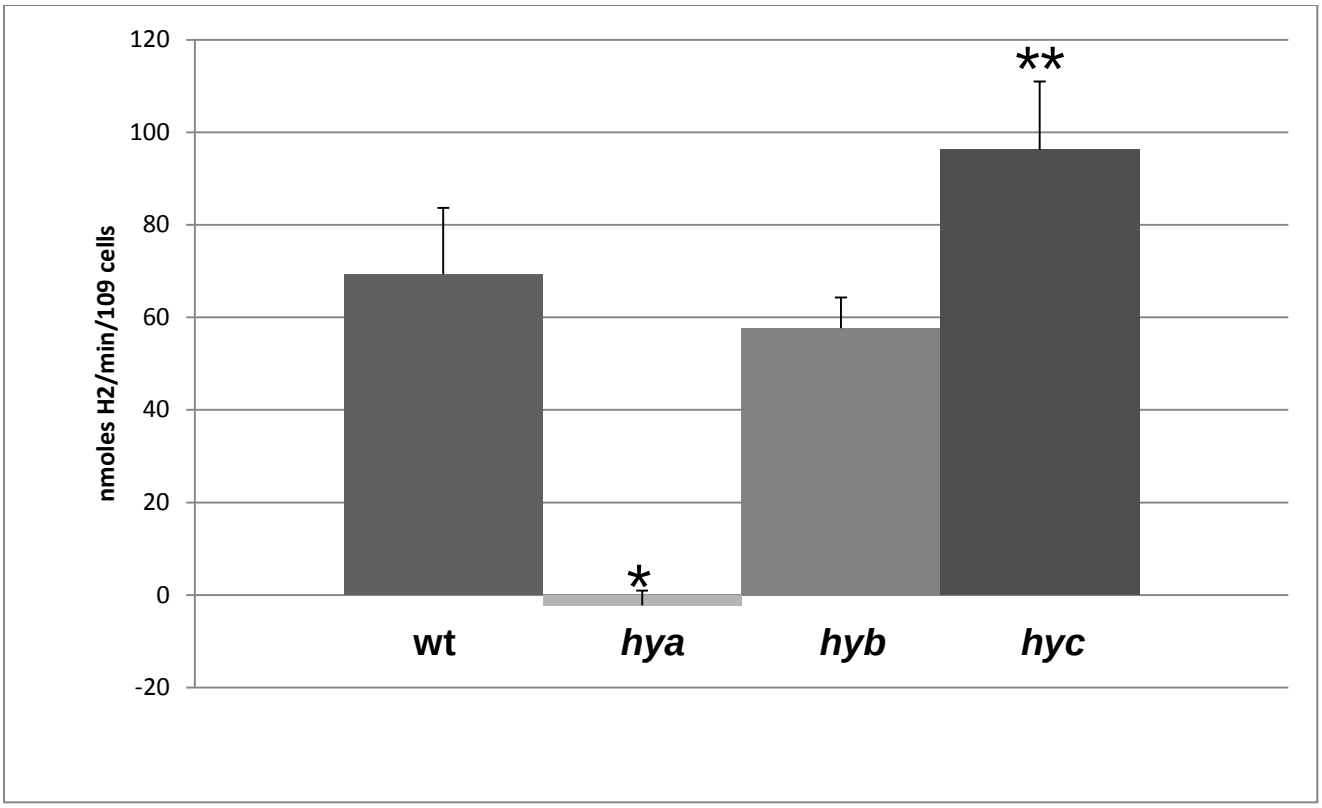

Figure 2.2 Microelectrode hydrogenase assay of *S. flexneri* strains. Hydrogenase activity was measured by hydrogen consumption under anaerobic conditions (atmosphere composed of 10% H_2, 5% CO_2, and 85% N_2). Strains are WT (2457T), *hya* (ALZ44), *hyb* (ALZ47), and *hyc* (MMM01). Standard deviation was determined from 5-8 replicate recordings of H_2 disappearance by individual strains, after inactivating H_2 evolution enzymes by brief O_2 treatment (32). Asterisk(s) indicate significant differences from wt at $P < 0.01$ (*) and $P < 0.05$ (**) based on Tukey HSD method**.**

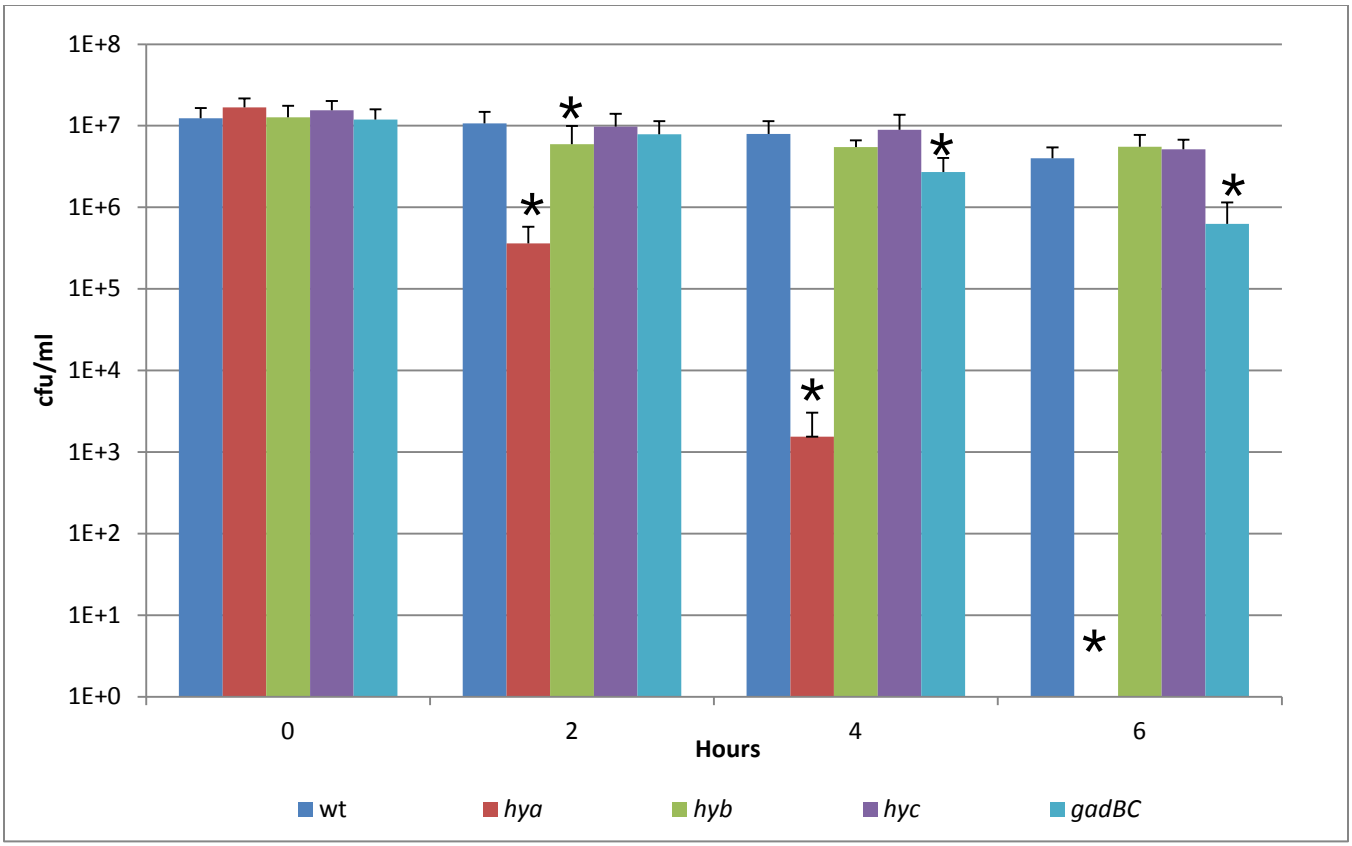

Figure 2.3 Viability upon acid challenge of *Shigella* cultures at pH 2.5. CFU yield from serial dilutions at post inoculation times are shown. Standard deviations are determined from at least 3 replicates from multiple dilutions. Control experiments in the same buffer but at pH 6.5 showed no viability loss for strains. Asterisk indicates a significant difference ($P < 0.01$) compared to wild-type calculated using Tukey HSD Test.

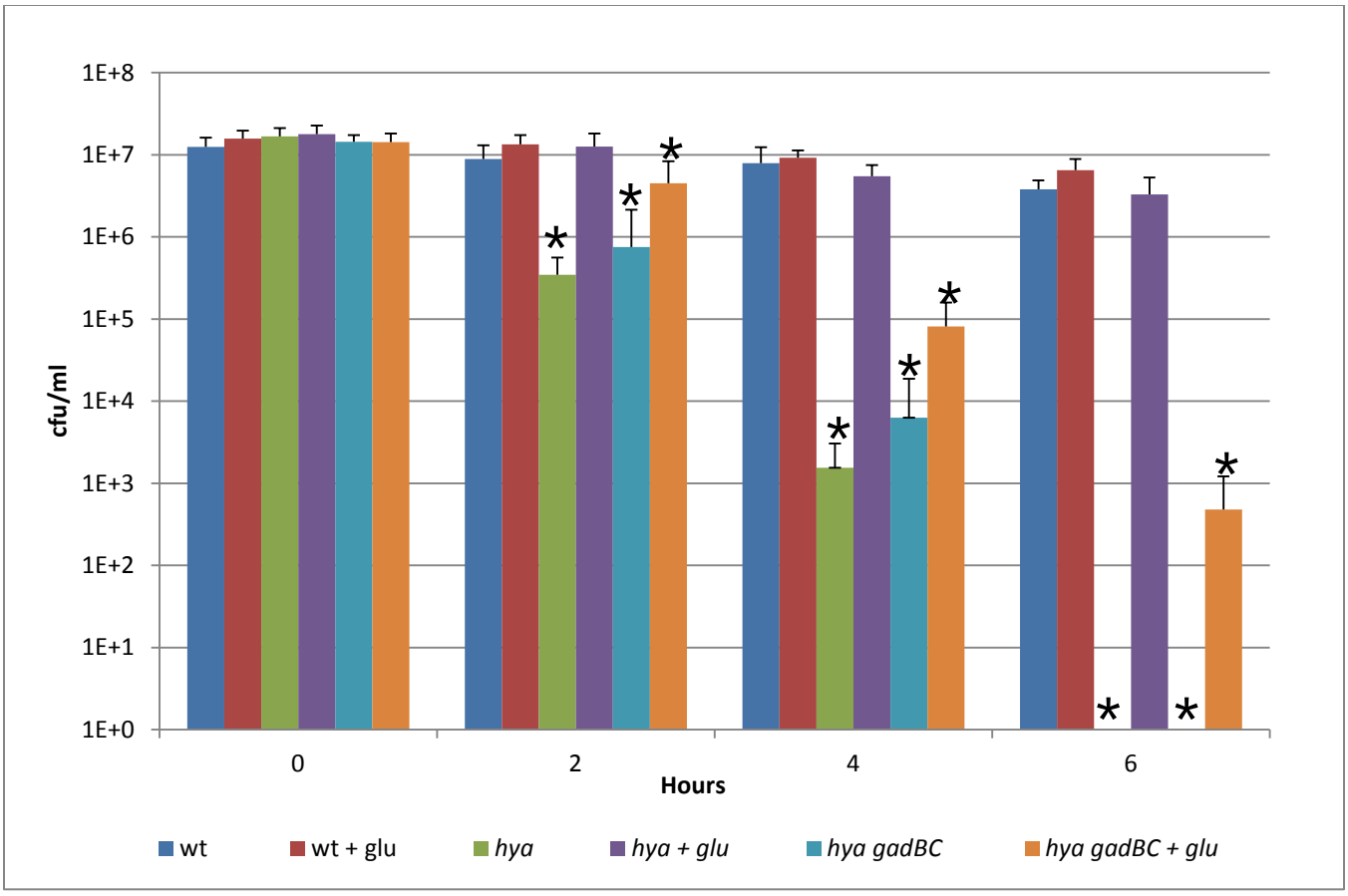

Figure 2.4 Influence of added glutamate to acid challenged *Shigella*. Acid challenge at pH of 2.5 was conducted as described in the text. 1.5mM glutamic acid was added to the challenge buffer where indicated. No *hya* or *hya/gadBC* isolates were recoverable at 6 hr post challenge (no-glutamate added samples). Standard deviations are determined from multiple dilutions and from 12 independent samples. Asterisk indicates a significant difference ($P < 0.01$) compared to wild-type calculated using Tukey HSD Test.

