HYDROGEN USE BY BACTERIAL ENTERIC PATHOGENS

by

ANDREA L. ZBELL

(Under the Direction of Robert J. Maier)

ABSTRACT

Uptake-type hydrogenases catalyze the oxidation of molecular hydrogen in an energy-conserving manner. Many prokaryotes use these hydrogenases in their central metabolism. It has been recently shown that pathogens can use H₂ as an energy source during host colonization. *Salmonella enterica* serovar Typhimurium contains three uptake-type hydrogenases: Hya, Hyb and Hyd. Since all of these hydrogenases can catalyze the oxidation of H₂, it was hypothesized that they are used under different conditions and are differentially regulated.

Studies with hydrogenase promoter fusions to *lacZ* revealed that *hya* is preferentially expressed during fermentative growth. Hya gene expression was activated by FNR and repressed by NarL. It may be involved in recycling anaerobically-produced H₂, since hydrogen evolution was not observed in a double mutant that only contained Hya. Studies using resolvase *in vivo* expression technology showed that the bacterial *hya* genes were also expressed in *S. enterica* serovar Typhimurium cells residing within macrophages and during mouse infection. Hya seems to be very important for pathogenesis, since it was necessary for acid-resistance and colonization of macrophages. Interestingly, the *hyd* genes were upregulated during aerobic growth and were repressed by ArcA. The *hyd* genes were highly expressed in bacteria residing within macrophages and in the mouse liver and spleen. The *hyb* genes were upregulated during
anaerobic respiration, and may be expressed in bacteria residing within macrophages. Hyb was repressed by IscR and in the presence of glucose, and was activated by ArcA and FNR. Growth studies indicated that the presence of hydrogen augmented growth during anaerobic respiration, and Hyb is an important hydrogenase for this phenomenon. Collectively, the in vivo and in vitro results demonstrate that each hydrogenase is differentially expressed and suggest that each has a unique role in the physiology of S. enterica serovar Typhimurium.

Shigella flexneri is another important enteric pathogen that contains two uptake-type hydrogenases, Hya and Hyb. Whole-cell hydrogenase assays showed that hydrogenase activity was higher when cells were grown via anaerobic respiration with fumarate, as compared to cells grown anaerobically on blood-agar. Activity was highest in anaerobic or microaerobic cultures, as compared to cells grown under atmospheric conditions. Studies with hydrogenase mutants indicated that Hya is important when cells are grown anaerobically on rich media, while Hyb is important in cells grown under anaerobic respiration conditions.

INDEX WORDS: hydrogenase, Salmonella, Shigella, hydrogen uptake, Enterobacteriaceae, anaerobic respiration, organ colonization, pathogenesis
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DEDICATION

This dissertation is dedicated to all of my teachers who encouraged me to pursue a graduate degree and those who helped me complete the degree. I would especially like to thank Dr. Linda Beck who was a wonderful mentor during my undergraduate career and Dr. Robert Maier who helped me throughout my time in graduate school.
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CHAPTER 1
INTRODUCTION AND LITERATURE REVIEW

Hydrogenases are thought to be ancient enzymes. They are found in species from all domains of life— from Bacteria and Archaea to the organelles of some primitive eukaryotes (128). These enzymes are very important for many of these organisms as the reactions they catalyze are intimately linked to their energy metabolism. Uptake-type hydrogenases catalyze the oxidation of H₂ to H⁺ and electrons in an energy conserving manner, while evolution-type hydrogenases catalyze the production of H₂, which can be used to dispose of excess reducing potential in the cell. Most of the organisms that have evolution-type hydrogenases also have uptake-type hydrogenases (129). There have been numerous studies on the biochemistry and cellular function of hydrogenases in many diverse prokaryotes including aerobic hydrogen oxidizers, cyanobacteria, photosynthetic bacteria, rhizobia, sulfate reducers, anaerobic fermenters, and extremophiles (128). In addition, there has been a surge of interest concerning the use of hydrogenases to produce hydrogen as an alternative energy source (36).

Although many pathogenic enteric bacteria contain hydrogenase genes in their genomes, the contribution of hydrogenase to bacterial pathogenesis has not been well studied, and is a fairly new field (71). Enteric pathogens are an important problem in both industrialized nations and in the developing world, causing about 2 million deaths annually (26). It has been recently shown that bacteria can use molecular hydrogen as an energy source during an infection (71). Studies with Helicobacter pylori, H. hepaticus, and the enteric pathogen Salmonella enterica serovar Typhimurium have demonstrated that the hydrogenase enzymes are important for
virulence in these bacteria (73, 91). Although some studies have been done on hydrogenases in enteric bacteria, much remains unknown about their complex regulation

\textit{in vitro} and \textit{in vivo}.

**OVERVIEW OF \textit{S. ENTERICA} SEROVAR TYPHIMURIUM AND \textit{S. FLEXNERI}**

**MICROBIOLOGICAL CHARACTERISTICS**

**Morphology and General Characteristics**

\textit{Salmonella enterica} serovar Typhimurium and \textit{Shigella flexneri} are in the class Gamma-proteobacteria and are members of the family \textit{Enterobacteriaceae} (NCBI). They are Gram-type negative short rods, about 1-5 µm in length. These bacteria are facultative anaerobes and can grow on a wide variety of substrates, with optimal growth at 37°C. Most \textit{Salmonella} strains are motile, possessing peritrichous flagella. \textit{Shigella} species are non-motile.

\textit{Salmonella} serovars and \textit{Shigella} species primarily reside within the intestinal tract of animals. However, both can survive for extended periods of time in groundwater, soil, or crops if conditions are favorable. The source of this contamination is often sewage wastewater.

According to the National Research Council, about 5 million dry Mg of sewage sludge is generated in the United States each year, and about 60% of that is applied to farmland as fertilizer (38). This fertilizer is a rich source of enterics, including \textit{Salmonella} and \textit{Shigella} species. This is an important problem as national alerts of enterobacterial contamination of produce entering the U.S. food supply are becoming commonplace.

**Genome and Classification**

The genus \textit{Salmonella} has a rather complicated taxonomy. \textit{Salmonella} contains two species, \textit{S. enterica} and \textit{S. bongori}. \textit{S. enterica} is further subdivided into seven subspecies: \textit{enterica} (I), \textit{salamae} (II), \textit{arizonae} (IIIa), \textit{diarizonae} (IIIb), \textit{indica} (IV), \textit{houtanae} (VI), and subspecies VII. \textit{S. enterica} subspecies I is responsible for about 99% of human salmonellosis cases (136). The
subspecies are further classified into serovars, determined by the Kauffman-White serotyping scheme (based on antigenic variation of surface molecules) (136). There are about 2,000 serovars, which are often named for their host specificity or for the region where they were isolated. The *Salmonella* serovars share over 90% sequence similarity (31). In addition, *Salmonella* species are closely related to *Escherichia coli*. About 55% of *S. enterica* serovar Typhimurium LT2 and *E. coli* K12 proteins are identical (136). However, most *Salmonella* serovars contain 5 large insertions in their genomes, which are not present in *E. coli* (45). Many of the genes in these insertion regions encode proteins used in virulence, therefore these regions are called pathogenicity islands.

The *Salmonella* serovar used in this study, *Salmonella typhimurium* strain LT2, has about a 4,800 kilobase (Kb) chromosome and a 9.4 kilobase (kb) virulence plasmid, with about 50% GC content (78). Most *Salmonella* serovars have around 6 integrated phages in the chromosome, along with around 30 – 75 transposase proteins and multiple transposable regions of the chromosome, which gives the genome some plasticity (31). Host specific serovars like *Salmonella enterica* serovar Typhi undergo frequent chromosome rearrangements while other serovars have fairly stable chromosomes (31).

*Shigella flexneri* serotype 2a strain 2457T used in this study has about a 4,600 kb chromosome and a GC content of about 51% (132). This strain also harbors a large virulence plasmid pINV2457T and three small plasmids pSf2, pSf4, and pSF-R27. *S. flexneri* is highly similar to the pathogenic *E. coli* strains like *E. coli* O157:H7 and non-pathogenic strains like *E. coli* K12. *E. coli* O157:H7, K12, and *S. flexneri* 2a share about 2,881 protein-encoding open reading frames (ORFs). Genome sequence comparison revealed that only 175 ORFs were unique to *S. flexneri*. In addition, around 1,200 *S. flexneri* proteins are 100% identical to *E. coli*
K12 proteins (132). However, there are several important differences between the *E. coli* and *S. flexneri* genomes. *S. flexneri* has deletions or disruptions in around 879 genes that are intact in *E. coli* K-12. *S. flexneri* contains about 37 pathogenicity islands, while *E. coli* O157 has around 100 (132). Some of the genes encoded on the *S. flexneri* islands are unique to this species and have not yet been described. Some of these new proteins are putatively annotated as enterotoxins and siderophores or have another possible function in virulence (132).

**Metabolism**

*Salmonella typhimurium* and *Shigella flexneri* are both facultative anaerobes and can utilize a variety of carbon and energy sources for growth. These organisms are capable of aerobic and anaerobic respiration and fermentation. Complex regulatory pathways are responsible for determining which mode of growth will be used, depending on which substrates are available (122).

*S. enterica* serovar Typhimurium and *S. flexneri*, like other enteric bacteria, will grow by aerobic respiration with oxygen as the terminal electron acceptor. They can also grow by anaerobic respiration with substrates such as lactate, succinate, NADH, or H$_2$ as the electron donor and oxidants such as nitrate, dimethyl sulfoxide (DMSO), or fumarate as the electron acceptor (122). There are three basic components in the electron transport chain that are necessary for either aerobic or anaerobic respiration: substrate-specific dehydrogenases, quinones, and terminal oxidoreductases. The dehydrogenases oxidize the electron donor, while the mobile quinones pass those electrons to the oxidoreductases. The oxidoreductases then reduce the electron acceptor with reducing equivalents from the quinones (122). Enteric bacteria have remarkable flexibility in their respiratory chains with a variety of dehydrogenases, quinones, and oxidoreductases available. This allows them to grow on a variety of substrates,
with respiratory chains as shown in Figure 1.1. However, multiple insertions and deletions have disrupted the function of several *S. flexneri* genes used in central metabolism, as compared to *E. coli*. Therefore, *S. flexneri* 2457T cannot utilize glycerol, acetate or various sugars as a growth substrate. In addition, they cannot utilize two-carbon compounds via the glyoxylate shunt, and require NAD for growth (65,132).

**FIG. 1.1** Components of respiratory chains in *E. coli*. Electron donors on the left pass electrons to dehydrogenases (ovals at left), then to quinones (center), and finally to reductases (ovals at right). The respiratory chains that are generated are based on the donors and acceptors that are present. Adapted from (122).
The respiratory chains conserve energy for the cell by generating a proton electrochemical gradient across the cell membrane that can be harnessed to do work. This electrochemical gradient is often directly generated by membrane-spanning proteins that either pump H\(^+\) out of the cell, or release H\(^+\) into the periplasmic space (125). When these protons re-enter the cytoplasm they can be coupled to processes that produce ATP, transport solutes across the membrane, or provide motility for the cell.