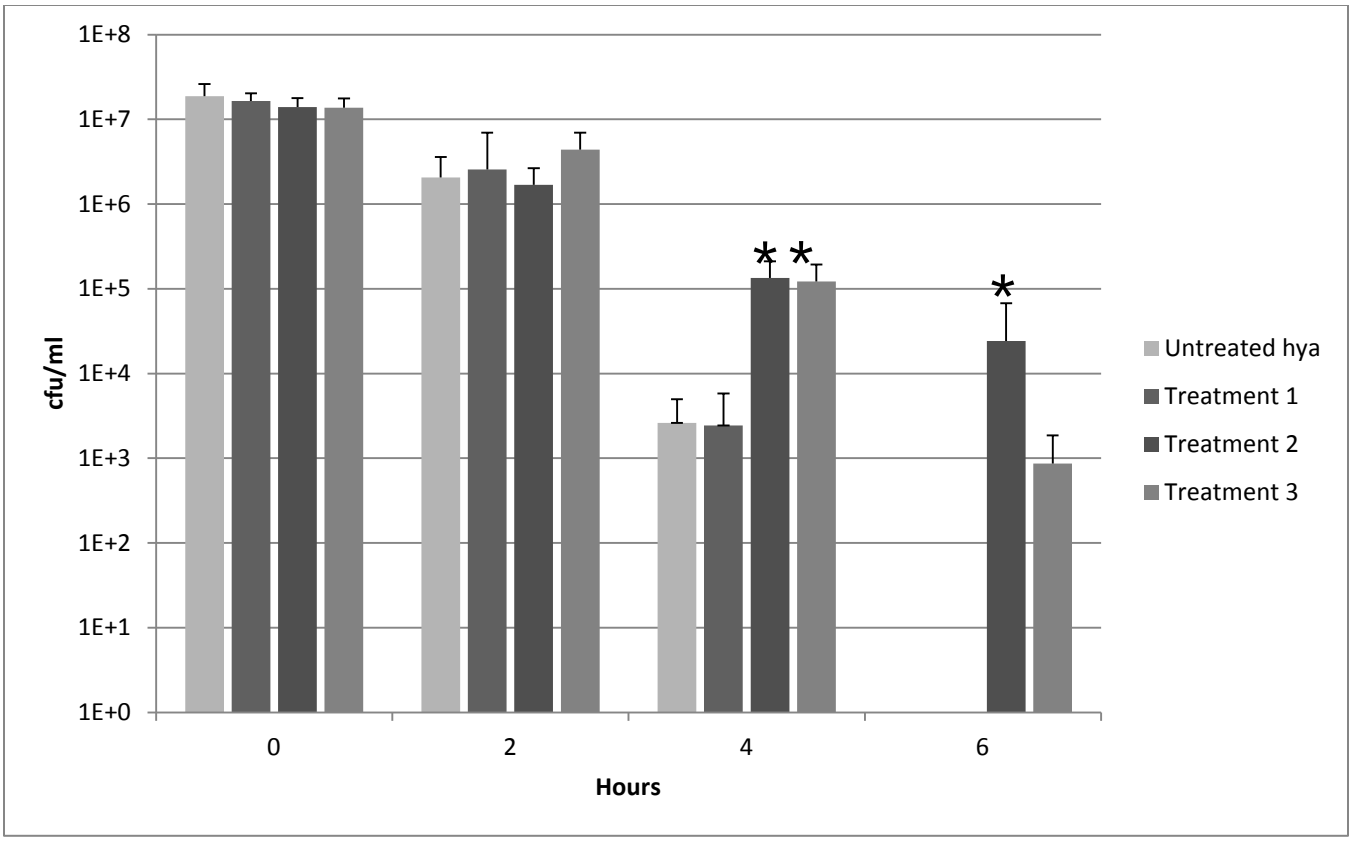

Figure 2.5 NaCl effects on acid tolerance of *hya*. Treatment 1: 1% NaCl was added to the challenge medium (pH 2.5). Treatment 2: 1% NaCl was added to the overnight growth medium (LB broth). Treatment 3: 1% NaCl was added to both overnight and challenge media. As with the glutamate acid rescue, *Shigella* strains were grown anaerobically in LB to stationary phase, and then added to pH 2.5 buffer. At the indicated times serial dilutions were plated. Significance of values (*) are based on comparison to the untreated Hya mutant. $P < 0.01$.

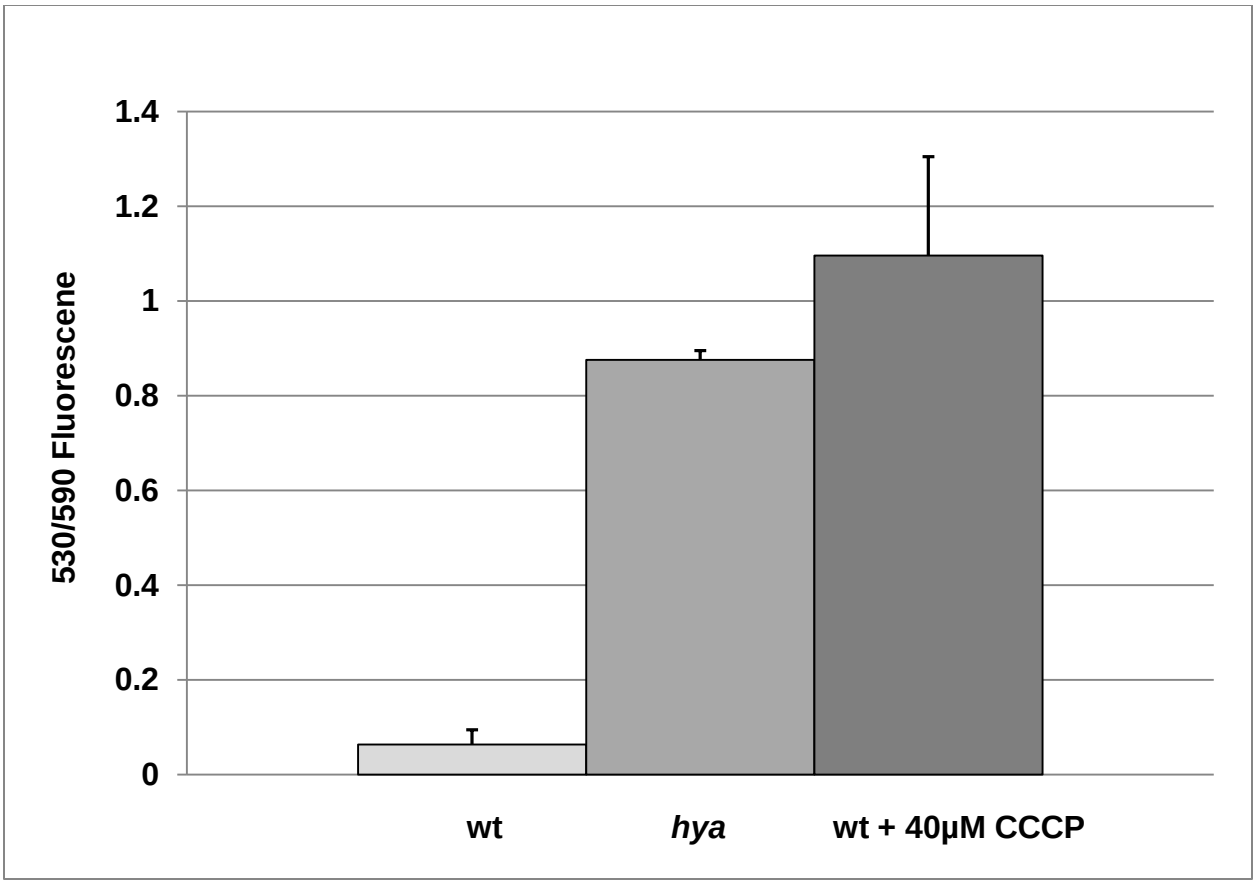

Figure 2.6 Comparative Δψ measurements. Ratios are fluorescence emission spectra for 530/590. A lower ratio indicates a greater Δψ. Strains were incubated with JC-1. For the negative control, CCCP (dissipates pmf) was added to wild-type *Shigella* for 3 minutes prior to adding JC-1 (10µg/ml). Standard deviations were determined from 5 fields (100 cells each). The wt result is statistically significantly less than *hya* or the CCCP control based on Students two-sample t-test at $p < 0.02$.

CHAPTER 3

THE PROTONOPHORE CCCP CAUSES *S. FLEXNERI* ACID SENSITIVITY

ABSTRACT

The Hya hydrogenase is a membrane bound enzyme that catalyzes the oxidation of molecular hydrogen. The products produced (H^+ and e^-) are used for ATP generation and proton motive force generation. CCCP is a protonophore that can dissipate the membrane potential. Treatment of H_2-oxidizing wild type *S. flexneri* with micromolar levels of CCCP induces acid sensitivity, but does not affect viability at pH 6.6. This acid sensitivity is comparable to that observed for a *hya* mutant challenged in pH 2.5. These results support a mechanism for Hya aiding in acid survival. This mechanism may involve buffering of the bacterial membrane from outside H^+ influx via proton production in acidic conditions.

INTRODUCTION

Bacteria have engineered a multitude of ways to acquire energy. Enteric bacteria are especially adapted to grow in diverse environments. *Shigella flexneri* is a facultative bacterium that can generate energy via aerobic/anaerobic respiration and fermentation. In stress conditions, metabolic flexibility likely becomes even more important. When *Shigella* enters the host it is bombarded by organic and inorganic molecules in the gastric environment. This acidic environment can range in pH from 1.0- 2.5 (1). Organic

components such as mucin and pepsin protect the gastric epithelial layer from the acidic medium, and break down bacterial proteins, respectively. Many ions are also present in the gastric juice including bicarbonate, hydrogen, chloride, and potassium. Hydrogen and chloride ions form HCl which is a contributing factor to the low pH of the gastric juice (2, 9).

Shigella infects the intestinal epithelial cells of the human, but to establish there the bacteria must first manage the acidic environment for upwards of 2 hours, until the stomach empties. The emptying of the stomach can vary depending on the volume and type of food digested, and can vary from 12 minutes from ingestion of water up to 6 hours for a meat-heavy meal (8, 9). Some bacteria such as *Shigella spp.* and *Escherichia spp*. activate acid resistance pathways (AR) to survive the acidity of the stomach. There are 3 known pathways that can provide protection for bacteria. Two AR pathways exist that depend on available free amino acids, glutamate and arginine to activate the process (5, 6). These amino acids can be provided most likely in the gastric juice because of the proteolytic enzymes that break down proteins into single amino acids.