*Shigella* and *Salmonella* species are also capable of growing anaerobically by fermentation. Fermentation can be defined as a type of anaerobic growth without an exogenous electron acceptor (16). This process generally begins by the oxidation of sugars like glucose to pyruvate, producing two NADH molecules (reducing equivalents). This oxidation typically occurs by the Embden-Meyerhof-Parnas pathway. The reducing equivalents must be regenerated (oxidized) in order for the process to continue. Enterics generally do this by reduction of metabolic intermediates (such as pyruvate) to produce ethanol and a mix of lactic, acetic, succinic and formic acids which is coupled to the oxidation of NADH (16). The fermentation pathways used by *E. coli* are shown in Figure 1.2. Fermentative growth yields far less ATP than respiratory growth, with only 2-4 ATP generated by substrate-level phosphorylation per glucose oxidized.

Hydrogenases are important for both fermentative and respiratory growth, and have been studied since the 1930s (121). The formate hydrogen lyase (FHL) complex and its associated hydrogenase (*hyc*) split formate into CO\(_2\) and H\(_2\) during fermentative growth (109). This process helps maintain a neutral pH inside the cell. In addition, the hydrogen-uptake hydrogenases (*hya*, *hyb*, and in *S. enterica* serovar Typhimurium *hyd*) can recycle the H\(_2\) produced by *hyc* to
conserves energy by contributing to the proton motive force (16). During respiratory growth H₂ can serve as an electron donor when the hydrogen-uptake hydrogenases split H₂ to yield protons and electrons (122).

**FIG. 1.2 Fermentation reactions in E. coli.** Abbreviations: ACK, acetate kinase; ADH, alcohol dehydrogenase; FHL, formate hydrogenlyase; D(-) LDH, NAD+-dependent D(-) LDH; PFL, pyruvate formate-lyase; PTA, phosphotransacetylase; P, phosphate; PEP, phosphoenolpyruvate. Adapted from (16).
PATHOGENESIS OF *S. ENTERICA* SEROVAR TYPHIMURIUM INFECTION

*S. enterica* serovar Typhimurium is a prominent enteric pathogen associated with food-borne illness. *Salmonella* species are responsible for about 30% of food-related deaths in the United States alone (80). The following section describes the location and types of *Salmonella* infections, important virulence factors, and the host immune response to *Salmonella* infection.

Location and types of *Salmonella* infections

*Salmonella* species cause different types of infections in a wide variety of hosts. The host specificity of various strains, types of infections, source and cause of infection and disease progression is described below.

(1) Host specificity of *Salmonella* species

*Salmonella* species cause disease in almost all types of animals, both warm-blooded and cold-blooded. In addition, some strains are highly host-specific, while others are generalists (31). *Salmonella bongori* is the species that specifically colonizes cold-blooded hosts and was originally isolated from a lizard (136). It has been recently shown that *S. bongori* lacks *Salmonella* pathogenicity island 2 (SPI-2) which may be needed to colonize warm-blooded hosts (20). The *Salmonella enterica* subspecies that preferably colonizes reptiles (subspecies IIIa arizonae) was found to have modifications in its SPI-2, as compared to other *S. enterica* subspecies (20). *S. enterica* subspecies I, IIIb, VI and VII only colonize warm-blooded hosts, while subspecies II and IV can infect either cold or warm-blooded hosts (136). Flagellar phase variation (allelic variation at the *fliC* locus) is present in the subspecies that colonize warm-blooded hosts and is deemed especially important to evade the immune system in these animals (62).
(2) Host specificity and infections caused by *S. enterica* subspecies I (enterica)

*S. enterica* subspecies enterica is responsible for about 99% of all human cases of salmonellosis. In addition, the serovars in this subspecies infect a variety of other warm-blooded animals (136). The *S. enterica* serovars can be classified as host-restricted, host-adapted, or non-host-adapted. Host-restricted serovars can only infect one species while host-adapted species prefer to infect a single species, but can cause disease in others as well. Non-host-adapted serovars cause disease in a wide variety of species. *S. enterica* serovar Typhimurium is a non-host-adapted serovar. Examples of each of these types of serovars are shown in Table 1.1.

*S. enterica* serovars can cause one of three types of infection: typhoid fever, bacteremia, or enteritis. The type of infection depends on the serovar and on the host species (107). For example, *S. enterica* serovar Typhimurium causes typhoid fever when in mice and enteritis when in humans. In humans, the serovars typically associated with typhoid fever are Typhi, Paratyphi A, Paratyphi B, and Paratyphi C. Enteritis is caused by serovar Typhimurium and Enteritidis, while bacteremia in humans is commonly caused by Choleraesuis or Dublin. Bacteremia is the rarest type of infection in humans. Typhoid fever is rare in the United States, but is common in Asia, Africa, and South America. However, enteritidis is common, with about 1.4 million illnesses in the United States per year (140).

*S. typhi* or *S. paratyphi* is typically spread by chronic asymptomatic human carriers (41). Typhoid fever is characterized by a relatively long incubation period (5-9 days) followed by fever. Diarrhea only occurs in about 1/3 of patients and is considered a minor symptom (140).
**Table 1.1 Host specificity of *S. enterica* subspecies enterica serovars.** The host range and specificity of example serovars is listed below. Adapted from (136).

<table>
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<th>Serovar</th>
<th>Host specificity</th>
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<tr>
<td>Typhi</td>
<td>Host-restricted</td>
<td>Humans</td>
</tr>
<tr>
<td>Gallinarum</td>
<td>Host-restricted</td>
<td>Birds</td>
</tr>
<tr>
<td>Abortusovis</td>
<td>Host-restricted</td>
<td>Sheep</td>
</tr>
<tr>
<td>Dublin</td>
<td>Host-adapted</td>
<td>Cattle</td>
</tr>
<tr>
<td>Choleraesuis</td>
<td>Host-adapted</td>
<td>Pig</td>
</tr>
<tr>
<td>Pullorum</td>
<td>Host-adapted</td>
<td>Bird</td>
</tr>
<tr>
<td>Typhimurium</td>
<td>Non-host-adapted</td>
<td>Not host specific</td>
</tr>
<tr>
<td>Enteritidis</td>
<td>Non-host-adapted</td>
<td>Not host specific</td>
</tr>
</tbody>
</table>

Constipation may also occur (107). Histological changes in the intestine are minor for typhoid fever as compared to enteritidis. During typhoid fever, the small intestine develops diffuse enteritis caused by the infiltration of mononuclear leukocytes, and the area around the Peyer’s patches may experience necrosis. Intestinal perforation or hemorrhage occurs in about 2% - 10% of cases (41). However, in most cases, the majority of the intestinal epithelium remains intact (140). *Salmonella* typically spreads to the mesenteric lymph nodes, liver, and spleen and causes the enlargement of these organs (107). Low level bacteremia may result and the disease is fatal in about 10% - 20% of untreated cases (41).

Enteritidis is often caused by ingestion of contaminated meat, dairy, or eggs containing *S. enterica* serovar Typhimurium or *S. enteritidis*. The incubation period for this disease is short, about 12-72 hours. Disease symptoms are intense diarrhea, vomiting and abdominal pain (107).
Salmonella cells localize to the terminal ileum and colon, causing major changes in these organs. An acute enteritis results from a rapid infiltration of polymorphonuclear leukocytes (predominantly neutrophils), which cause necrosis of the uppermost mucosa (76, 140). Studies using the calf model of enteritidis (with S. enterica serovar Typhimurium) indicated that the prognosis of the disease depends on the infectious dose. An infectious dose of $10^4$ to $10^7$ colony-forming units (CFU) resulted in diarrhea for 2 to 8 days while an infectious dose of $10^8$ to $10^{11}$ CFU resulted in death (140). In humans the disease is often self-limiting; however, in immunocompromised patients the mortality of untreated cases is about 0.1% (41).

Bacteremia is probably the most dangerous type of Salmonella infection, with a mortality rate of around 20% in patients infected with serovar Dublin (35). The source of S. dublin or S. choleraesuis is often contaminated pork or unpasteurized milk. The characteristic symptom is a high spiking fever. Diarrhea occurs in about 1/3 of cases. In contrast to typhoid fever and enteritis, the intestinal tissue of infected patients is often relatively unaffected (107). In calves, S. dublin enters the lymphatic system through the mesenteric lymph nodes and is disseminated throughout the body in the lymph (98). The invading bacterial cells are primarily extracellular, not dwelling in lymphocytes. Meningoencephalitis, polyarthritis, osteomyelitis, or pneumonia may develop in infected calves (140). In humans, Salmonella can be isolated from blood in 75% to 91% of cases.

**S. enterica serovar Typhimurium virulence factors**

Upon translocation to the host intestine, S. enterica serovar Typhimurium encounters the epithelial cell membrane. Bacterial cells pass through the epithelial cell layer and colonize
lymphocytes. *Salmonella* must survive and grow intracellularly in order to cause a successful infection. The virulence factors that are important during each of these stages of infection are outlined below.

(1) **Uptake into the epithelial cell**

*S. enterica* serovar Typhimurium cells generally enter the intestinal submucosa through M cells located in the Peyer’s patches. They can also enter via intestinal epithelium cells, or be shuttled across the epithelium by immune cells that are sampling the intestinal lumen, although these routes are not as common (133). When *Salmonella* encounters a non-phagocytic cell such as an M cell or epithelial cell, it induces its own uptake. It does this by the trigger mechanism. The type III secretion system (TTSS-1) encoded on pathogenicity island-1 in the genome is used to inject effectors into the host cytoplasm (43). The two *Salmonella* proteins SipB/C form a pore on the host cell through which other effectors are transferred into the host cytoplasm (23). Next, several *Salmonella* effector proteins rearrange the host cytoskeleton by altering host transduction pathways or by directly affecting actin rearrangement (141). SopE, SopE2, and SopB affect host signal transduction, while SipA and SipC are actin-binding proteins (141). The combined affect of these proteins is to stimulate dramatic membrane ruffling on the host cell, and uptake of the invading bacteria in a macropinocytic pocket (23). *Salmonella* TTSS-1 mutants cannot enter epithelial cells, but can enter phagocytic cells such as macrophages by host-mediated phagocytosis (43).

(2) **Formation and maturation of the *Salmonella*-containing vacuole (SCV)**

Once *Salmonella* enters a phagocytic or non-phagocytic cell, it is enclosed in a membrane-bound vesicle called a *Salmonella*-containing vacuole (SCV). This SCV is modified by *Salmonella* for
the first 2-3 hours post-infection and then the bacterial cells begin to replicate (43). The initial modifications of the SCV are different depending on the host cell type.

Based on studies in HeLa cells, the SCVs first acquire proteins from early host-cell endosomes, probably from a fusion of the SCV with these endosomes. Proteins from late endosomes are then acquired, including lysosomal glycoproteins, vacuolar ATPase, and lysosomal-acid phosphatase. However, the SCV does not fuse with late endosomes or mature lysosomes. The bacterium induces transcription of genes needed to grow in the SCV at this time. Structures called Salmonella-induced filaments (Sifs) aggregate around the SCV just prior to bacterial cell replication (43).