It is known that hydrogenases play a role in acid survival for *Shigella* and related bacteria (6, 7). The *Shigella flexneri* Hya (H_2-oxidizing) was found to contribute to energy generation and was also postulated to aid in proton repulsion (in low pH) at the periplasmic/cytoplasmic interface (6). The *hya* mutant was acid sensitive while a wild-type strain was acid resistant at pH 2.5.

It has been previously shown that the *S.flexneri hya* mutant is incapable of producing a detectable membrane potential (Δψ) (6). The cationic dye JC-1 was used to indicate polarization/depolarization of the membrane (4). As a control, the protonophore chemical carbonyl cyanide m-cholorophenylhydrazone (CCCP) was used to show complete depolarization of the membrane. CCCP is a hydrophobic weak acid, that can bind protons and transport them to the inner membrane for release

The aim of this study was to further investigate the effects of membrane depolarization and acid survival by use of a protonophore. By addition of CCCP to *S.flexneri* cells, and subsequent pH 2.5 challenge, we provide evidence that Hya's membrane potential productivity is correlated with acid resistance.

METHODS

Bacterial Strains and Growth Conditions

Wild-type (wt) strain *Shigella flexneri* 2a 2457T (ATCC 700930) was used. The *hya* mutant, *hya* (ALZ44) construction was described in (6). Both strains are grown in Luria Bertani broth (LB, pH 7.0, NaCl 10 g/L) and supplemented with 0.4% vol/vol glucose to stationary phase (16-18 hours). 70 ml capped vials are used for growth and acid challenge assays. 10mL liquid volume was saturated with anaerobic mix (85% N_2, 10% H_2, 5% CO_2).

Acid Challenge Assay

Acid challenge was done according to the method described in (6). Briefly, *Shigella* cells are grown to stationary phase in LB with added glucose 0.4% vol/vol. Stationary cells

($1x10^7$) challenged in pH 2.5 citric acid-phosphate media in an anaerobic environment (described above) over 120 minutes. Carbonyl cyanide m-cholorophenylhydrazone (CCCP) (Sigma C2759) dissolved in DMSO was added at 2.5, 5, 10, 20 µM (where indicated) to cells before addition to pH 2.5. Controls: no CCCP added and 20 µM CCCP was added to pH 6.6. This was done to assess the effect of CCCP on viability when the strains were not acid challenged. At indicated intervals aliquots of each concentration were serially diluted in PBS on LB Agar. P-values were calculated using the Student's T-test.

RESULTS AND DISCUSSION

Wild-type (wt) *S. flexneri* challenged at pH 2.5 up to 120 minutes shows a dose dependent response (viability loss) to CCCP addition (Fig 3.1). At 30 min post inoculation, recovery of cells treated with 20 µM CCCP was significantly less ($p< 0.003$) than untreated cells. The poor survival of the 20 µM CCCP sample was more evident at later time points. CCCP (20 µM) treated *S. flexneri* at pH 6.6 showed no signs of acid sensitivity over the 120 minute period (Fig 3.2a).

The *hya* mutant strain was also challenged at pH 2.5 with different levels of added CCCP. The *hya* mutant is known to be acid sensitive in pH 2.5 (6). It is hypothesized that this sensitivity is due to a depolarized membrane (6). Over 120 minutes a 1,000-fold difference in cell recovery was observed in the *hya* mutant not treated with CCCP compared to the 2.5 µM CCCP treated *hya* mutant (Fig 3.2b). In the *hya* strain treated with 5 and 10 µM CCCP, cells were unable to be recovered at 120 minutes. The addition of CCCP to the mutant further decreased *S. flexneri*'s ability to

survive acid treatment. The most probable cause is that the protonophore makes the membrane permeable to ions; decreasing the time it takes the protons to flood the cytoplasmic portion of the membrane.

Survival of wt *S. flexneri* treated with 10 and 20 µM CCCP (Fig 3.2a) is comparable to the acid challenge results of 2.5 µM treated *hya* cells (Fig 3.2b). A 100 and 10,000-fold difference in cell recovery was observed in 10 and 20 µM, respectively. In the *hya* mutant at 2.5 µM a 10,000-fold difference in cell recovery was observed after 120 minutes. However what is evident is that higher concentrations of the protonophore are needed to affect the acid sensitivity of the wt strain. At 5 µM CCCP there is little effect on sensitivity of wt while in *hya* this treatment results in zero cell recovery. This result strengthens the argument that the contribution to acid resistance is in part due to a functional Hya hydrogenase, and that Hya's role in production of Δψ is correlated with acid resistance.

It has been shown in the enteric bacteria *Yersina* that activation of a type III secretion system is dependent on a pmf (3, 10). *Shigella* requires a type III secretion system to deliver effector proteins into host cells for successful uptake and dissemination. It is hypothesized that the lack of a pmf in the *hya* mutant could have an effect on this strain's ability to cause infection in the host.

The Hya hydrogenase's enzymatic role is to oxidize molecular hydrogen into protons and electrons. These acid challenge experiments with CCCP have shown that the maintenance of a membrane potential is paramount in aiding acid survival for *S.*

flexneri. The addition of CCCP to wild type *Mycobacterium smegmatis* to pH 5 also resulted in acid sensitivity. Treatment with CCCP creates pores in the bacterial cytoplasmic membrane. This study has helped define an additional biochemical role of *S. flexneri* Hya in acidic conditions. The knowledge gained can transfer into innovative medical strategies to control *S. flexneri* infection.

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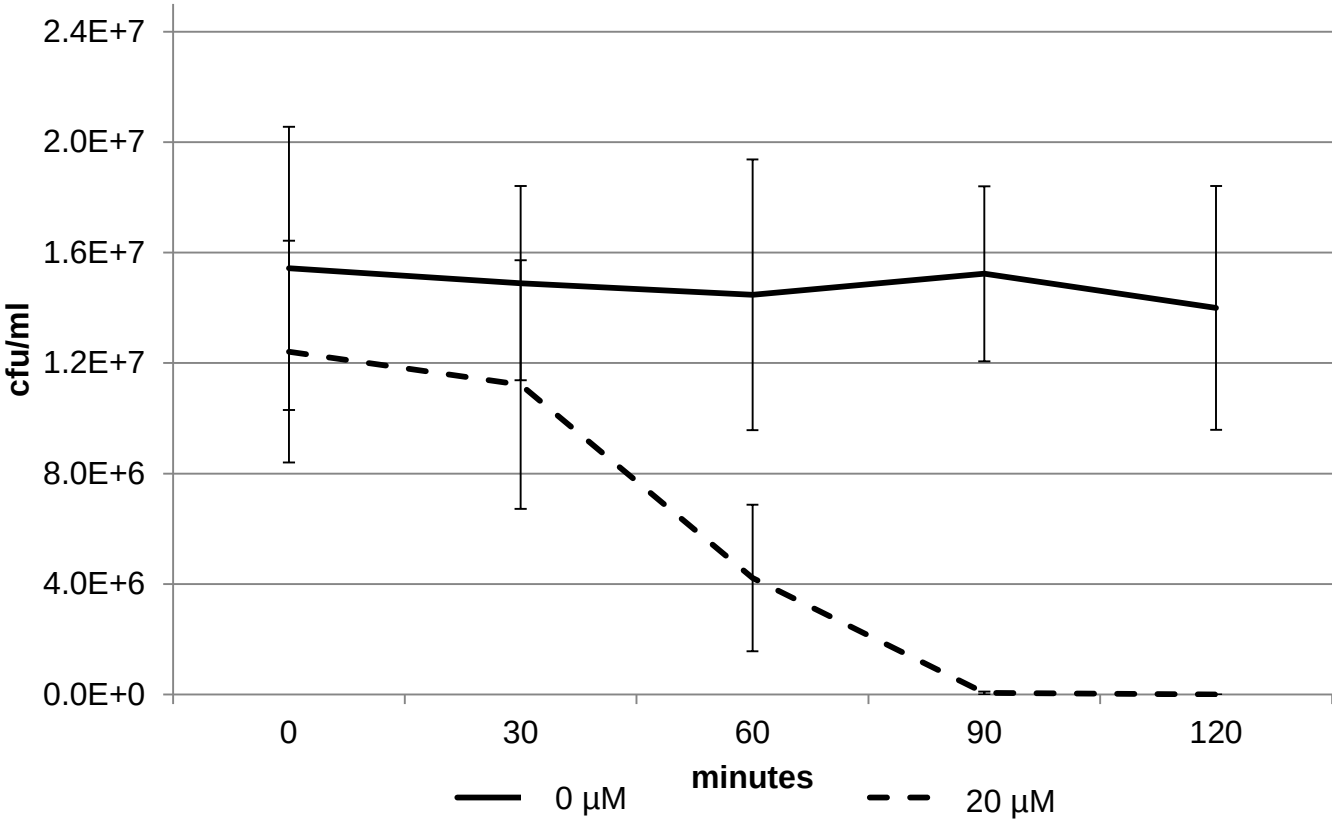
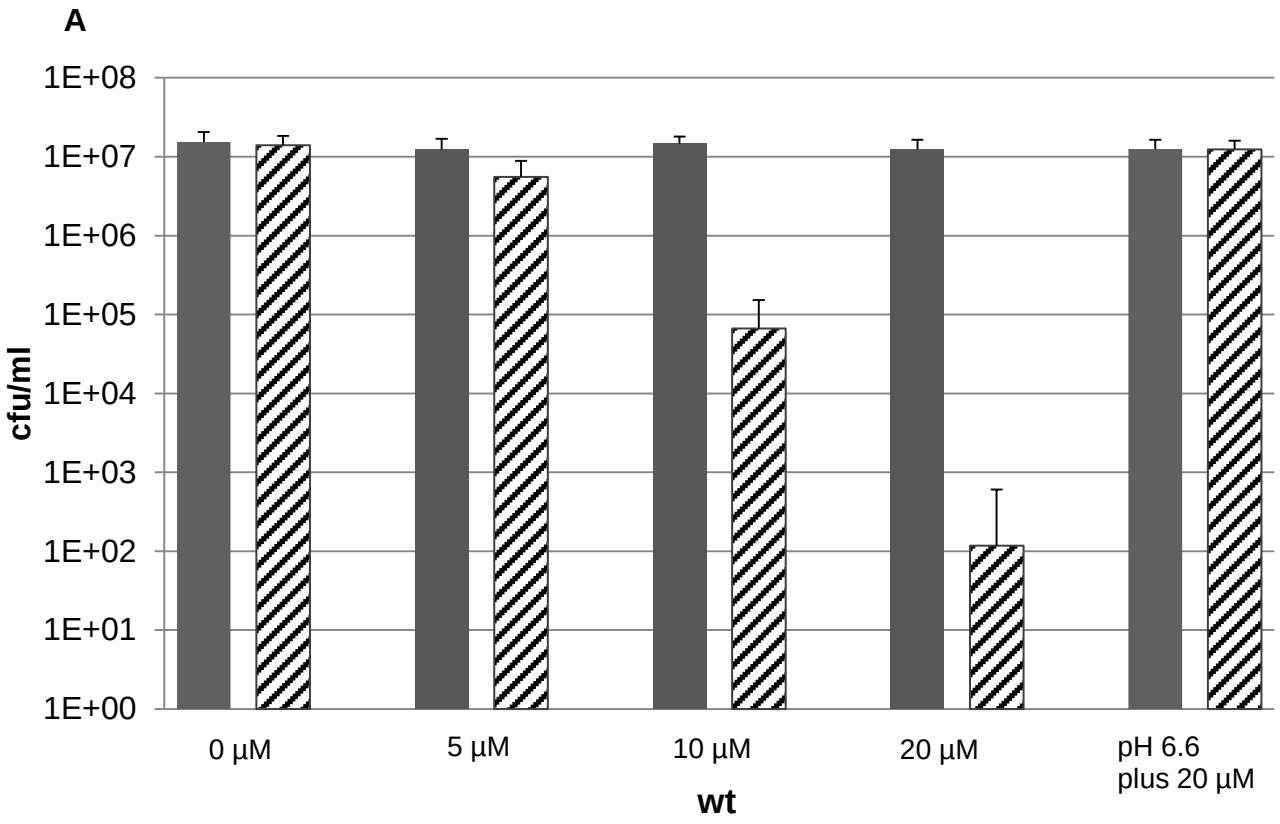


Figure 3.1. Effect of CCCP on viability of wild type *S. flexneri*. The strain was treated with CCCP in anaerobic conditions (85% N_2, 10% H_2, 5% CO_2) at pH 2.5. Solid line (0 µM) and dashed line (20 µM). The cultures were serially diluted at the indicated times. Means and standard deviations represent a composite of 5 independent experiments. Significance of treatment with 20 µM CCCP compared to untreated wt cultures: $p<0.003$ at t=30 minutes and $p<0.0001$ at t= 60, 90, 120 minutes.


A
1E+08
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cfu/ml
0 µM
5 µM
10 µM
20 µM
pH 6.6
plus 20 µM
wt

B

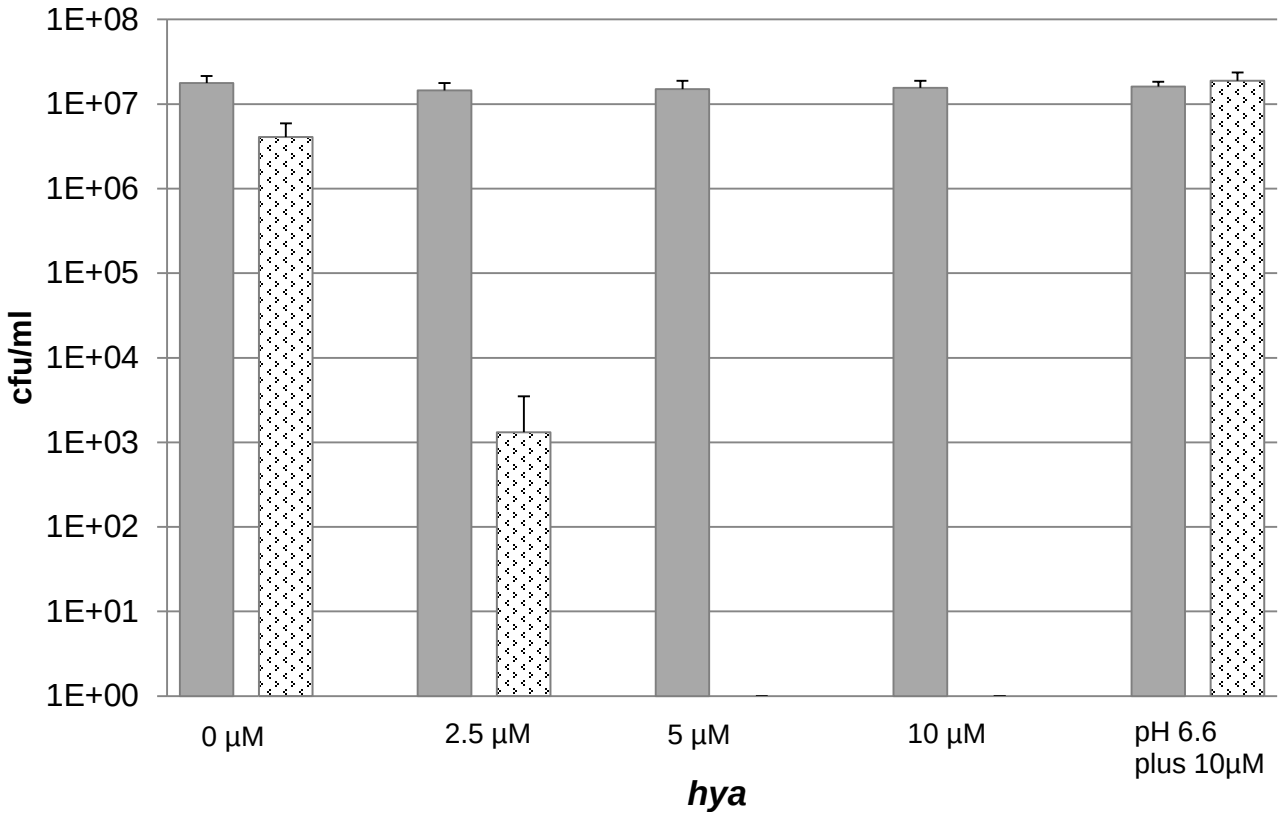

Figure 3.2. Viability comparisons upon CCCP treatment at 0 min versus 120min. WT (A) and *hya* (B) were acid challenged (pH 2.5) for 120 minutes in anaerobic conditions (85% N_2, 10% H_2, 5% CO_2) with CCCP added at designated concentrations. 0 and 120 minutes are represented by solid and patterned bars respectively. *Hya* treated with 5 and 10µM could not be recovered at 2 hours post inoculation. Means and standard deviations are a representative of 5 independent experiments

CHAPTER 4

PROTEOMIC ANALYSIS OF PROTEIN EXPRESSION IN A *SHIGELLA FLEXNERI HYA* MUTANT

ABSTRACT

Expression of bacterial genes and the subsequent protein products are normally dependent on environmental factors and the availability of substrates. Peptide fingerprinting was used to analyze protein expression in a *S.flexneri hya* mutant strain. Global changes in protein expression were identified upon with the deletion of the respiratory hydrogenase Hya. Proteins that increased in expression compared to the H_2-oxidizing parent strain were of the fatty acid biosynthesis, energy generation, amino acid biosynthesis and pyridoxal 5'-phosphate (PLP) biosynthesis and salvage pathway groups. Proteins involved in synthesis of glycine, cysteine, lysine, tryptophan, and isoleucine were decreased in expression. In addition, SodB was decreased in expression in the *hya* strain. We conclude that the deletion of the Hya hydrogenase causes significant metabolic disruption and an increase in expression of growth related proteins that compensate for the loss of the active enzyme.

INTRODUCTION

Comparative proteomic expression studies can provide a general view of metabolism and alterations to the metabolic pathways between bacterial strains. This approach can

be beneficial when formulating new research projects and hypotheses because of the amount of data generated (1). These analyses can also help determine biological and metabolic roles of specific proteins in an organism. Today many techniques including microarray technology, RNA-seq, two-dimensional gel electrophoresis or peptide fingerprinting can be used to analyze transcriptome or proteome expression profiles (10, 30, 35). Peptide fingerprinting was the chosen method for global protein expression.

Peptide fingerprinting is done in multiple steps. First the cultures are fractionated into desired groups, and then subjected to SDS-PAGE (1). Trypsin is used to digest the proteins into their unique peptides fragments. From there the fragments are separated and charged for subsequent mass spectrometry (MS/MS). The resulting mass spectra of the peptide fragments are further analyzed to determine the amino acids sequence (30). A database (biocyc.org) of polypeptides can then be used to identify proteins. Statistical measurements are done using peptide sequence proteomic software.

Comparative analysis of the wild type and *hya* mutant was done to determine if the deletion of the uptake hydrogenase would have a global effect on protein expression in late exponential phase. Previously, it was shown that deletion of Hya affected the ability of *S. flexneri* to produce a membrane potential (24). Membrane potential is the electrical component of the proton motive force, which contributes to ATP generation in the bacteria. Respiratory hydrogenases in *Salmonella enterica* serovar Typhimurium have been shown to facilitate uptake of amino acids (18). Therefore, it was predicted that the most affected proteins in the *hya* mutant would be involved in energy generation and transport.

METHODS

Wild-type (wt) strain *Shigella flexneri* 2a 2457T (ATCC 700930) was used. The *hya* mutant was previously constructed in (24). Growth was performed in Luria-Bertani Broth (LB) in 165 ml serum stopper-sealed vials. Total liquid volume was 20 ml and the atmosphere contained anaerobic mix (10% H_2, 5% CO_2, 85% N_2). Initial cell concentrations were measured at OD_{600} ~0.02. Thereafter, the vials were placed in 37°C shaking at 200 rpm. 1 ml aliquots from each vial were measured spectrometrically at 600nm at indicated times.

S. flexneri protein lysate samples were then prepared as follows: *S. flexneri* was grown in saturated anaerobic conditions (85% N_2, 10% H_2, 5% CO_2) at 37°C (shaking, 200 rpm) to late exponential phase. The anaerobic environment was maintained in a 2000 mL closed top flask. Glucose was added at 0.4% g/vol to 250 mL LB broth. Cultures were then pelleted using centrifugation (4°C, 6000rpm). Pellets were washed with PBS followed by addition of 1mM PMSF (phenylmethylsulfonyl fluoride) to prevent protease activity. Total cell lysate was separated into cytoplasmic and membrane fractions using sonication, and ultra centrifugation (4°C, 3 hours, 60000 rpm). Protein quantification was done on both fractions using BCA assay. 15 µg of protein was loaded on to a precast 4-15% gradient gel (Bio-Rad, Criterion TGX). The protein gel was then stained using Coomassie Blue.

The protein gel was divided into 12 sections of 6 cm in length for excision (Fig 4.1). Each division was cut into 1 mm cubes, and placed in a microcentrifuge tube. The gel bands were washed using 50% acetonitrile and 100 mM ammonium bicarbonate.

After dehydration, 10 mM DTT/100 mM ammonium bicarbonate was added to remove disulfide bonds. 55 mM iodoacetamide/100 mM ammonium bicarbonate was added to reduce the sulfhydryl to S-carboxyamidomethyl-cysteine which cannot be reoxidized (12). Trypsin (Trypsin Gold, Promega) was added in a 1:15 trypsin to protein ratio to the washed gel cubes to digest the proteins. After digestion, peptide fractions were dried and stored at -80°C until proteomic analysis. Proteomic analysis was performed using a Finnigan LTQ Linear Ion Trap Mass Spectrometer. ProteoIQ software was used to compare relative protein abundance in the *hya* mutant with wild type as a reference group. Proteins increased or decreased log 2 compared to wild type were selected for analysis (19, 29).

RESULTS

Peptide fingerprinting and subsequent analysis of relative protein abundances revealed differences in protein expression in the *hya* mutant compared to the wild type strain. It was expected that the absence of a PMF would result in differences in energy producing proteins and transporters requiring PMF. While no growth defects were observed in the *hya* mutant (Fig. 4.2), an increase in these types of proteins could compensate for the lack of electrical potential energy in the form of a PMF. These alterations in protein expression can explain the *hya* mutant's ability to grow at similar rates to the wild type strain.

Glycolytic Proteins

Proteins that produce intermediates which are fed into the energy generating glycolysis pathway were increased in the *hya* mutant (Fig. 4.3; Table 4.1). 6-phosphofructokinase I

(PfkA) and fructose-bisphosphate aldolase (Fba) can drive metabolic reactions where the products feed into the so called “energy investment phase” of glycolysis or the phase where an input of energy in the form of ATP is needed for activation (31) (Fig. 4.3). PfkA drives the metabolism of fructose 1, 6-bisphosphate from the products ATP and D-fructose-6-phosphate (26). Fba yields fructose 1, 6-bisphosphate or D-glyceraldehyde-3-phosphate, precursors to pyruvate biosynthesis. Phosphoglycerate kinase (Pgk) catalyzes the energy producing reaction of 1, 3-bisphospho-D-glycerate and ADP to yield 3-phospho-D-glycerate and ATP. Products from Pgk can be further converted to pyruvate (2).