Studies of SCV maturation in macrophages have yielded conflicting results. Some researchers concluded that the SCV in macrophages fuses with lysosomes, while others argue that this does not happen. Most researchers agree that SCV maturation in macrophages is similar to what happens in epithelial cell lines (43, 101). However, it was recently reported that the mechanism of Salmonella entry into macrophages (host-mediated phagocytosis or bacterial-mediated membrane ruffling) determines the environment inside the SCV. The results from this study indicate that the SCV acidifies, with a pH between 4.5 and 6, whether the bacteria mediated uptake or not (30). Other studies have shown that Sifs do not form near SCVs in macrophages and that the genes from Salmonella pathogenicity island-2 (SPI-2) are necessary to survive in the SCV and to avoid bacterial death by NADPH oxidase in macrophage (43). Genes involved in oxidative and nitrosative stress repair, heat shock, and DNA repair including soxS, katE, katG, ibpA, ibpB, and msrA are upregulated while Salmonella resides in macrophages (54).
(3) Systemic infection with *Salmonella* or clearance of infection

Susceptible mouse strains (such as BALB/c) develop typhoid fever-like symptoms upon infection with *S. enterica* serovar Typhimurium. In a typhoid fever-like infection, infected dendritic cells and macrophages carry *S. enterica* serovar Typhimurium through the lymph to the liver and spleen (133). Massive infiltration of leukocytes into the liver and spleen causes enlargement of these organs and concomitant inflammation. Neutrophils respond to this inflammation and cause necrotic lesions in the liver, which become granulomas. In addition, *Salmonella* induces apoptosis of the infiltrating phagocytes (103). Mice typically die from liver damage within 8 days p.i.

In humans, *S. enterica* serovar Typhimurium causes enteritis. Once the bacterial cells have entered macrophages and epithelial cells, they replicate in the SCV. However, the infection is localized to the intestine and mesenteric lymph nodes and does not spread systemically (140). There is a rapid influx of neutrophils into the intestine that seem to combat the infection. The production of reactive nitrogen and reactive oxygen species from inducible nitric oxide synthase and NADPH phagocyte oxidase, respectively, is deemed especially important in killing *Salmonella* by neutrophils (127). Most of the time the infection is cleared; however, death can occur from dehydration and renal failure (usually in people more than 74 years old or in the very young) (26).

**Immune response to *Salmonella* infection**

The innate immune response is the first line of defense against *Salmonella* infection, while the acquired immune response combats the infection during the latter stages. The following section describes these two types of immune response to *Salmonella* challenge.
(1) Innate immune response to *Salmonella* infection

The innate immune defense system in the intestine is comprised of the epithelial cell layer (a barrier to infection in most cases), phagocytes that reside in the submucosa, and soluble products such as antibodies, cytokines, and complement components (133). As described in the previous section, *S. enterica* serovar Typhimurium has the capacity to cross the intestinal epithelium. However, the bacteria induce a proinflammatory response from the epithelial cells when this occurs. Bacterial flagellin is an important signal to trigger this response (120). It has also been suggested that *Salmonella* lipopolysaccharide (LPS) can activate the host immune system, since mice that cannot recognize LPS are especially susceptible to *Salmonella* infection (49). In response to these signals, epithelial cells release cytokines and chemokines (such as IL-8) on the basolateral membrane that recruit phagocytes to the intestinal lumen (133).

Several types of phagocytes are recruited to the intestine and spleen upon *Salmonella* invasion of the intestinal epithelium. Neutrophils and macrophages are recruited to the mesenteric lymph nodes, Peyer’s patches, and the spleen in mice after oral infection with *Salmonella*. Neutrophils are probably the first cells recruited, since they increase in abundance before macrophage numbers increase (133). Although *S. enterica* serovar Typhimurium can survive and grow inside these phagocytes, the initial respiratory burst generated when bacteria first encounter the phagocytes is thought to kill some of the bacterial cells. This probably helps to control the infection before the acquired immune response has time to mount an attack.

In addition to the contribution of phagocytes, soluble antimicrobial chemicals released by epithelial and phagocytic cells help combat the infection. Epithelial cells in the intestine secrete the antimicrobial peptides called β-defensins that are thought to kill bacteria by creating pores in the bacterial membrane. Upon contact with invading bacteria, Paneth cells of the small
intestine secrete large quantities of antimicrobial molecules such as phospholipase, lysozyme, and α-defensins (88). In addition to their antimicrobial properties, many of the α and β-defensins are chemokines that will recruit cells involved in the adaptive immune response such as dendritic cells and T cells. Complement proteins are constantly circulating in the bloodstream and contribute to initial killing of Salmonella during an infection.

(2) Adaptive immune response to Salmonella infection

The innate immune response is often not enough to control S. enterica serovar Typhimurium infection in mice or humans, and Salmonella cells migrate to the mucosa-associated lymphoid tissues (MALT). The adaptive immune response is then activated, although it typically takes several days for an effective response to develop (29). It is thought that dendritic cells in the intestinal submucosa present Salmonella antigens that activate a T cell response in the Peyer’s patches. However, there is probably some suppression of this by the bacteria, since some studies have shown that Salmonella can inhibit antigen presentation by these cells (120). The resulting activated CD4 T cells are important to control Salmonella infection while CD8 T cells are involved in controlling a second challenge with S. enterica serovar Typhimurium. The CD4 T cells are activated in the mesenteric lymph nodes as early as 3-6 hours p.i. and are generally of the T_{H1} type (120). Therefore, the response from these cells is primarily a cellular-mediated response, not an antibody-mediated response. Memory T cells are generated and create immunity to S. enterica serovar Typhimurium.

PATHOGENESIS OF S. FLEXNERI INFECTION

Shigella species are important enteric pathogens, causing about 164.7 million cases of dysentery per year worldwide (59). S. flexneri is the predominant species associated with these infections. In addition, it has become more difficult to treat these outbreaks. S. flexneri infections cannot be
treated solely with oral rehydration and *Shigella* has become increasingly resistant to antimicrobial drugs in the past 50 years (59). The development of Shigellosis, virulence factors, and the immune response involved in *Shigella* infection are discussed in the following section.

**Characteristics of Shigellosis**

*Shigella* species cause infection exclusively in humans and primates (114, 132). *Shigella* causes dysentery, characterized by bloody, mucoid diarrhea. The following section describes the species of *Shigella* and the progress of infection.

(1) *Shigella* species

*Shigella* species belong to the family *Enterobacteriaceae*. They are divided into the subgroups *S. flexneri*, *S. sonnei*, *S. dysenteriae*, and *S. boydii* based on biochemical, serological, and clinical phenotypes (114). In addition, they are divided into serotypes based on the composition of the O-antigen polysaccharide located on the cell membrane (94). *S. flexneri* and *S. sonnei* cause the majority of bacillary dysentery infections. However, *S. dysenteriae* is responsible for epidemic outbreaks and causes the most fatalities (114).

Sequence comparisons have revealed that *Shigella* is closely related to *E. coli*. There is currently some debate whether *Shigella* should be re-classified as a strain of *Escherichia coli*, since the sequence divergence between *S. flexneri* and *E. coli* K-12 is only about 1.5% (114). *Shigella* species have genomes altered for entry into and life inside a host, as compared to non-pathogenic *E. coli*. Compared to the latter, it seems they have gained a virulence plasmid and pathogenicity islands that are located both on the plasmid and in the chromosome. While gaining some virulence attributes, *Shigella* has lost the ability to synthesize functional flagella and some catabolic pathways that are deemed unnecessary for intracellular growth (114).
(2) Characteristics of *Shigella* infection

*Shigella* infections are most common in developing countries with about 160 million cases annually, mostly in very young children (under the age of 5). These children are also the most likely patients to die of shigellosis. *S. flexneri* and *S. dysenteriae* are common in these countries, probably representative of the level of hygiene in endemic areas. *Shigella* can also cause traveler’s diarrhea, resulting in a more serious infection than that caused by enterotoxigenic *E. coli*. *S. sonnei* is most associated with disease in developed countries (94). *Shigella* is also associated with chronic diarrhea in HIV patients (59).

Most of our knowledge of *Shigella* infection is a result of studies on *S. flexneri*. Shigellosis develops after the ingestion of as few as 10 - 100 bacteria, typically by the fecal-oral route. *Shigella* species are highly acid-tolerant, therefore, low numbers of bacteria are sufficient to cross the acidic stomach (10, 114). It has been shown that *Shigella* isolates can survive pH 2.5 for at least 2 hours, while *Salmonella* cannot (94). After ingestion of *Shigella*, bacterial dysentery develops. The bacteria cause severe tissue destruction of the intestinal epithelium, resulting in bloody stools. There is an impaired absorption of water, nutrients, and solutes in the intestine, causing watery diarrhea, and mucus is observed in the stools as well. *S. dysenteriae* serotype 1 also produces Shiga toxin, which creates lesions in the colon, kidney, and central nervous system (114). Potentially life-threatening complications can develop including hypoglycemia, bacteremia, or septicemia, septic shock, and dehydration leading to renal failure or even malnutrition in chronic cases. No vaccine is available, and rehydration treatment alone is not effective. Many strains are resistant to first-line antibiotics such as ampicillin and tetracycline, so new-generation antibiotics such as fluoroquinolones are often used to treat infections (94).
**Shigella virulence factors**

*Shigella* cells must cross the epithelial cell layer, evade the host immune system, and enter and replicate in epithelial cells in order to mount a successful infection (55). The virulence factors important in each stage of infection are outlined below.

1. **Crossing the epithelial cell layer**

   Upon ingestion of an infectious dose of *Shigella* cells, the bacteria migrate to the intestine. Unlike *Salmonella*, *Shigella* does not mediate its own uptake into epithelial cells from the apical side. It is thought that the mucin layer that coats the apical side of the epithelial cells is a barrier to *Shigella* invasion (55). Instead, *Shigella* enters the M cells in the colon. M cells constantly sample the luminal content of the intestine and take up *Shigella* during this process. *Shigella* can mediate their uptake into M cells as well, with membrane ruffling, described in the following sections (55). Following entry into M cells, the bacteria are passed to an intraepithelial pocket and enter into the submucosa (114). Here, they encounter leukocytes including macrophages, dendritic cells, B and T lymphocytes.

   There are also several M cell-independent methods that *Shigella* can use to cross the intestinal epithelium. Recent studies have demonstrated that *S. flexneri* can manipulate and pass through the tight junctions between intestinal epithelium cells in order to access the submucosa, which is not done by *S. enterica* serovar Typhimurium (55). Later in the infection, PMNs also destroy tight junctions, allowing *Shigella* to pass through epithelial tissue (114). In addition, PMNs and dendritic cells can cross the epithelial layer and phagocytize *Shigella*, which effectively translocates the bacteria to the submucosa.

   When *Shigella* encounters a host cell, the type III secretion system (T3SS) (which is needed later in the infection), encoded by the *mxi-spa* genes on the plasmid-located
pathogenicity island (PAI) is activated. The translocator proteins IpaB, and IpaC are released from the chaperone IpgC. IpaD is released from the tip of the T3SS, unplugging the needle, which allows the translocation pore to assemble from IpaB and IpaC (114). The T3SS is then ready to secrete effectors into macrophages or the basolateral side of epithelial cells.

(2) Escape from phagocytes

When *Shigella* passes into the submucosa it encounters phagocytes. Shortly after phagocytosis by macrophages, *Shigella* induces apoptosis of these cells, which releases the still-viable bacteria (114). In this process, *Shigella* must first escape the phagocytic vacuole. It secretes IpaB through the T3SS, which lyses the membrane of the vacuole. It is thought that *Shigella* can escape from the vacuole within 15 minutes (114). IpaB then binds to caspase-1, which induces apoptosis (65). This apoptosis also stimulates an immune response with the release of IL-1β and IL-18 (55). These cytokines lead to the influx of PMNs into the submucosa, which further promotes the passage of *Shigella* across the epithelium, as described above.