Anaerobic respiration and other glucose oxidizing/anabolic pathways

It was not surprising to see alterations in anaerobic respiratory enzymes because the present proteomic expression study was done using anaerobically grown strains. Nitrate reductases, NarG and NapA (Table 4.5) were decreased in protein expression. In *E.coli* it was shown that *narG* was maximally expressed in elevated levels of nitrate; while *napA* was expressed in low nitrate conditions (37). The results of low protein abundance of these nitrate reductases indicate that the *hya* mutant will not preferentially use nitrate as an electron acceptor in anaerobic respiration.

Other proteins decreased in expression included D-lactate dehydrogenase (LdhA) and phosphoenolpyruvate carboxykinase (PckA) involved in mixed acid fermentation and gluconeogenesis, respectively (Fig. 4.4; Table 4.5). *Shigella flexneri* can perform mixed acid fermentation in absence of an electron acceptor. The conversion of pyruvate to lactate consumes a reducing equivalent (NADH).The *hya*

mutant may need to conserve this energy source in absence of the electrons that Hya could provide to the bacterium. It is still not clear as to why the *hya* mutant would show a difference in expression of these proteins. We hypothesize that the mutant conserves energy substrates in the form of ATP and NADH. This is supported with the decreased expression of LdhA and PckA. LdhA consumes NADH and PckA uses ATP to catalyze its metabolic reaction.

Pentose Phosphate Pathway

The pentose phosphate pathway (PPP) is an alternative pathway in which glucose can be partially oxidized to produce reducing equivalents and anabolic metabolites. Transaldolase B (TalB; Fig.4.3; Table 4.1) is involved in the non-oxidative branch of the PPP (36). TalB favors production of D-fructose-6-phosphate and D-erythrose-4-phosphate (14). D-erythrose-4-phosphate can be converted back into D-glyceraldehyde-3-phosphate (glycolysis metabolite).

Vitamin B_6 metabolism

The cofactor pyridoxal 5'-phosphate (PLP) the active form of Vitamin B_6 is required for transamination and decarboxylation of amino acids (32). Pyridoxal phosphate biosynthetic protein (PdxJ; Fig. 4.5; Table 4.2) was increased in expression in the *hya* mutant. This protein produces pyridoxine-5' phosphates (PNP), H_2O, and H^+ from 1-deoxy-D-xylulose 5-phosphate and 3-amino-1-hydroxyacetone-1-phosphate. PNP can later be converted to PLP. The biosynthesis of this vitamin can indicate the necessity to breakdown amino acids into citric acid cycle substrates.

Fatty acid biosynthesis

3-oxoacyl-[acyl-carrier-protein] synthase I (FabB), 3-oxoacyl-[acyl-carrier-protein] synthase III acetyl CoA ACP transacylase (FabH), and enoyl-[acyl-carrier-protein] reductase (NADH) (FabI) were increased in expression compared to the wild type strain (Fig. 4.6; Table 4.3). These proteins are involved in biosynthesis of fatty acids which are necessary for maintenance of a fluid membrane. Fatty acids also contribute to lipid A synthesis, vitamin derived molecules, and membrane proteins (22). FabB is involved in the anaerobic biosynthesis of unsaturated fatty acids (33). FabI and FabH are involved in the synthesis of saturated fatty acids. Both FabB and FabI are important in fatty acid chain elongation control (22). All three proteins are involved in palmitate biosynthesis, a straight-chain fatty acid (15). A significant portion of the bacterial membrane contains palmitate, which can also be found in eukaryote membranes.

Amino acid biosynthesis and related proteins

Alterations to several amino acid biosynthetic pathways were observed in the *hya* mutant. Relative protein abundance compared to wild type was 4.6 fold more for 3-phosphoserine aminotransferase (SerC) in the mutant. SerC can catalyze reactions to produce serine, glycine, or cysteine as an end product (Fig. 4.7; Table 4.4) (6). Several proteins involved in amino acid biosynthesis were decreased in expression in the *hya* mutant (Table 4.6). Among those decreased in expression are cysteine synthase A (CysK) and serine hydroxymethyltransferase (GlyA) leading to cysteine and glycine synthesis, respectively (Fig. 4.7; Table 4.6). Both amino acids share the requirement of

a serine precursor for amino acid biosynthesis. Dihydroxyacid dehydratase (IlvD) is involved in isoleucine synthesis which uses glutamate as a metabolite in its biosynthetic pathway. Tryptophan synthesis was also limited in the *hya* mutant by the decrease in protein abundance of the trp repressor binding protein (WrbA).

In addition to alterations in amino acid biosynthetic pathway a marked increase in the *hya* mutant glutamate tRNA synthetase (GltX) was observed (Table 4.4). GltX transports a glutamate molecule to a peptide chain and is necessary for proper translation of nucleotide sequences to proteins (5, 8). Biosynthesis and degradation of nucleotides and proteins had altered expression in the *hya* mutant. Guanine-hypoxanthine phosphoribosyltransferase (Gpt) (Table 4.7) was decreased in expression in the *hya* mutant. Gpt can salvage purines for nucleotide biosynthesis (27). Aminoacyl-histidine dipeptidase (PepD) (Table 4.4) can remove an N-terminal amino acid usually from carnosine, a dipeptide (3). PepD was minimally increased in protein abundance of the *hya* mutant compared to the wild type. Though the biochemical role of PepD has not been characterized in *Shigella* yet, it is hypothesized that it is involved in protein degradation and cell cycle control (3, 4).

Detoxification Protein

Among the proteins that were low in abundance in the *hya* mutant is superoxide dismutase (SodB, Table 4.7). SodB was of most interest because of its importance in *S. flexneri* infection. This protein detoxifies the superoxide environment that *S. flexneri* would encounter in macrophage engulfment. Previously it had been shown that SodB

contributes to *S. flexneri* pathogenicity (9). A *sodB S. flexneri* mutant was unable to cause damage to the intestinal cells in a rabbit ileal loop animal model (9).

DISCUSSION

Data from the present study support a route for energy generation in the absence of a PMF in the *S. flexneri hya* mutant. Glycolytic proteins including Pgk can catalyze the production of 2 ATP and 3-phospho-glycerate. Substrate-level phosphorylation in this reaction can bypass the lack of protons available to drive ATP synthesis via the ATP-synthase. The lack of reducing equivalents from the inactivation of Hya is apparently compensated for by the activity of the PPP. This pathway can produce the reducing product NADPH which *Shigella* uses while in the anabolic stages of growth (16).

The data presented herein support multiple routes of serine production and conservation. 3-phospho-glycerate produce by Pgk (Fig. 4.3) can be used as a substrate for serine biosynthesis. An increase in protein abundance of the SerC (Fig. 4.7) protein involved in serine production was observed in the mutant. In addition, proteins involved in the synthesis of serine requiring amino acids were decreased in expression.

Why is it that the *hya* mutant builds up and conserves serine pools? We can infer that serine's involvement in diverse metabolic pathways requires copious pools of this amino acid for wild type level of growth. Serine can be a substrate for pyruvate biosynthesis via a deamination event (28). SerC can provide the intermediate 4-phospho-hydroxy-L-threonine in PLP biosynthesis (17). PLP is the active form of

vitamin B_6 which is an important cofactor for metabolic activity in bacteria. The deamination of serine to pyruvate is catalyzed by L-serine dehydratase (SDH) a PLP dependent enzyme (34). This allows for the *hya* mutant to use serine to feed into the TCA in presence of an anaerobic electron acceptor. Biosynthesis of serine requires conversion of glutamate to oxoglutarate. Oxoglutarate (α-ketoglutarate) is an intermediate in the citric acid cycle. This product can be converted into succinyl CoA (NADH produced) and then succinate with 1 ATP produced.

Previously, we determined the amino acid pools of both the wild type and *hya* mutant (chapter 2). This method is described in chapter 2 pg. 28. Serine concentrations were approximately 17 and 35 nmole per 10^8 cfu for the wild type and *hya* mutant, respectively. The by-product of serine deamination, ammonia was elevated in the *hya* mutant at 440 nmole compared to the wild type at 324 nmole per 10^8 cfu. Glutamate is used as a substrate for serine biosynthesis and this was also in higher concentrations in the mutant compared to the wild type 16 and 3 nmole 10^8 cfu respectively. There is some indication that the *hya* mutant is maintaining serine pools for metabolic activity.

The amino acid serine is also utilized in *E.coli* phospholipid synthesis (6). The pentose phosphate pathway is used when the bacterium is in an anabolic state, thus the production of reducing equivalents that the bacterium can use to build cell membranes. TalB can contribute to fatty acid biosynthesis via the PPP (D-fructose-6-phosphate product) (Fig 4.3) or to vitamin B_6 synthesis by providing D-erythrose-4-phosphate (Fig. 4.5). In addition to serine pools, glutamate appears to play a role in the *hya* mutant's

growth. The increase in relative protein abundance of the glutamate tRNA synthetase infers a requirement of glutamate for protein synthesis.

The proteomics data of the present study was compared to a previously reported protein expression analysis of *S. enterica* (7). In this study *S. enterica* was grown in anaerobic conditions. The results were comparable to the *S. flexneri hya* mutant (7) protein expression profiles. The shifts in protein expression between the anaerobic and aerobic cultures were compared (7). An increase in glycolysis related proteins including Pgk was observed in the anaerobically grown culture. Also TalB involved in the PPP pathway was increased in that same culture. The need for an alternative carbon energy source was apparent in *S. enterica* as evidenced by the increase in AspA (aspartate ammonia-lyase) and AspC (aspartate aminotransferase) (7). AspC requires PLP as a cofactor and converts glutamate to aspartate (11). *Salmonella* cannot directly use aspartate as a carbon source but the production of fumarate via AspA allows for some carbon to be utilized (13, 23). In *Shigella*, there was no indication of a protein abundance difference between the wild type and the *hya* mutant in proteins involved in aspartate biosynthesis. However, similar to *Salmonella*'s use of aspartate, the *hya* mutant can use serine to serve as an energy precursor.

Conclusion

This work suggests that the deletion of the Hya hydrogenase renders a bacterium that increases energy producing proteins and decreases large energy consuming pathways. ATP generating Pgk and the precursor to pyruvate, and D-glyceraldehyde-3-phosphate (Fba) were both increased in expression in the *hya* mutant. The *hya* mutant had a

preference for serine which can be used as a substrate for pyruvate metabolism. Accordingly, proteins that use serine as a precursor to produce other amino acids were decreased in expression. While Hya is not essential, the organism must find other pathways to produce ATP and reduced carriers. With a functioning Hya the protons produced could be used to drive ATP synthesis via the ATP synthase or the electrons could be donated to carriers to contribute to pmf. Future work will involve further analysis of the roles of these proteins in the *hya* mutant metabolic pathways including additional biological replicates, RNA transcript analysis, and enzymatic assays. Nevertheless, these studies of altered protein expression in the *hya* mutant compared to the wild type have added another piece to the understanding of the contribution of the Hya hydrogenase to *S. flexneri* metabolism.

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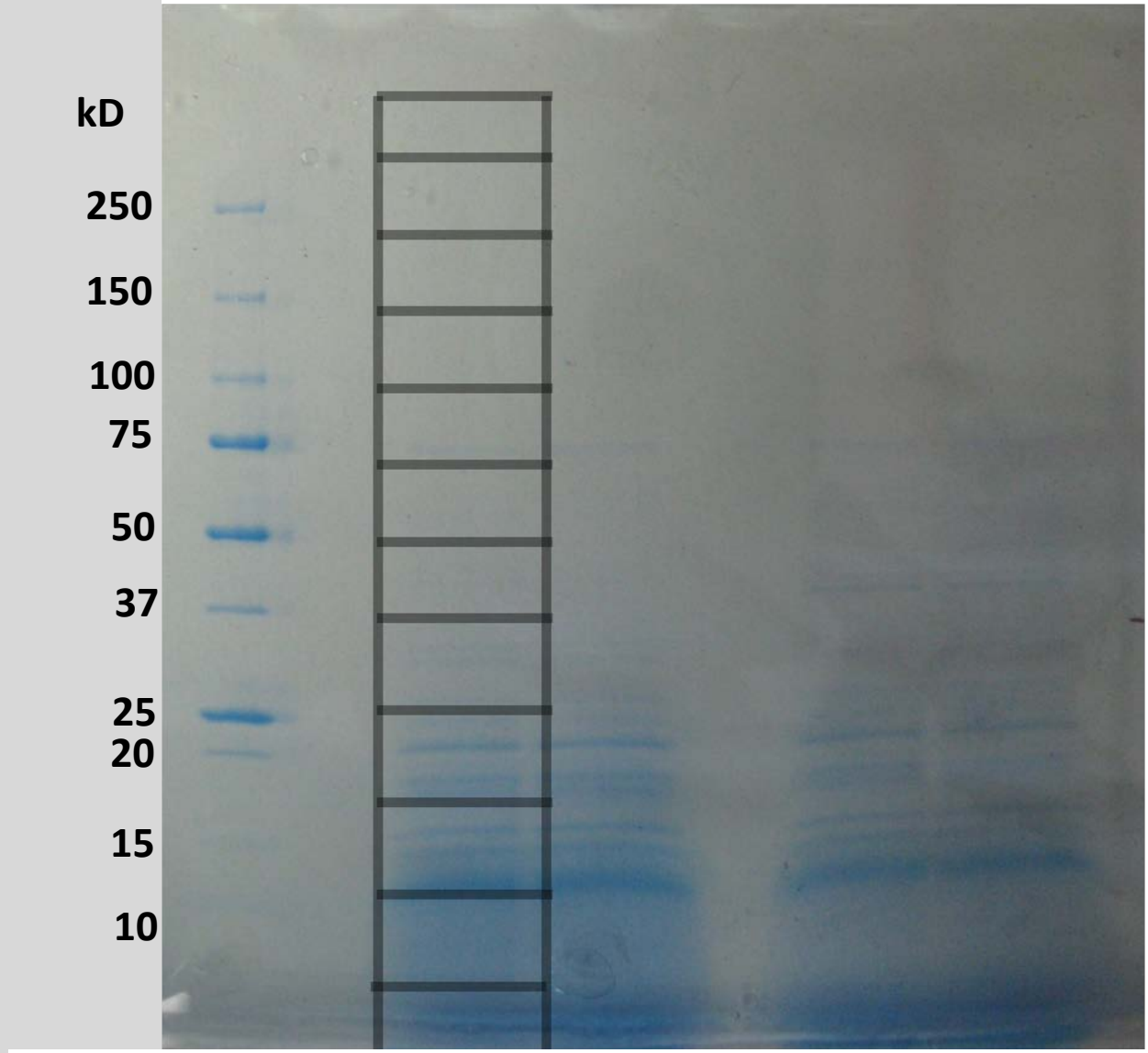

Figure 4.1 Polyacrylamide gel of *S. flexneri* membrane proteins. Proteins were stained with Coomassie Blue. Gray segments indicate portions that were cut into 1 mm cubes for subsequent dye removal and trypsin digestion.

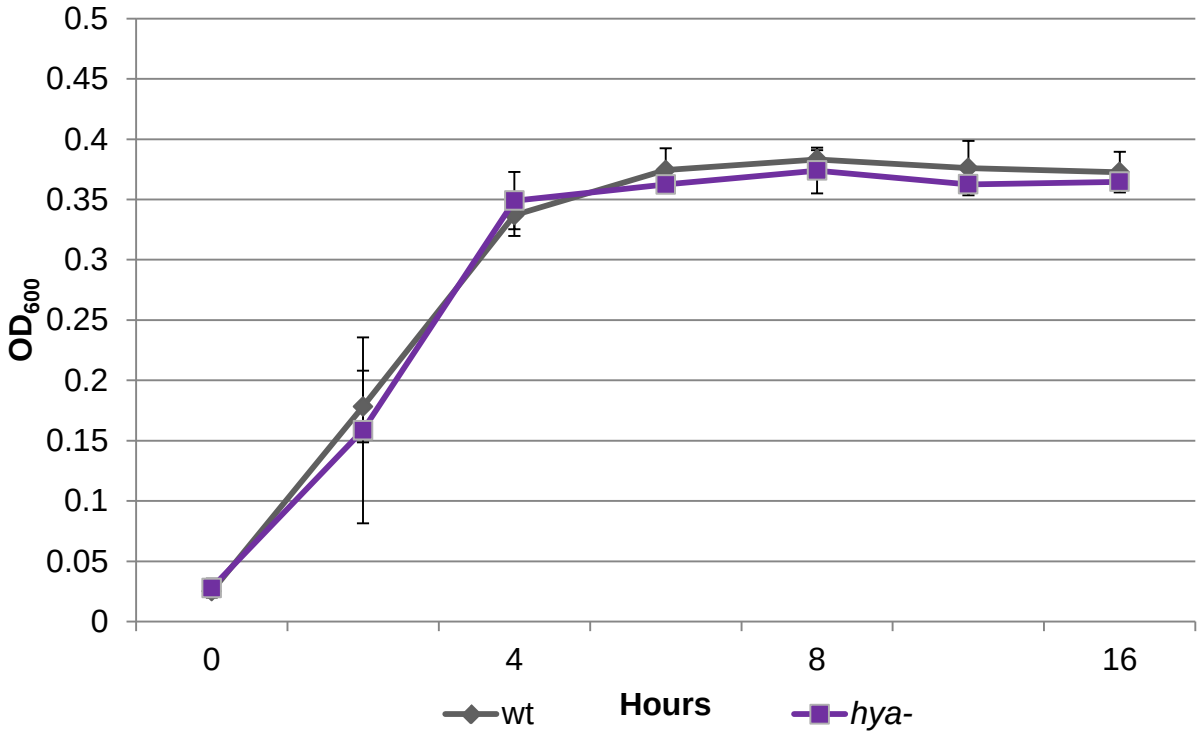

Figure 4.2 Anaerobic growth curve of wt and *hya* mutant. Strains were grown in Luria Bertani broth. Anaerobic conditions (85% N_2, 10% H_2, 5% CO_2) were maintained in 70 ml closed bottles. Means and standard deviation is a compilation of 3 independent experiments.

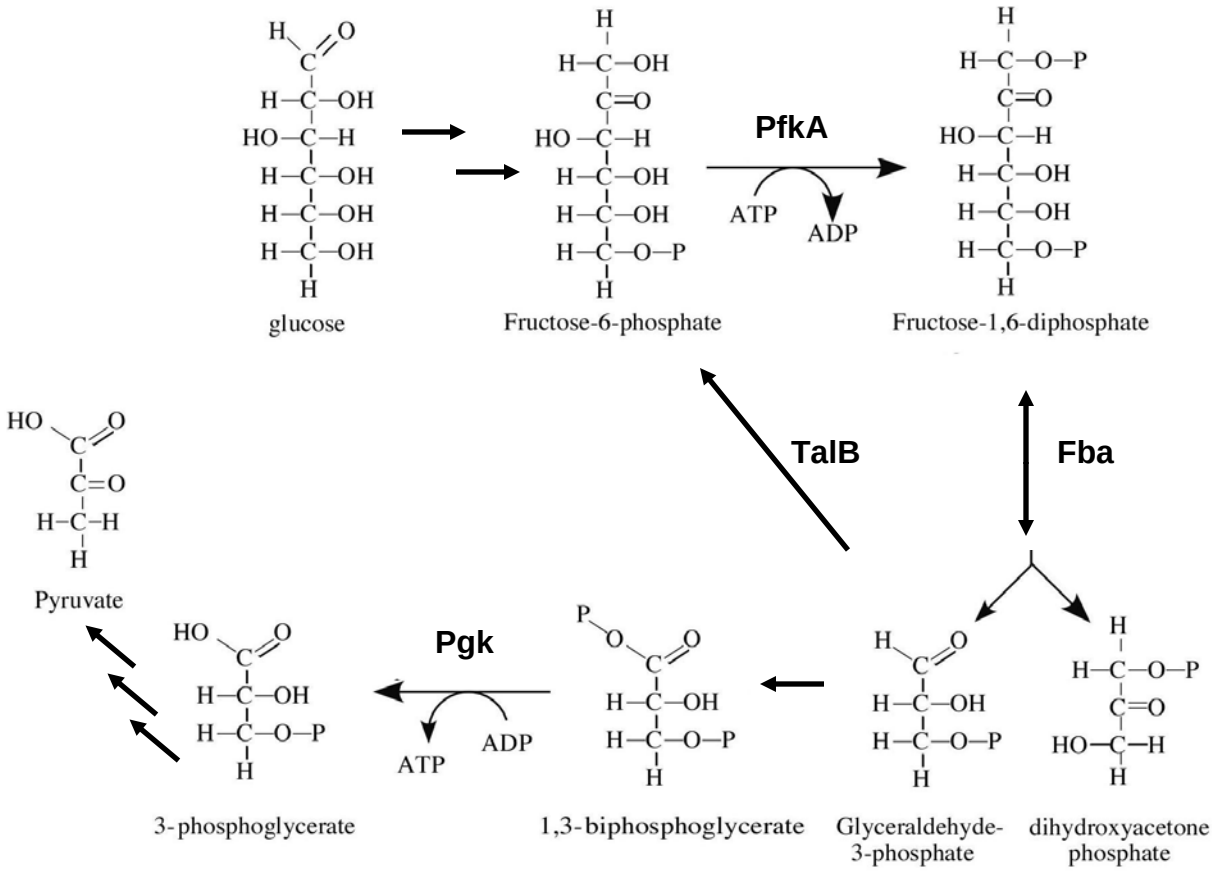

Figure 4.3 Glycolysis pathway. Glycose oxidation facilitated by glycolytic (PfkA, Fba, and Pgk) and pentose phosphate pathway protein (TalB); which were found to be increased in expression in the *hya* mutant in comparison to the wild type. Figure adapted with permission from Dr. José Bonner (http://courses.bio.indiana.edu/L104-Bonner/)

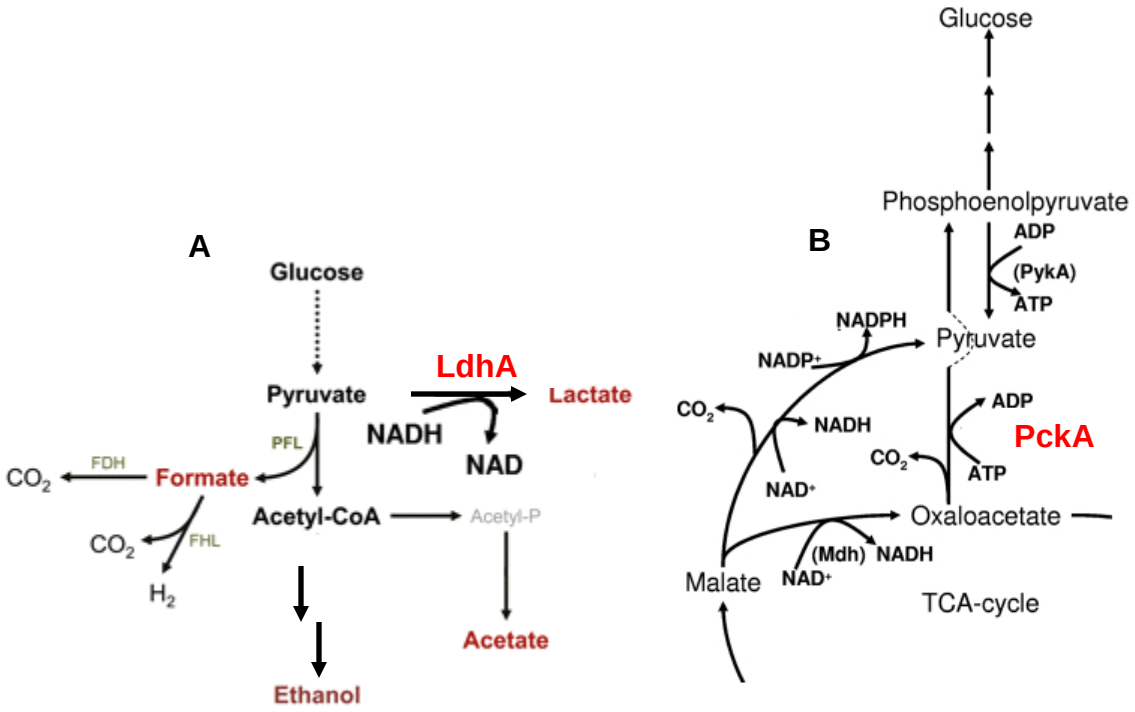

Figure 4.4 Mixed acid fermentation and gluconeogenesis pathways. Proteins in red (LdhA and PckA) indicates decrease in protein abundance in the *hya* mutant. Mixed acid fermentation (A) figure adapted from Lü *et al*., (21). Gluconeogenesis schematic adapted from Mulley *et al*., (25).