A recent microarray study found that several *S. flexneri* genes are induced upon entry into U937 human macrophages. Acid-induced genes such as *ydeO* and *asr* were upregulated, as well as genes that respond to oxidative stress such as *sbp, soxS, ibpA, ibpB*, and *ycfR*, and *msrA*. Low levels of SOS-response genes were induced including *uvrY, umuCD, sulA, recA*, and *lexA*. However, *katG* and *katE* were not induced, indicating that *S. flexneri* is likely not exposed to peroxide in macrophages. Genes that are important in iron, magnesium, and phosphate acquisition were also upregulated, including *sitABCD, mgtA, phoRB*, and *proVWX* (65). All of these genes probably ensure the survival of *Shigella* cells before they can escape from the phagocyte.
(3) Entry into and replication in epithelial cells

After escaping macrophages or dendritic cells, *Shigella* encounters the basolateral membrane of the epithelial cells. Like *Salmonella*, *Shigella* uses the trigger mechanism to enter the epithelial cell. The TTSS is important for this uptake. The bacterial proteins IpaB/C form a pore in the host cell that delivers effectors into the eukaryotic cell (23). *S. flexneri* recognizes host cells by the abundance of cholesterol (lipid rafts) in their plasma membrane and adheres to the CD44 and α5β1 receptors with the IpaBCD complex (114). A macropinocytic pocket is formed by rearrangement of the actin cytoskeleton. The effectors VirA and IpaC are secreted into the host cytosol and instigate the depolymerization of actin microtubules and the nucleation of actin, respectively. IpaC also activates host cell Rho GTPases which trigger the formation of filopodial extensions around the bacteria. IpgD, a phosphoinositide phosphatase disassociates the actin cytoskeleton from the plasma membrane (55). The Arp2/3 complex and Abl tyrosine kinases are responsible for actin re-polymerization to form the pocket. The bacterial cells then enter the macropinocytic pocket and actin is depolymerized to close it. IpaA is important in this process (23).

Unlike *Salmonella*, *Shigella* escapes the phagocytic vesicle with the secretion of IpaB, in the same way that it escaped the vesicle in macrophages. *Shigella* can then replicate within the host cell cytoplasm. Like *Listeria* and *Rickettsia*, *Shigella* travels throughout the host cell by actin-based motility (23). *Shigella* secretes the outer membrane protein IcsA (VirG), which is localized exclusively at one pole of the bacterium, with the help of DegP, SopA/IscP and PhoN2 (55, 114). IcsA interacts with the host neural Wiskott-Aldrich syndrome protein (N-WASP) and vinculin, which stimulates Arp2/3-mediated actin polymerization (55). An actin tail forms behind the *Shigella* cells, which propels the cell through the host. VirA degrades the
cytoskeleton in front of the *Shigella* cells, creating a tunnel for the bacteria to move through (114). *Shigella* also uses this actin tail to enter neighboring epithelial cells. When *Shigella* encounters the lateral membrane in the epithelial cell, the propulsion from the actin tail pushes the bacteria through the membranes and into the neighboring cell. The membranes surrounding *Shigella* are lysed by IpaB and IpaC, and VacJ is also used to release *Shigella* into the host cytoplasm. The bacteria are then free to replicate in the new host cell (55). This movement of bacteria from cell to cell on the lateral side is also responsible for tissue damage in the host.

A microarray study concluded that the transcriptional profile of *S. flexneri* in HeLa epithelial cells was similar to that in U937 macrophages, except for a few notable differences. Acid resistance genes were not induced in HeLa cells, as compared to U937 cells. Genes necessary for energy production, such as NDH-1, cytochrome bo3, and succinate dehydrogenase were expressed later in HeLa cells than in macrophages, which the researchers concluded meant that *Shigella* is better able to replicate in epithelial cells, as compared to macrophages (65).

**Immune response to *Shigella* infection**

*Shigella* infection is often contained in the intestine and gut-associated lymphoid tissue and does not cause a systemic infection. The innate and acquired immune response to *Shigella* infection is described below.

**(1) Innate immune response to *Shigella* infection**

It has proven difficult to find an appropriate animal model to study the immune response to *Shigella* infection. Mice are resistant to *Shigella* infection, and the natural host is exclusively humans and primates. The best animal model involves macaque monkeys, where animals are infected with a high dose (10⁹ CFU) of *Shigella*, however these studies are costly. Rabbit ileal loops can be infected with *Shigella* and are often used to assess the virulence of *Shigella*
mutants. In addition, the keratoconjunctivitis assay in guinea pigs is often used to assess invasiveness of mutant bacteria. The most commonly used mouse models are the mouse pulmonary infection model and the newborn mouse model (inoculated orally) (94).

The innate immune system is the first response to ingestion of Shigella from contaminated food or another source. During infection, the bacteria first encounter the low pH of the stomach which they can survive well, since Shigella is extremely acid resistant. This acid resistance is probably due to several mechanisms, one involving RpoS and another involving glutamate decarboxylase (63).

Once they reach the intestine, they encounter antimicrobial peptides. Defensins and cathelicidins are common antimicrobial peptides in humans. However, it has been shown that Shigella can inhibit the production of these compounds (94). The glycoprotein lactoferrin does have antimicrobial action against Shigella. This protein impairs Shigella’s ability to invade the intestinal epithelium by inducing degradation of IpaB and IpaC (94).

When Shigella is translocated across the intestinal epithelium it encounters resident macrophages. These cells are not effective at killing Shigella, and the bacterial cells induce apoptosis of the macrophages, as described in previous sections. However, the apoptosis and tissue destruction resulting from Shigella infection induces the production of cytokines. IL-8 released from macrophages recruits natural killer cells and T lymphocytes, which produce IFN-γ. IFN-γ activates macrophages and fibroblasts, which can combat the infection. In addition, the IL-8 recruits PMNs, which are able to phagocytize and kill Shigella. PMNs are thought to confine Shigella infection to the intestinal mucosa and submucosa, and prevent systemic spread of the bacteria (55). Epithelial cells also sense the bacterial LPS and activate NF-κB, which also results in the secretion of IL-8 and the induction of the inflammatory response (94). The
resulting inflammation is intense and persistent. It has been found that the inflammation induced by shigellosis can continue for over a month (55).

(2) Adaptive immune response to *Shigella* infection

Not as much is known about the cellular immune response to *Shigella*, in comparison with the literature available for other intracellular bacterial pathogens. There is ample information about the humoral response. It is known that T lymphocytes are recruited upon *Shigella* infection, and may be both T_{H1} and T_{H2} types. However, mice deficient in T cells were still able to resist challenge with *Shigella* in the mouse pulmonary model (55). It was recently shown that αβ T cells and natural killer cells help control Shigella infection during the acute stages of the disease by amplifying the inflammatory response (94).

Although the cellular immune response may not be very important, it is believed that the humoral immune response is critical in controlling shigellosis. A recent study showed that the delayed antibody production in children, as compared to adults, made them more susceptible to shigellosis (100). Secretory IgA, IgG, and IgM all seem to be important in humoral immunity to *Shigella* and are secreted in response to LPS or virulence plasmid encoded proteins. IgA can coat the *Shigella* cells, reducing their ability to attach to epithelial cells and can promote cell-mediated killing of the bacteria (55). IgG and IgM may be involved in complement-mediated killing or cellular cytotoxicity of *Shigella*.

*Shigella* infections are often resolved within 5-7 days (www.cdc.gov). Death, if it occurs, is usually from complications including bacteremia and dehydration (94). Even if the infection is resolved, inflammation can persist for a month, and normal bowel function may not occur until then. In addition, successful adaptive immunity to a certain *Shigella* strain may only occur after
several infections with that strain (94). Because of this and the limited treatment options for 
shigellosis, a successful vaccine would be the best weapon against this disease.

**STRUCTURE AND FUNCTION OF HYDROGENASES**

The hydrogenase field has seen a renewed interest in the structure and function of hydrogenases lately, with expectation that hydrogenases can be used to produce H₂ as an alternative energy source. Over 100 hydrogenase enzymes have been characterized from organisms in all domains of life (128). These hydrogenases often have central roles in energy production for the organisms. Hydrogenases can be divided into three types, the metal-free H₂ases, the [NiFe]-H₂ases, and the [FeFe]-H₂ases. An overview of the structure and roles of each type of hydrogenase in these diverse organisms is presented below.

**Classes and distribution of hydrogenases**

Most of the hydrogenases are found in the domains Archaea and Bacteria, but some are found in primitive Eucarya as well. The distribution of each of the three types of hydrogenases is discussed in the following section.

(1) **Metal-free hydrogenases/ Fe-only hydrogenases**

Until recently, it was thought that all hydrogenases were iron-sulfur proteins that contained either a Ni and a Fe atom at the active site or two Fe atoms. In 1990 the Thauer group published an article describing a new type of hydrogenase from methanogenic archaea called “H₂-forming methylenetetrahydromethanopterin dehydrogenase” or Hmd (142). This enzyme catalyzes the transfer of H⁺ from H₂ to methenyl-H₄MPT⁺ to form methylene-H₄MPT (128). It was originally thought that Hmd did not contain any metal atoms, since they do not contain any iron-sulfur clusters. However, the Thauer group later discovered that there is an iron-containing cofactor in Hmd, and the hydrogenases were re-classified as “iron-sulfur cluster free” H₂ases (69).
Not much is known about the distribution of iron-sulfur cluster free \( \text{H}_2 \)ases. However, it is known that this class of hydrogenases is phylogenetically distinct from \([\text{FeFe}]\) and \([\text{NiFe}]\)-\( \text{H}_2 \)ases (129). So far, they have been found only in certain methanogenic archaea growing on \( \text{H}_2 \) and CO\(_2\), including *Methanothermobacter marburgensis* (68).

2) \([\text{NiFe}]\) - hydrogenases

The \([\text{NiFe}]\) – \( \text{H}_2 \)ases are the most common type of hydrogenase, and also the most thoroughly studied. These hydrogenases have both a large and a small subunit and contain both Ni and Fe atoms in the active site. They can be divided into four groups and are found in both bacteria and archaea (but not in eukaryotes) (129). Groups are designated based on the function of the hydrogenase and also follow the phylogeny of the hydrogenases to some extent (128). \([\text{NiFe}]\)-hydrogenases either catalyze the oxidation of \( \text{H}_2 \), or produce \( \text{H}_2 \) gas.

The Group 1 \([\text{NiFe}]\) – \( \text{H}_2 \)ases are called “uptake-type” respiratory hydrogenases because they catalyze the oxidation of \( \text{H}_2 \) to the reduction of various substrates (128). In this way, the cell is able to use \( \text{H}_2 \) as an energy source either by contributing to the proton gradient across the membrane or by the reduction of sulfate. These hydrogenases are membrane-bound enzymes connected to the electron-transport chain. The hydrogenase is anchored to the plasma membrane by the small subunit, which contains a twin-arginine element for translocation to the membrane. These hydrogenases are widely distributed, being found in groups as diverse as Proteobacteria, nitrogen-fixing bacteria, and methanogenic archaea. The nomenclature of these hydrogenases is also varied. In Proteobacteria such as *E. coli*, they are called Hya or Hyb. In the nitrogen-fixing bacteria such as *Rhizobium leguminosarum* and *Rhodobacter capsulatus* they are generally called Hup or Hox. The archaeal Group 1 \([\text{NiFe}]\) hydrogenases from organisms such as *Archaeoglobus fulgidus* are named Vho and Vht. The soluble \([\text{NiFe}]\) hydrogenases in Group 1
are generally found in sulfate-reducers such as *Desulfovibrio desulfuricans* and are called Hmc, Hyn, or Hyd (128).

The hydrogenases in Group 2 include enzymes from cyanobacteria and nitrogen-fixing bacteria. The Group 2 hydrogenases (Hup) in cyanobacteria such as *Nostoc* and *Anabaena* are located on the cytoplasmic side of the plasma membrane and are used as uptake-type hydrogenases under N$_2$-fixing conditions (129). Group 2 hydrogenases in *B. japonicum*, *R. capsulatus*, and *R. eutropha*, called HupUV and HoxBC are soluble enzymes found in the cytoplasm. They function as H$_2$-sensing hydrogenases, and trigger the production of the uptake-type hydrogenases (128).

Bidirectional [NiFe]-hydrogenases that are bound to soluble cofactors are clustered in Group 3. These enzymes are linked to cofactor 420, NAD, or NADP (129). These enzymes (called Frh, Frc, Fru, or Mvh, Vhu, Vhc) are found in methanogenic archaea such as *Methanosarcina mazei* or *Methanothermobacter marburgensis* and are used in an energy conserving manner. Group 3 enzymes (Hyh or Shy) are also present in hyperthermophiles such as *Pyrococcus furiosus* and *Thermococcus litoralis* and are used to couple the oxidation of H$_2$ to S$\textsuperscript{2-}$ reduction (128). Certain bacteria and cyanobacteria also contain Group 3 hydrogenases, called Hox, which may function to dispose of electrons during the initiation of photosynthesis (129).

The Group 4 [NiFe]-H$_2$ases are membrane-associated H$_2$-evolving enzymes. This group includes the well-characterized Hyc (and the less studied Hyf) from *E. coli* and other Proteobacteria. Group 4 hydrogenases are most commonly found in archaea and are present in methanogens and thermophiles such as *M. barkeri* and *P. furiosus*. These hydrogenases are called Eha, Ehb, Ech or Mbh respectively (128). *Rhodospirillum rubrum* contains a Group 4
hydrogenase CooLH which is induced by the presence of carbon monoxide. All of the Group 4 enzymes are used to dispose of reducing equivalents generated during the oxidation of C_1 organic compounds such as formate or CO under anaerobic growth (129).

(3) [FeFe] - Hydrogenases

In 1984 Huynh et al. demonstrated that not all hydrogenases contain nickel; some are iron-only hydrogenases (51). The [FeFe]-H_2ases are generally less abundant than [NiFe]-H_2ases and are different in several important ways. [FeFe]-H_2ases are typically monomeric (although they can be found as dimers, trimers, and tetramers) and do not contain Ni (129). They can be found in anaerobic prokaryotes and are the only type of hydrogenase present in eukaryotes. These enzymes generally function anaerobically and are used to produce H_2 (although a [FeFe]-H_2ase from *D. vulgaris* may be used to oxidize H_2).

Although functional hydrogenases are only found in a few eukaryotes, genes that are homologous to [FeFe]-H_2ases (called laminin-binding NARFs) are found in many eukaryotes (46). The NARF genes function in Fe-S metabolism. Bona-fide eukaryotic H_2ases (called Hyd) are found in special organelles called hydrogenosomes or in chloroplasts in anaerobic flagellates and cilates such as *Chlamydomonas, Trichomonas* and *Giardia*. They may help these organisms conserve energy under anaerobic conditions (129).

[FeFe]-H_2ases are present in prokaryotes such as clostridia and sulfate reducers. These enzymes can use a variety of electron donors or acceptors. They also have a variety of subunits and accessory domains, depending on the organism (128). *Clostridium pasteurianum* and *Megasphaera elsdenii* are two prokaryotes that do not contain [NiFe]-H_2ases, but only have [FeFe]-H_2ases (called Hyd). These prokaryotes are strict anaerobes and conserve energy by
fermentation. The hydrogenases, which are cytoplasmic, are thought to dispose of excess reductant produced by fermentation (2).

**Structure of hydrogenase active site and reactions catalyzed**

Hydrogenases are generally composed of one or two structural proteins called the large and small subunit. They either contain a Ni ion and iron-sulfur clusters, iron-sulfur clusters only, or just Fe in the catalytic subunit, based on the type of enzyme. All hydrogenases catalyze the production or oxidation of molecular hydrogen (H₂). The structure of the active site and type of reaction catalyzed by each type of hydrogenase is described in the following section.

(1) **Fe-only H₂ase active site structure and catalytic function**

The crystal structure of the Hmd holoenzyme from *Methanocaldococcus jannaschii* has recently been obtained (117). The Hmd apoenzyme structure has also been analyzed (96). However, most of the information known about this enzyme has been generated from IR, Mössbauer, and X-ray absorption spectra analysis.

The Hmd hydrogenase is a homodimer with a molecular mass of 76 kDa (118). It does not contain iron-sulfur clusters, but instead has one iron molecule per monomer (143). The iron at the active site of Hmd is associated with the methenyl-H₄MPT⁺ cofactor that is important for enzyme activity (117). In addition, the Fe is probably bound to two CO ligands, one S, and one or two O/N ligands (116, 118). It is thought that the Fe binds to H₂, and catalyzes the transfer of H⁻ to methenyl-H₄MPT⁺ to form methylene-H₄MPT (117, 118). This enzyme does not catalyze the reaction H₂ ↔ H⁺ + e⁻, unlike the [FeFe] and [NiFe]-H₂ases (143).

(2) **Structure and catalytic function of the [NiFe]-H₂ase active site**

Much work has been done to characterize the structure and active site of [NiFe]-H₂ases. In the early 1980s, it was shown that some hydrogenases contained nickel, and the nickel may be
important in the catalytic activity of these enzymes (5, 44). Numerous Mössbauer, electron
paramagnetic resonance (EPR), and Fourier transform infrared (FTIR) spectroscopy studies;
along with the crystal structures of purified hydrogenase have revealed the structure of these
enzymes and their catalytic action (reviewed by (129)).

[NiFe]-H$_2$ases are generally composed of a large and small subunit, with the active site
present in the large subunit. The small subunit often contains a membrane-anchoring component
(97). The small subunit also contains three [4Fe-4S] clusters (one of which can be a [3Fe-4S]
cluster) (118). The active site is dinuclear, containing a Ni and a Fe atom. The nickel atom is
connected to the Fe atom by two cysteiny1 sulfur ligands. Two other sulfur ligands bind to the
Ni as well. Two CN$^{-}$ ligands and one CO bind to the Fe atom (50, 95, 130). These ligands make
the Fe and Ni more catalytically active.

[NiFe]-H$_2$ases catalyze the oxidation or production of H$_2$. H$_2$ may enter the enzyme
through a hydrophobic gas channel that leads to the active site (87). The hydrogenase must be
reduced in order to be active (129). H$_2$ then binds to the Ni atom, is split and electrons are
passed to the nearby [4Fe-4S] clusters (37). The binding / splitting reaction is oftentimes referred
to as H$_2$ activation. The reduced Fe-S clusters then transfer the electrons to other electron
acceptors. The proton is released, leaving a metal-bound hydride. The hydride is then oxidized
and released as H$^{+}$ and the enzyme is ready to accept another H$_2$ (7, 129). Isotope exchange
assays with D$_2$ gas indicated that the H$_2$ generating reaction occurs by the same pathway, except
presumably backwards (79).

(3) [FeFe]-H$_2$ase active site structure and catalytic function
The [FeFe] - hydrogenases were first described in the 1930s (121). In the late 1930s to early
1940s it was known that these hydrogenases contained Fe linked to a sulfur ligand (118, 131).
Later studies using a variety of spectroscopic methods combined with crystal structure analysis have contributed to our knowledge of the [FeFe]-H₂ase enzyme and active site structure.

[FeFe]- Hydrogenases have a unique cluster of Fe atoms at the active site called the H cluster (2). There are two Fe atoms in the active site that are attached to each other via two sulfur ligands and carbon monoxide. Each Fe atom also has a CO and CN⁻ ligand. One of the active site Fe atoms is bound to a [4Fe-4S] cluster (known as the F cluster) by a cysteinyll sulfur. The hydride/dihydrogen is thought to bind the other Fe in the active site (90, 93, 129).

These [Fe-Fe] enzymes most often catalyze the H₂ formation reaction, rather than H₂ oxidation. In the catalytic cycle for H₂ oxidation, the enzyme is first activated by the oxidation of the H-cluster. H₂ binds to the Fe atom distal to the [4Fe-4S] cluster. Electron transfer reactions from the H cluster to the F cluster ([4Fe-4S]) result in the generation of a hydride and a proton (2). When the enzyme catalyzes the production of H₂, electrons reduce the F cluster, which reduces the H cluster. The H cluster then reduces protons and generates H₂ (1).

**Cellular location and role of hydrogenases**

Hydrogenases usually serve one of two purposes. They generate H₂, which disposes of excess reductant produced by fermentation, or they oxidize H₂ and couple the generated electrons to a variety of terminal electron acceptors in respiratory pathways. A third, less common use has also been described; some hydrogenases sense the presence of H₂ and start a regulatory cascade which influences the abundance of other proteins. The location of these hydrogenases is related to their role in the cell. The specific role of each type of hydrogenase and their location in the cell is discussed below.
(1) Role of Fe-only hydrogenases and cellular location

As discussed above, the Fe-only hydrogenases catalyze the reaction methenyl-H₄MPT⁺ + H₂ → methylene-H₄MPT + H⁺ (118). These hydrogenases are cytoplasmic and have only been found in some methanogenic archaea. Organisms which contain these hydrogenases conserve energy by reducing CO₂ with H₂ to form methane. Hmd is used to catalyze the above reaction, which is an important step in the production of methane (reviewed in (124)). However, the action of two other enzymes can catalyze the reduction of methenyl-H₄MPT⁺; therefore not all methanogens contain Hmd. The enzymes Frh (F₄₂₀-reducing [NiFe]-hydrogenase) and Mtd (F₄₂₀-dependent methylene-H₄MPT dehydrogenase) catalyze the reduction of F₄₂₀ with H₂ and the reduction of methenyl-H₂MPT⁺ with F₄₂₀H₂, respectively (118).

Since there is more than one pathway to reduce methenyl-H₄MPT⁺ in these organisms, Hmd is only used under certain conditions. It was found that Hmd is used under low nickel conditions, which is logical since Frh is a [NiFe]-hydrogenase (3). It was recently found that this regulation is transcriptional, and that Hmd expression is not affected by H₂ concentration (4). The apparent redundancy in enzyme activity indicates that this pathway is important in the energy conserving reactions of these organisms. Methanogenesis will occur when nickel is abundant or scarce, because of these two independent pathways.

(2) Function of [NiFe]- hydrogenases and cellular location

[NiFe]- hydrogenases are the most abundant and widely distributed type of hydrogenase. These hydrogenases have different uses in each type of organism and are found in various cellular locations. The function and location of Group 1, 2, 3 and 4- type [NiFe]-hydrogenases are discussed below.
Group 1 [NiFe]-hydrogenases catalyze the oxidation of H₂ gas to protons and electrons (128). These hydrogenases are membrane-bound. H₂ is used as an energy source, as electrons are passed to neighboring quinones and cytochromes while protons are used to maintain the membrane potential. This hydrogen oxidation is coupled to the reduction of various electron acceptors, from O₂ in nitrogen-fixing bacteria, photosynthetic organisms, and probably some facultative anaerobes (19, 73), to anaerobic electron acceptors like nitrate, sulfate, fumarate or CO₂ in proteobacteria and anaerobes (128). These enzymes are used in different modes of metabolism by a variety of organisms. Methanogens use them to oxidize H₂ to CO₂ with the production of methane. Nitrogen-fixing bacteria use these hydrogenases to reclaim energy from H₂ produced by the nitrogenase enzyme. Sulfate-reducers use these enzymes to couple the oxidation of H₂ to sulfate reduction. Enteric bacteria couple the oxidation of H₂ with fumarate or nitrate reduction. Overall, the Group 1 hydrogenases oxidize H₂ which is used as an energy source by a chemiosmotic mechanism (128).