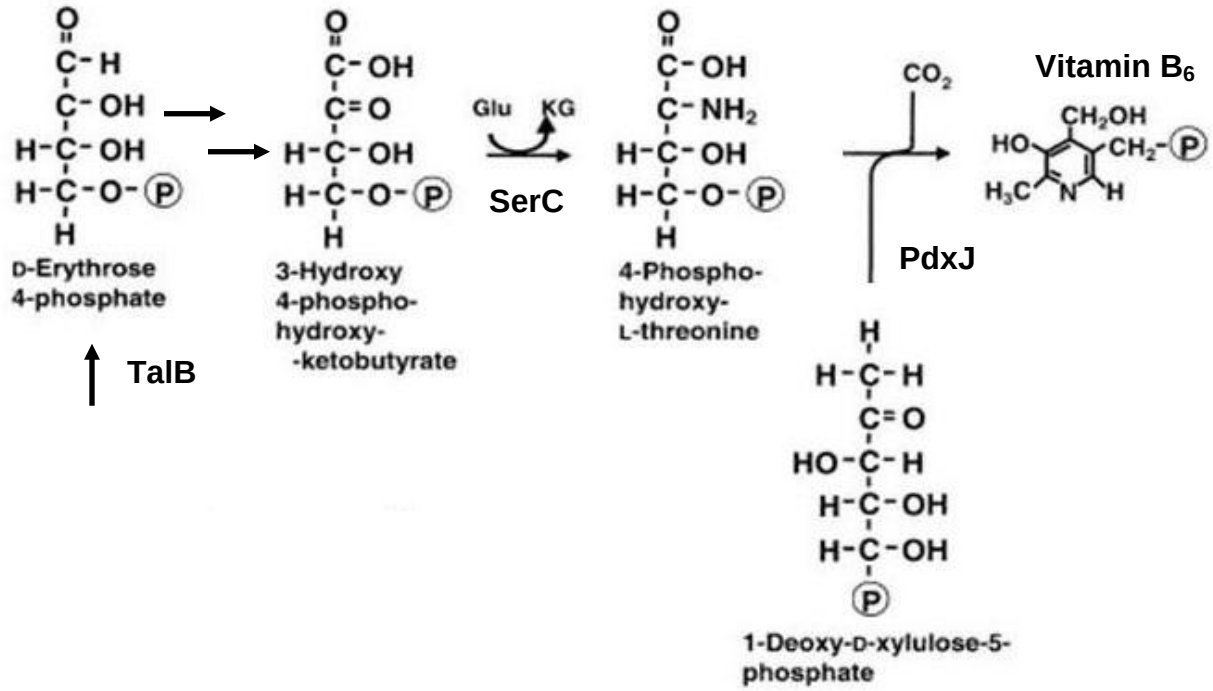

Figure 4.5 Vitamin B_6 synthesis. Labeled proteins leading to de novo synthesis of pyridoxal 5'-phosphate (vitamin B_6) were increased in expression in the *hya* mutant. Figure adapted from Sakai *et al*., (32).

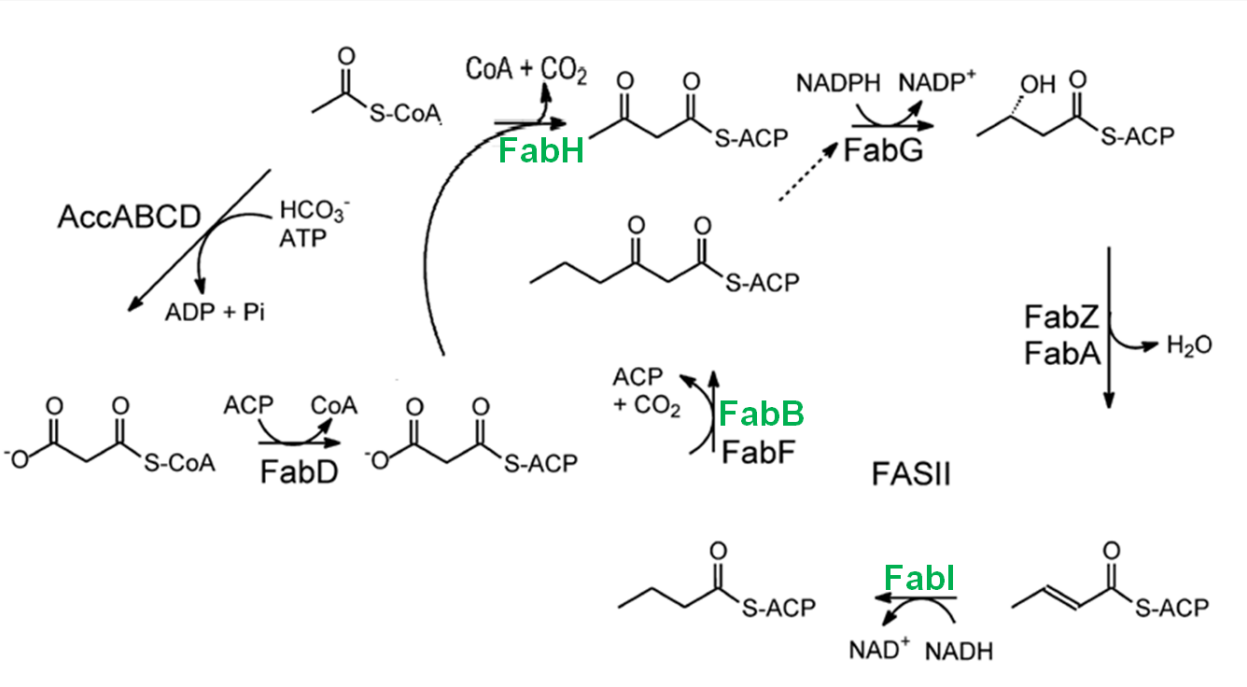

Figure 4.6 Fatty acid biosynthesis in bacteria. Schematic adapted from Lu and Tonge, (20). Proteins in green (FabB, FabH, FabI) were increased in abundance in the *hya* mutant. FASII: fatty acid biosynthesis pathway (II-bacterial).

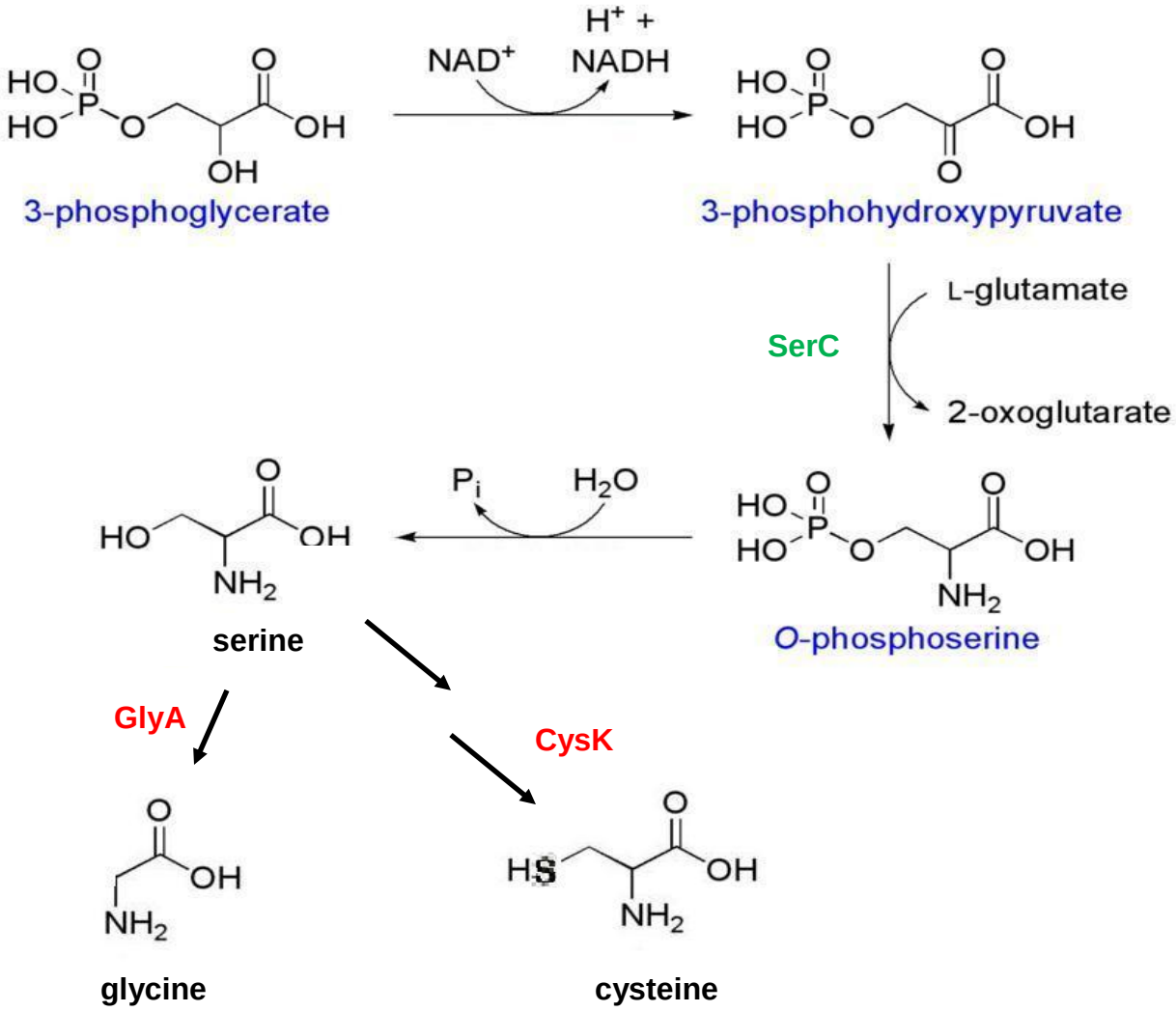

Figure 4.7 Serine biosynthetic pathway. Serine metabolism including abbrevaiated pathways for glycine and cysteine biosynthesis. Green colored protein(SerC) indicates an increase in protein abundance. Red colored proteins (GlyA and CysK) indicate a decrease in protein abundance in the *hya* mutant. Image adapted with permission from Calvero at http://commons.wikimedia.org/wiki/File:Serine_biosynthesis.png.