Group 2 [NiFe]-hydrogenases do not contain a signal peptide in the small subunit. The enzymes in this group are either uptake hydrogenases from cyanobacteria or H₂-signaling hydrogenases in B. japonicum, R. capsulatus, and R. eutropha (128). In cyanobacteria such as Anabaena and Nostoc, it is thought that the hydrogenase oxidizes H₂ and transfers electrons to mobile carriers in the electron-transport chain. The cyanobacterial hydrogenase does not have a signal peptide, suggesting that the small subunit is not membrane-bound. However, its function suggests that the hydrogenase should be in the membrane. It has been hypothesized that another small peptide may be associated with the hydrogenase and anchors it to the membrane (123). This hydrogenase probably oxidizes the H₂ produced by nitrogenase. It also couples H₂ oxidation to O₂ reduction, lowering the partial pressure of O₂ (which inhibits nitrogenase...
function) (47, 123). The H2- sensing hydrogenases are cytoplasmic. They sense the presence of H2 and de-repress the uptake-type hydrogenase. The H2-sensing hydrogenase and the HupT/HoxJ histidine kinases control the phosphorylation of HupR/HoxA. HupR/HoxA are transcriptional activators that activate the uptake-type hydrogenase (128). The H2 signaling hydrogenase is not used for energy conservation, but is instead involved in this regulatory cascade.

Hydrogenases in Group 3 are cytoplasmic, reversible, and bind soluble cofactors (such as F420, NAD or NADP). The hydrogenases that are associated with F420 are found in methanogens. The Methanosarcinales use this hydrogenase to generate a proton-motive force, much like the function of the Group 1 hydrogenases. The F420H2 oxidation is coupled to heterodisulfide reduction (28). In hydrogenotrophic methanogens, the oxidation of F420H2 by this hydrogenase is the only electron source for CO2 reduction (128). Hyperthermophiles also contain bidirectional Group 3 hydrogenases. The cytoplasmic hydrogenase from P. furiosus is thought to dispose of excess electrons generated during fermentation and is coupled to S" reduction (70, 128). Bidirectional NAD(P) hydrogenases are found in cyanobacteria, R. eutropha, and in photosynthetic bacteria (T. roseopersicina) (129). In cyanobacteria, this hydrogenase is light-stimulated, and may be used to dispose of electrons released by photosynthesis (128, 129). However, it may have other functions that are debatable. It may be part of a respiratory complex or involved in disposing of excess electrons generated during fermentation (123).

Membrane-associated H2-evolving [NiFe]- hydrogenases are called Group 4 hydrogenases. These hydrogenases are found in enterics like E. coli, S. flexneri, and Salmonella species, and also in Rhodospirillum rubrum. They have also been found in Archaea (128). In E. coli, the Group 4 enzyme Hyc is used to couple formate oxidation to H2 production. The E. coli
Hyf (another Group 4 enzyme) is thought to produce H₂ and link formate oxidation to respiratory pathways (6). In *R. rubrum* the Group 4 hydrogenase together with the CO dehydrogenase catalyze the production of H₂ and oxidation of CO to CO₂. Group 4 hydrogenases from Archaea also evolve H₂. They are used during acetate oxidation in *Methanosarcina barkeri* and *P. furiosus* (128). Overall, the Group 4 hydrogenases are used to dispose of reducing equivalents produced during anaerobic fermentation (129).

(3) **Role of [FeFe]- hydrogenases and cellular location**

[FeFe]- hydrogenases are found in anaerobic prokaryotes, a few unicellular eukaryotes, and in *Desulfovibrio vulgaris* (129). They are cytoplasmic proteins in anaerobic prokaryotes. In eukaryotes, they are found in hydrogenosomes or chloroplasts, while they are periplasmic in *D. vulgaris* (2). The [FeFe]- hydrogenases serve various purposes in different organisms. The anaerobic bacterium *Clostridium pasteurianum* contains two [FeFe]- hydrogenases. One is bidirectional and generates H₂ in order to dispose of excess reductant produced during fermentation, while the other oxidizes H₂ (21). *Megasphaera elsdenii* has a [FeFe]- hydrogenase that generates H₂ during fermentation. The [FeFe]- hydrogenase in *D. vulgaris* oxidizes H₂ and plays a role in hydrogen recycling (2, 129). The hydrogenases in the eukaryote *Chlamydomonas reinhardtii* probably produce H₂ in order to dispose of reducing equivalents generated in fermentation, like the [FeFe]- hydrogenases in anaerobic prokaryotes (89).

**TRANSCRIPTIONAL REGULATION OF ENTERIC HYDROGENASES**

The hydrogenases found in enteric bacteria are [NiFe]- hydrogenases that are classified as Group 1 or Group 4-type (128). The Group 1-type hydrogenases are called *hya* and *hyb* in *E. coli* and *Shigella* (128). In *Salmonella* they are called *hya*, *hyb*, and *hyd* (138). These hydrogenases oxidize H₂ in an energy-conserving manner. The Group 4 hydrogenases in enterics are called
*hyc* and *hyf*. They are used to generate H₂ during fermentation (128). Since these hydrogenases are linked with the metabolic processes used by the organism at any particular time, they are subject to complex regulation. Hydrogenase regulation by the aerobic/anaerobic response, pH, catabolite repression, affect of metabolite concentration, and regulation by environmental factors is discussed in the following section.

**Regulation of enteric hydrogenases by the aerobic/anaerobic response**

Enteric bacteria like *E. coli* have maximal growth rates when oxygen is present. When oxygen becomes scarce, enzymes involved in fermentation or anaerobic respiration are activated, depending on the metabolites present. Enzymes that are used for aerobic respiration are repressed, since they are no longer needed. In addition, proteins that are sensitive to oxygen are expressed only under anaerobic conditions (60). Most enteric hydrogenases are preferentially expressed anaerobically, however some are expressed under aerobic conditions (102, 138). Several transcription factors are involved in the regulation of hydrogenases during the aerobic/anaerobic switch and are discussed below.

The Fumarate Nitrate Respiration (FNR) protein is an important global anaerobic transcription factor. FNR is involved in the regulation of about half the genes involved in the anaerobic/aerobic switch (106). It contains iron-sulfur clusters which are used to sense the oxygen levels in the cell. The active form, present under anaerobic conditions, is a dimer containing [4Fe-4S] clusters. As the concentration of O₂ increases, the dimer breaks into monomers that contain [2Fe-2S] clusters or breaks into apoenzyme monomers (57). The monomers cannot bind DNA and are inactive. The dimer binds DNA at a specific recognition site (TTGAT-N₄-ATCAA) and often activates transcription of anaerobic genes, although it can
also act as a transcriptional repressor (126). FNR alters the transcription of genes through DNA bending and interaction with the $\alpha$-subunit of RNA polymerase (135).

In *E. coli*, FNR was shown to have an indirect effect on the expression of *hya* and *hyb*, perhaps by altering available nickel levels in the cell (102) through the expression of the nickel transporter genes *nikABCDE* (104, 137). It was also found that FNR activates the expression of *hypBCDE*, which are required for hydrogenase maturation (60, 83). This result suggests that *hyc* and *hyf* are probably indirectly regulated by FNR as well. In *S. enterica* serovar Typhimurium, some of the uptake-type hydrogenases were less abundant in an FNR mutant, suggesting that they may be regulated by FNR (53). Our studies have shown that FNR activates *hya* and *hyb* transcription in *S. enterica* serovar Typhimurium (138).

Aerobic Respiration Control (Arc) is another global anaerobic transcription factor that is involved in hydrogenase expression. ArcA and ArcB work as a two-component system to repress genes involved in aerobic respiration. This system also activates a few genes in *E. coli*, namely cytochrome d oxidase and pyruvate formate lyase (60). The Arc proteins are abundant under both aerobic and anaerobic conditions, but are only active under low oxygen or anaerobically. The sensor kinase ArcB detects the redox state of the cell and under anaerobic conditions phosphorylates the response regulator ArcA. Under oxidizing (aerobic) conditions, ArcB contains two disulfide bonds that inhibit kinase activity. When the oxygen level decreases in the cell, the bonds are reduced by quinones, and the sensor kinase regains activity (75). Phosphorylated ArcA binds to DNA at the consensus sequence $(A/T)\text{GTTAATTA}(A/T)$ and interacts with other DNA-binding proteins to block transcription. Alternatively, ArcA can bind to other DNA sequences and promote transcription by DNA bending and interactions with the $\alpha$ C-terminal domain of the RNA polymerase (67).
In *E. coli*, ArcA probably directly regulates *hya* and *hyb*, since sequence analysis revealed binding sites in the regulatory region of both promoters. Studies with *lacZ* fusions to the *hya* and *hyb* promoters suggest that ArcA activates *hya* expression and represses *hyb* expression (18, 102). In addition, ArcA regulates the production of formate, since both FNR and ArcA/B activate pyruvate formate lyase (PFL) expression (108). This regulation likely influences formate hydrogen lyase and *hyc* activity. Our studies have shown that ArcA activates *hyb* and represses *hyd* expression in *S. enterica* serovar Typhimurium (138).

*E. coli* has another transcription factor involved in the aerobic/anaerobic switch, called AppY (acid phosphatase protein). AppY is an anaerobic growth-phase dependent transcriptional activator and is probably a DNA-binding protein. It is a member of the AraC family of transcriptional activators and regulates cytochrome *bd* oxidase expression (8, 9). The expression of *hya* is upregulated by this protein in *E. coli* (18, 102). However, it does not appear that *S. enterica* serovar Typhimurium has a homolog of AppY.

IscR (Iron-sulfur cluster regulator) influences the expression of about 40 genes in *E. coli*, including the iron-sulfur cluster synthesis genes *iscRSUA* and genes for anaerobic respiratory proteins that contain iron-sulfur clusters (39). This protein is an unusual regulator of anaerobic respiration proteins because it functions aerobically. It contains a [2Fe-2S] cluster and is a member of the Mar/Sox/Rob family of transcriptional regulators. It has been hypothesized that IscR is only functional when it contains the [2Fe-2S] cluster. Therefore, when there are many [Fe-S] proteins present, free [Fe-S] clusters would be limiting and IscR would be inactive. The Isc proteins would produce more [Fe-S] clusters, thereby activating IscR and feedback inhibition would result (115). A recent study reported that *E. coli* *hya* and *hyb* genes were repressed by IscR under aerobic conditions, but not anaerobically (39). Our results indicate that *S. enterica*
serovar Typhimurium \textit{hyb} is repressed by IscR aerobically; however the \textit{hya} genes do not seem to be regulated by IscR (138).

**Expression of enteric hydrogenases under different growth conditions and affect of metabolite availability**

Enteric bacteria such as \textit{E. coli} have multiple hydrogenases that catalyze the same reaction. These hydrogenases are used under different growth conditions and are optimally expressed under the condition that they are most used. Their expression is influenced by the presence of certain metabolites. For example, if nitrate is available, the expression of certain hydrogenases is repressed. The rationale from the cell’s point of view may be that nitrate catabolism yields more energy than hydrogen metabolism, or that H\textsubscript{2} oxidation via that particular (repressed) hydrogenase is energetically best coupled to other terminal acceptors. Hydrogenase expression under different growth conditions, catabolite and other metabolite repression is discussed in the following section.

**(1) Expression of hydrogenases under different growth conditions**

In \textit{E. coli}, both \textit{hya} and \textit{hyb} are maximally expressed under anaerobic respiration conditions with glycerol and fumarate as the terminal electron acceptor (102). It is fairly well accepted that \textit{hyb} is used to oxidize hydrogen in an energy-conserving manner during anaerobic respiration (11, 82, 109). However, the role of \textit{hya} is not as clear. It has been hypothesized that \textit{hya} in \textit{E. coli} is used to recycle H\textsubscript{2} generated by \textit{hyc} and \textit{hyf} during fermentative growth (109, 111). Sawers \textit{et al.} measured hydrogenase enzyme abundance in \textit{E. coli} and demonstrated that \textit{hya} is present at high levels during fermentative growth, which supports the idea that \textit{hya} is used to recycle the H\textsubscript{2} produced fermentatively (109). The H\textsubscript{2}-generating hydrogenase \textit{hyc} is most abundant under fermentative conditions with formate, as is expected due to its role in fermentative growth (109).
The regulator FhlA is involved in the activation of \textit{hyc} in the presence of formate (113). HycA is a repressor that may bind to FhlA in the formate-binding site and repress FhlA activity(119). In \textit{E. coli}, \textit{hyc} expression is $\sigma^{54}$- dependent, while \textit{hya} and \textit{hyb} expression is $\sigma^{70}$- dependent (61, 66).

\textit{S. enterica} serovar Typhimurium has three H$_2$- oxidizing enzymes, \textit{hya}, \textit{hyb}, and \textit{hyd}. Researchers in the 1980s measured the activity associated with two of these enzymes, based on homology to the \textit{E. coli} uptake-type hydrogenases. Isoenzyme 2 (Hyb) was present under all growth conditions tested, except when nitrate was present in the growth media. Hyb was necessary for anaerobic respiration-dependent hydrogen uptake, since this activity was greatly reduced in a strain lacking that isoenzyme (112). Our study found that \textit{hyb} was maximally expressed when cells are grown with glycerol and fumarate (138). Sawers et \textit{al.} reported that \textit{S. enterica} serovar Typhimurium isoenzyme 1 (analogous to both Hya and Hyd) was present in high levels under fermentative growth (112). Our study clarified this result, since we demonstrated that \textit{hya} was expressed under fermentative growth, while \textit{hyd} was maximally expressed under aerobic conditions (138). Hyc activity was greatest in cells grown fermentatively with glucose, as was expected (112).

\textbf{(2) Catabolite repression of hydrogenase genes}

Catabolite repression is a phenomenon where the expression of certain genes is lower in the presence of a preferred catabolite, such as glucose. This occurs in the following manner for glucose in \textit{E. coli}. First, the presence of glucose inhibits adenyl cyclase. This results in lower cyclic AMP (cAMP) levels. Promoters that are regulated by cAMP-CRP (cyclic AMP receptor protein) are no longer activated, and are expressed at lower levels than before. The overall affect
is the preferential expression of genes used to break-down glucose at the expense of other metabolic pathways (42).

The *E. coli* *hyb* is subject to catabolite repression, while *hya* is not. Expression of *hyb* was higher when cells were grown on ribose than in media containing glucose, indicating that the presence of glucose repressed the expression of *hyb*. This repression was mediated by the cAMP-CRP system, since *hyb* had lower expression in *crp* and *cy a* mutant cells (102). In a *S. enterica* serovar Typhimurium study measuring isoenzyme presence or absence, *hyb* was repressed by glucose. This repression was mediated by CRP (53). In our study, the gene expression of each hydrogenase was measured in the presence of different sugars. Our results agree with the Jamieson study in that the *hyb* genes were repressed when cells were grown with glucose. We also found that *hya* and *hyd* are not repressed by glucose (138).

(3) Nitrate repression of hydrogenase genes

In enteric bacteria, the presence of nitrate often represses the expression of genes involved in other anaerobic respiration pathways. The two-component regulatory systems NarQ/P and NarX/L are involved in this regulation. NarQ and NarX are membrane-bound proteins that sense the presence of nitrate or nitrite. They phosphorylate NarL and NarP, which are DNA-binding proteins (99, 134). NarL and NarP bind to the heptamer TACYYMT (Y=C or T, M= A or C) in target promoters. In order for NarP to bind to the sequence, there must be a pair of heptamers with 2 bp in between sequences (7-2-7 arrangement). NarL will bind at 7-2-7 heptamer pairs, or some other arrangement of heptamers (25).

Several biochemical studies demonstrated that *E. coli* Hya and Hyb protein levels are repressed in the presence of nitrate (11, 109). A reporter fusion study examined this repression further. This study revealed that both *E. coli* *hya* and *hyb* gene expression is repressed by
nitrate, mediated by both NarL and NarP (102). In *S. enterica* serovar Typhimurium, hydrogen uptake was abolished in cells grown with nitrate (112). Nitrate also represses formate hydrogen lyase (FHL) expression (12). We found that nitrate repressed *hya* expression in *S. enterica* serovar Typhimurium. This repression was mediated by NarL, but not by NarP. There is a potential NarL heptamer recognition sequence in the promoter region of the *hya* operon. The heptamer was not in the 7-2-7 arrangement, as described by Darwin et al. (25), which provides further evidence that NarL was involved in the observed repression, but NarP was not. When this site was mutated, gene expression was no longer repressed by nitrate (138).

**Effect of pH on hydrogenase expression**

Enteric pathogens encounter a significant drop in pH (as low as pH 2 in the stomach) upon entry into the host digestive tract. This drop in pH influences the expression of a variety of genes, including those involved in the acid tolerance response (ATR) and genes needed to survive while inside the host (105). The pathogen also encounters a low pH if it resides inside phagocytes. The pH of the *Salmonella*-containing vesicle in macrophages and other phagocytes is around 5 (24, 30).

*E. coli* and *S. flexneri* have glutamate and arginine decarboxylase systems that are used to survive in pH as low as 2 (77). *S. enterica* serovar Typhimurium does not have this system and cannot survive pH lower than 4 for long periods of time (105). When *S. enterica* serovar Typhimurium encounters acid, it changes the transcription of multiple genes including those involved in cell surface maintenance, general stress responses, and efflux pumps. The regulators *rpoS, fur, phoPQ, ompR*, and *envZ* are induced by acid stress and are used to influence the transcription of the ATR genes (105).
Enteric bacteria also encounter low pH outside of the host. When they grow by mixed-acid fermentation, the pH of the media drops. Hyc produces H$_2$ during fermentative growth, which disposes of excess reductant and can help maintain pH homeostasis inside the cell (86). Both $hyc$ and $hyf$ expression are upregulated under acidic conditions in $E. coli$ (110). The presence of formic acid is especially important in the transcriptional activation of these operons by FhlA. King and Przybyla found that the $E. coli$ $hya$ expression is also upregulated under acidic conditions (pH 5), while $hyb$ expression is highest under alkaline conditions (pH 8.5) (58). The acid induction of $hya$ was not mediated by RpoS, but may be affected by ArcA. The expression of hydrogenases is necessary for survival at acidic pH, since an $E. coli$ hypF mutant (that had no hydrogenase activity) was sensitive to acid in stationary phase anaerobic cultures (48).

In $S. enterica$ serovar Typhimurium, Park et al. found that one of the NiFe hydrogenase promoters was acid-inducible (92). However, it is unclear whether this was the $hya$ or $hyd$ promoter. Our studies have shown that in $S. enterica$ serovar Typhimurium, $hya$ is acid-induced, while $hyb$ is maximally expressed under alkaline conditions, like in $E. coli$. Hyd expression was not influenced by pH (139, unpublished results). In addition, $hya$ is necessary for survival under acid stress (pH 4) (139). Hya may be used to recycle the H$_2$ produced by Hyc under low pH. This would help $S. enterica$ serovar Typhimurium cells survive acidic pH because Hya would contribute to energy conservation, as was suggested by Sawers et al. (112).

**Hydrogenase regulation by other environmental factors**

The expression and activity of hydrogenases can be influenced by factors such as the concentration of metal cofactors in the growth environment. Enteric hydrogenases contain nickel and iron cofactors. In addition, the formate dehydrogenase component of formate
hydrogen lyase contains selenium (selenocysteine) and molybdenum (40). There is a complex maturation pathway that is responsible for incorporating the metal centers into hydrogenase enzymes. The hyp genes are important in this pathway. Hydrogenase activity can depend on the availability of these metals, which is determined by their concentration in the extracellular milieu or by the regulation of metal uptake and efflux systems. Alternatively, the regulators that respond to metal availability can directly regulate hydrogenase expression. Fur and NikR are two of the most well studied regulators that respond to metal availability in E. coli.

The ferric uptake regulation (Fur) protein regulates the expression of genes in response to iron levels. When intracellular iron is available, it binds to Fur, which then can bind DNA at the Fur box in target promoters (a minimum of 3 repeats of NATA/TAT). This binding inhibits transcription. When Fe²⁺ is present in low concentrations in the cell, it is released from Fur, and transcription can occur (33). Fur regulates genes necessary for iron storage and metabolism and for the production of siderophores. However, Fur also regulates genes necessary for virulence, such as acid-resistance in S. enterica serovar Typhimurium and Shiga toxin in Shigella (64, 105). In H. pylori, microarray analysis has shown that hydrogenase expression is repressed by iron-free Fur (32). It is unknown whether Fur regulates hydrogenase expression in enteric bacteria. However, hydrogenase expression does respond to iron availability through IscR, as described in a previous section.

Nickel is essential for hydrogenase function, and it is also necessary for the function of glyoxalase I in E. coli (22). NikR is the E. coli transcriptional regulator that responds to nickel concentration in the growth media (27). The relationship between NikR and hydrogenase is complicated. NikABCDE is a nickel transporter whose function is essential for hydrogenase maturation. NikR binds to nickel and represses transcription of nikABCDE. The expression of
NikR is autoregulated and partially controlled by FNR (27). The (multi-component) hydrogenase assembly pathway may also regulate NikR function, either by sequestering nickel within the cell or by blocking NikR function when nickel is limiting (104). Therefore, hydrogenase influences NikR function and FNR plays a role in both NikR and hydrogenase regulation.

RcnR is another protein involved in the regulation of nickel levels in *E. coli*. It binds the *rcnA* (nickel exporter) promoter in the absence of nickel, which causes the repression of *rcnA* expression. When *rcnA* is deleted, NikR represses *nikABCDE*, lowering the amount of nickel available in the cell and reducing Hyc activity (52). In wild-type cells, RenA, RcnR, NikR and hydrogenase are all probably competing for nickel and influence nickel availability in the cell. Uncovering the interrelationships between these nickel binding components, transcriptional regulators, and hydrogenases should be a fruitful but challenging research endeavor in the next 10 years.