Proteins increased in expression

Fold change = relative protein abundance

Table 4.1 Energy generation

Gene number	Description	Fold Change
S3111	phosphoglycerate kinase (***pgk***)	8.6
S3110	fructose-bisphosphate aldolase, class II (***fba***)	5.9
S3753	6-phosphofructokinase I (***pfkA***)	5.0
S0008	transaldolase B (***talB***)	3.0

Table 4.2 Salvage of substrates

Gene number	Description	Fold Change
S2799	pyridoxal phosphate biosynthetic protein (***pdxJ***)	5.7

Table 4.3 Fatty acid biosynthesis

Gene number	Description	Fold Change
S1375	enoyl-[acyl-carrier-protein] reductase (NADH) (***fabI***)	4.3
S2534	3-oxoacyl-[acyl-carrier-protein] synthase I (***fabB***)	3.5
S1175	3-oxoacyl-[acyl-carrier-protein] synthase III acetyl CoA ACP transacylase (***fabH***)	3.0

Table 4.4 Amino acid associated factors

Gene number	Description	Fold Change
S2605	glutamate tRNA synthetase, catalytic subunit (***gltX***)	15.0
S0966	3-phosphoserine aminotransferase (***serC***)	4.6
S0306	aminoacyl-histidine dipeptidase (peptidase D) (***pepD***)	2

Proteins decreased in expression

Table 4.5 Anaerobic respiration and other oxidizing/anabolic pathways

Gene number	Description	Fold Change
S1311	nitrate reductase 1 alpha subunit (***narG***)	-4.0
S4341	phosphoenolpyruvate carboxykinase (***pckA***)	-3.9
S2420	probable nitrate reductase 3 (***napA***)	-2.9
S1459	fermentative D-lactate dehydrogenase (***ldhA***)	-2.1

Table 4.6 Amino acid biosynthesis

Gene number	Description	Fold Change
S0030	dihydrodipicolinate reductase (***dapB***)	-5.0
S3913	dihydroxyacid dehydratase (***ilvD***)	-2.5
S2615	cysteine synthase A, O-acetylserine sulfhydrolase A (***cysK***)	-2.3
S2770	serine hydroxymethyltransferase (***glyA***)	-2.0
S1077	trp repressor binding protein (***wrbA***)	-2.0

Table 4.7 Miscellaneous proteins

Gene number	Description	Fold Change
S1816	superoxide dismutase (***sodB***)	-8.1
S0307	guanine-hypoxanthine phosphoribosyltransferase (***gpt***)	-8
S4563	GroES, 10 Kd chaperone (***mopB***)	-3.0

CHAPTER 5

DISSERTATION SUMMARY AND FUTURE DIRECTIONS

Dissertation Summary

Shigella flexneri is an important enteric bacterium because of its ability to cause shigellosis with ingestion of just a few organisms (1). Increasing incidence of antibiotic resistance and lack of an effective vaccine has made *Shigella* prevention a priority in many countries (8). Study of the hydrogenases was done because of the enzymatic role of molecular hydrogen oxidation in other pathogens. It has been shown in *S. Typhimurium* that deletions of all H_2-oxidizing hydrogenases produced an avirulent strain (14).

The current study has shown that the Hya hydrogenase in *S. flexneri* contributes to acid resistance. *S. flexneri* has an additional uptake hydrogenase called Hyb. However, acid resistance assays with the *hyb* deletion did not render an acid sensitive mutant. Hydrogenase activity assays with the hydrogen evolution (Hyc) mutant showed that Hya is the active enzyme in acidic conditions. Also, in absence of Hya, the strain evolved H_2 connecting the recycling of H_2 generated by Hyc to Hya (15, 22).

Additional studies using the fluorescent dye JC-1 demonstrated that the deletion of *hya* correlated with undetectable levels of membrane potential. To further support

these findings wild type *S. flexneri* was challenged in pH 2.5 with the protonophore CCCP. This treatment resulted in an acid sensitive strain, thus inferring that Hya is important for energy generation, particularly in acid conditions.

A global survey of the *hya* mutant‘s protein expression was analyzed using peptide fingerprinting. The *hya* mutant was grown to late exponential phase, since this is when the bacterium was transferred to pH 2.5 in the acid challenge assays. An increase in protein expression of fatty acid and amino acid biosynthesis as well as key enzymes in glycolysis were up expressed in the *hya* mutant.

My findings support the hypothesis that the utilization of hydrogen is an important metabolic process for optimal function of *S. flexneri*.

FUTURE DIRECTIONS

In-depth analysis of global protein changes in the *hya* mutant

Chapter 4 (pg. 60) describes the proteomic expression data set of the wild type and *hya* mutant strains. Alterations in protein expression were observed in the *hya* mutant compared to the wild type strain. Additional biological replicates are necessary to support the initial data set and to calculate significance values. Peptide fingerprinting and subsequent analysis using ProteoIQ gives relative protein abundance in the strains. The overview of proteomic changes can be the basis of studies focusing on proteins that exhibited alterations to their expression relative to the wild type strain.

Transcriptional analysis of glycolytic proteins in the *hya* mutant

The *hya* mutant was hypothesized to show expression profile changes in energy generation proteins, because of its inability to produce a pmf (Ch. 4). The proteomic expression study supported this hypothesis in that proteins involved in glucose metabolism exhibited relative protein abundance changes compared to the wild type strain. Western blotting can provide expression profiles to support LC-MS/MS data described in Ch. 4. We can also estimate protein intensity using densitometry. Antibodies to glycolytic proteins (Pgk, Fba, PfkA; Fig. 4.3) should show increased expression in the *hya* western blot compared to the wild type.

The determination of enzymatic activity would be necessary to assign a physiological role for the identified proteins. Relative protein abundance (Ch. 4) does not give insight to protein activity as some proteins increased in abundance could be in their inactive forms. The Pgk protein would be an ideal enzyme to study because of its production of ATP in glycolysis. One approach to study this protein would be to detect and determine the relative concentration of 3-phospho-D-glycerate (the product of Pgk) in the *hya* hydrogenase mutant. A method using both liquid chromatography and electrospray-tandem mass spectrometry has been described for detection of glycolysis intermediates in bacteria (2, 18). Alternatively, measurement of ATP using a luminescence assay could be used to determine if the *hya* mutant shows wild type levels of ATP production (3). This is an indirect way to implicate Pgk as a significant contributor to ATP levels because of the various metabolic reactions that can produce ATP.

qPCR can be used to quantify the mRNA from glycolysis genes in the wt and *hya* strain. This method can give insight into quantities of RNA translated into proteins. The experimental results can support the relative protein abundance fold changes described in ch. 5. In addition qPCR can be modified to measure relative protein abundance and then correlate qPCR protein profiles with mRNA transcript measurements (19).

Alterations to membrane composition in the *hya* mutant

LC-MS/MS revealed that the *hya* mutant contained a higher abundance of fatty acid synthesis proteins than the wild type strain. The proteins identified FabB and FabI, and FabH, are involved in elongation of fatty acids and initial fatty acid synthesis, respectively (13) (Fig. 4.6). Abundance of various membrane lipids could indicate activity of these fatty acid proteins. In *E.coli* an overproduction of FabB resulted in an increased amount of palmitoleic acid synthesis and incorporation of cis-vaccenic acid (13). FabB overexpression provided *E.coli* antibiotic resistance against thiolactomycin. Overexpression of FabH led to an increase in myristic acid and shorter fatty acid chains (13). FabI is involved in the final reaction of fatty acid elongation (11). Liquid chromatography can be used to assess the membrane composition (12) of the wt and *hya* strains. Antibiotic susceptibility challenging the *hya* mutant with thiolactomycin and other chemical derivatives can determine if the increase in fatty acid protein abundance can provide antibiotic resistance.

Analysis of the *hya* mutant's viability in macrophages

Shigella macrophage invasion and apoptosis can be analyzed by use of J774 and RAW 264.7 (17). Macrophage assays can be used to study the ability of the bacteria to survive anti-bacterial substrates located within the immune cell. Proteomic analysis revealed that the *hya* mutant had a decreased amount of SodB. This protein is critical for detoxification of oxygen radicals that *S. flexneri* would encounter in phagocyte engulfment. SodB has been shown in *Shigella* to be important for infection (6, 7). The mutant can be subjected to macrophage killing and bacterial cell recovery can be compared to the wild type. Alternatively, a disk assay using menadione can test for the *hya* mutant's sensitivity to reactive oxygen species (4, 20).

Determination of serine pools and glutamate containing proteins

Proteomic analysis of the Shigella strains uncovered differences in amino acid biosynthetic protein abundance. Enzymes producing glycine or cysteine (requires serine as a precursor for synthesis) were decreased in expression. In lieu of this SerC responsible for serine production was increased in expression in the *hya* mutant. Conservation of serine pools appear to be important for this mutant. The energy intermediate, pyruvate can be produced from the deamination of serine. Determination of serine and pyruvate pools in the wild type and mutant are necessary to support the hypothesis of maintenance of serine pool for energy generation.

The GltX protein in the *hya* mutant is hypothesized to show a significant increase in abundance based on relative protein abundance. Using bioinformatics we could determine if any proteins in *Shigella flexneri* contain significant amounts of glutamate.

These proteins could be cross-referenced to protein abundance profiles (Ch. 4) to determine if the proteins are shown to be increased in the *hya* mutant.

Use of polymyxin B as a membrane pore generator in *S. flexneri*

The bactericidal antibiotic, polymyxin B (PMB) is a source of membrane disruption in bacteria. In *E.coli* it was reported that increasing concentrations of PMB contributed to cytoplasmic membrane depolarization (5). Between 2-20 µg/ml PMB increased the outer membrane permeability, while the cytoplasmic membrane remained intact. Measurement of K^+, H^+, TPP^+ and PCB^- movement was monitored to predict cytoplasmic and outer membrane permeability (5).

S. flexneri wild type in pH 2.5 buffer was treated with PMB at concentrations 2.5-15 µg/ml. A control of PMB added to cells at pH 6.5 was also done to determine if PMB was an effective antibiotic at neutral conditions. There was no difference in challenging the wt at pH 2.5 with PMB compared to no added PMB. However, in pH 6.6, PMB had a bactericidal effect. This is most likely because PMB is a lipopeptide, and optimal conditions for activity may be in the neutral range (21).

Proteomic studies have indicated that deletion of *hya* results in an increase in fatty acid biosynthetic proteins (Chapter 4). This could indicate that Hya contributes to membrane integrity. Similar to how Daugelavicius *et al.* determined membrane disruption via PMB we could use microelectrodes specific for ions to deduce stability of the inner and outer membrane. The electrode TPP^+ (tetraphenylphosphonium) can be used to assess membrane depolarization in standard conditions (9, 10, 16). This method of membrane measurement could continuous give measurements over time

(16). Once set up the electrode only needs to be placed in the bacterial culture for measurement (9). TPP^+ can be added to the bacterial mix and it will accumulate in the cytoplasm if the inside is negative or at normal membrane potential conditions. If not the ion will leave the cells and can be detected with the TPP specific electrode (16).

Concluding Remarks

Shigella flexneri's genome has been annotated but still so many questions remain as to how the genes and subsequent products work to create a functioning bacterium. The proteomic work presented in chapter 4 gives an overview on the global changes in the *hya* mutant. Aforementioned future directions presented can describe the genomic and metabolic changes that occur when Hya is deleted. This work done is just a glimpse into the intricate pathways that allows *S. flexneri* to be a potent pathogen. Additional roles of the hydrogenases, especially Hya, have been revealed from the studies herein.

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