**ROLE OF HYDROGENASES IN PATHOGENESIS**

Hydrogen gas is produced by intestinal prokaryotes that live in animal hosts. This gas is not used by the host, but instead is used as an energy source by other microorganisms living in the gut. However, the natural intestinal flora are not the only prokaryotes that can use H₂. Many bacterial pathogens contain genes that code for uptake-type hydrogenases. It has been recently shown that bacterial pathogens can use H₂ as an energy source while inside the host (71). The generation of H₂ by commensal prokaryotes and the use of this hydrogen by *Helicobacter* spp. and *S. enterica* serovar Typhimurium will be discussed in the following section.
Production of H₂ gas in the animal host

When cellulose and starch are ingested by animal hosts, they are not completely broken down in the upper digestive tract. The natural flora in the rumen or colon hydrolyze the undigested cellulose and starch to produce glucose oligosaccharides (85). The glucose is typically catabolized by the Ebden-Meyerhoff-Parnas pathway. The pyruvate that is produced is then broken down further by fermentation. Fermentation products are a mix of short-chain fatty acids such as acetate, propionate, succinate, butyrate and lactate. The major products in the human intestine are acetate, propionate and butyrate (84). Formate and ethanol are also produced, and the formate is further broken down to H₂ and CO₂. These compounds are produced by intestinal anaerobes in the genus Bacteroides, Selenomonas, Succinivibrio, Succinomonas, Butyribrio, Eubacterium, Lachnospira, Bifidobacterium, Butyribrio, and Lactobacillus. Organisms such as Ruminococcus albus can also produce H₂ from NADH or pyruvate (85). The amount of H₂ produced is dependent on the diet of the animal. In humans, H₂ is produced at high levels when non-digestible oligosaccharides (such as cellulose) are eaten (13).

The H₂ that is produced is removed from the animal in several different ways. It can be lost in feces or flatus, or can be absorbed into the bloodstream and then exhaled through the lungs (71). About 20% of the H₂ produced in the colon is released into the lungs (17). Methanogenic prokaryotes in the rumen or gut can also utilize and remove H₂. They use H₂ and CO₂ to make methane (85). The H₂ that is not used by methanogens or lost as flatus can diffuse into various organs in the animal. Measurements using microelectrodes have revealed that the H₂ concentration is 43 µM in the mouse stomach, from 118-239 µM in the small intestine, and about 50 µM in the liver and spleen of live mice (73, 74, 91). This H₂ can be used as an energy source by some pathogenic bacteria that colonize these organs.
Use of hydrogen as an energy source during pathogenesis

Studies have estimated that there are $10^8$ prokaryotes/mL in the ileum and $10^{11}$ prokaryotes/g in the gut (comprising 400-500 different species) (14). The majority of the prokaryotes in the gut are obligate anaerobes. These organisms compete with each other and with the host for nutrients. A pathogenic bacterium that enters this environment must compete with the resident flora for nutrients. Since the host cannot use H$_2$, it would seem to be an optimal energy source for both the pathogen and the resident flora. The use of H$_2$ by Helicobacter species and S. enterica serovar Typhimurium has been characterized and is discussed below.

1. Use of H$_2$ by Helicobacter species

*H. pylori* is a bacterial pathogen of humans that resides in the stomach. It causes peptic ulcers and has been linked to a variety of gastric cancers (15). *H. hepaticus* colonizes the lower intestine and livers of mice and may be associated with liver disease in mice and in primates (71). *Helicobacter* spp. have a [Ni-Fe] uptake-type hydrogenase that is encoded by the hya genes (sometimes annotated as hyd in *H. pylori*) (13, 81).

*H. pylori* and *H. hepaticus* are somewhat fastidious organisms. They cannot tolerate high concentrations of oxygen and are classified as microaerophiles. *H. hepaticus* may be able to grow anaerobically as well. They conserve energy by respiration with O$_2$ as the terminal electron acceptor, although *H. hepaticus* may also use nitrate as a terminal electron acceptor. *Helicobacter* spp. will use organic acids, but glucose and other sugars do not support much growth (13). These organisms are routinely cultured on rich media that contains sheep’s blood or bovine serum. Since the number of growth substrates is somewhat limited, H$_2$ is seen as an important energy source for these pathogens.
The hydrogenase in *H. pylori* and *H. hepaticus* is membrane-bound. It is coupled to the respiratory chain and oxidizes H$_2$ with oxygen as the terminal electron acceptor (72, 74). These enzymes were able to reduce a variety of electron transport proteins such as cytochrome *c*, ferricyanide, and methylene blue, indicating that they are able to pass electrons to mobile electron carriers after splitting H$_2$ (74). The $K_m$ of each hydrogenase was 2.5 µM and 1.8 µM for *H. hepaticus* and *H. pylori*, respectively (13). The hydrogenases are probably saturated with H$_2$ when the bacteria are inside the animal, since H$_2$ levels were above 40 µM for every location tested.

Mouse infection studies have shown that hydrogenase is important for effective colonization and persistence by *H. pylori* and *H. hepaticus*. *H. pylori* hydrogenase mutants only were able to colonize 9 out of 38 mice inoculated with the strain, while the wild-type strain colonized every mouse. In addition, lower numbers of bacteria were isolated from the 9 mice that were colonized by the mutant strain, as compared to wild-type colonization numbers (91). Hydrogenase has a different, yet still important affect on the colonization of mouse livers with *H. hepaticus*. There was no significant difference in the abundance of wild-type and hydrogenase mutant *H. hepaticus* cells isolated from infected mice, as determined by qPCR. However, there was more inflammation and tissue damage in livers containing wild-type cells, indicating that hydrogenase may be necessary for full progression of the liver disease (81).

Hydrogen utilization is presumably an energy source for *Helicobacter* spp. The energy gained from H$_2$-splitting may be used for amino acid transport. The presence of H$_2$ led to increased amino acid uptake in *H. hepaticus*, which would help the organism grow more effectively (81). In addition, the protons produced when H$_2$ is split likely help maintain transmembrane potential, which allows the cell to do work in a variety of ways. Sequence
analysis has revealed that other *Helicobacter* spp. contain uptake-type hydrogenases (13). Future studies will be useful to determine if these hydrogenases are used for energy conservation while the bacteria dwell in animal hosts.

(2) **Hydrogen use by *S. enterica* serovar Typhimurium**

The genes encoding hydrogenase structural subunits and accessory proteins are present in the genomes of many enteric pathogens, including *Salmonella enterica* serovars Typhi and Typhimurium, *E. coli* 0157, *Shigella flexneri* and *S. sonnei*, and *Campylobacter jejuni* (71). From gene sequence analysis it can be confidently predicted that other pathogens, such as *Legionella* and *Actinobacillus* also contain H₂-oxidizing hydrogenases. Like in *Helicobacter* spp., these hydrogenases may be used to oxidize H₂ and provide energy for the bacterium during host colonization. Recent experiments have been done to determine if hydrogenase is important for *S. enterica* serovar Typhimurium virulence in the murine model.

*S. enterica* serovar Typhimurium has five [NiFe]-hydrogenases. Three of these hydrogenases are “uptake-type” and oxidize H₂. The Hyb large subunit amino acid sequence is about 94% homologous to the *E. coli* Hyb. The *S. enterica* serovar Typhimurium Hya and Hyd large subunits are both homologous to *E. coli* Hya, with 91% and 66% homology, respectively (138). *S. enterica* serovar Typhimurium also contains two H₂-evolving hydrogenases, called Hyc and Hyf, which are homologous to *E. coli* Hyc and Hyf. The hydrogen-uptake activity of wild-type *S. enterica* serovar Typhimurium (measured amperometrically in the presence of O₂) is about 13.3 nmol H₂/min/10⁹ cells.

It was recently found that hydrogenase is important for *S. enterica* serovar Typhimurium virulence in mice. The removal of all three uptake-type hydrogenases in a triple mutant resulted in a strain that was avirulent and unable to colonize the mouse liver or spleen. However, the
presence of any one hydrogenase on a low copy number plasmid restored some virulence to the triple mutant. In addition, double-mutant strains that had a deletion in two hydrogenases were less virulent than the parent strain (73).

Hydrogen uptake activity is coupled to energy conservation in the cell. The electrons derived from H$_2$ oxidation are probably passed down the electron transport chain to acceptors such as oxygen. O$_2$ use was coupled to H$_2$ use in *S. enterica* serovar Typhimurium, as determined by a Clark-type electrode (73). The gut environment probably fluctuates between microaerobic and anaerobic, since *E. coli* mutants lacking high affinity cytochrome *bd* oxidase could not persist in mouse intestines (56). The protons from H$_2$ oxidation are also used to conserve energy, since they contribute to the proton motive force across the inner membrane.

Since hydrogenase is important for virulence and *S. enterica* serovar Typhimurium contains three uptake-type hydrogenases, we wanted to determine when and where each was used during an infection. Resolvase-*In Vivo* Expression Technology (RIVET) was used to measure hydrogenase promoter activity during a mouse infection. We found that *hyd* and *hya* are expressed differently in the mouse. The *hyd* genes are preferentially expressed in the livers and spleens of mice early in the infection, while *hya* genes were expressed in low levels at all locations tested, including the ileum. In addition, both *hya* and *hyd* were expressed in murine macrophages and human polymorphonuclear leukocytes. qRT-PCR results suggested that *hyb* is also expressed in phagocytes (139).

Since *hyd* is upregulated aerobically, this hydrogenase may be coupled to O$_2$ oxidation during the respiratory burst in macrophages. Since *S. enterica* serovar Typhimurium primarily resides within macrophages in the liver and spleen, it is reasonable that *hyd* is expressed highly in these tissues (133). We also found that *hya* is important for survival in macrophages, and may
play a role in acid-resistance inside these cells (139). Further studies will be useful to determine the exact role of Hya in acid-resistance and the role of Hyd in withstanding the macrophage.

The closely related bacterium, *S. flexneri*, also contains two uptake-type NiFe hydrogenases that are homologous to *S. enterica* serovar Typhimurium and *E. coli* uptake-type hydrogenases. Gene sequence alignments from the TIGR website have shown that the *S. flexneri hya* genes are homologous to *E. coli hya* genes. Likewise, the *S. flexneri hyb* genes are homologous to *E. coli hyb* genes (www.tigr.org). The *S. flexneri* uptake-type hydrogenases have not been studied. However, since *S. flexneri* is very similar to *S. enterica* serovar Typhimurium and colonizes a similar environment, it can be predicted that *S. flexneri* uptake-type hydrogenases will be important for host colonization and infection.

Not much is known about the type of nutrition used by enteric pathogens during an infection. There have been a few studies on respiration and carbon usage by enterohemorrhagic *E. coli* in the mouse intestine (34, 56) and H₂ use by *Helicobacter spp.* in mouse stomachs and livers (71). Since there are billions of resident microbes in the gut, pathogens face fierce competition for scarce nutrients. Metabolic flexibility is important in this environment. Therefore, H₂ use by enteric pathogens may be important for a variety of organisms and may be a major mode of nutrition for these pathogens.

**SCOPE OF THIS STUDY**

*Salmonella* and *Shigella* species cause debilitating and sometimes fatal gastrointestinal disease worldwide. These pathogens must out-compete natural flora in the gut in order to mount a successful infection. The hydrogen produced by natural flora is not used by the host, and is an
important energy source for pathogens such as *H. pylori* (71). However, not much is known about the hydrogenase enzymes used by *S. enterica* serovar Typhimurium and *Shigella spp.* within the host.

A few studies from the 1980s measured *S. enterica* serovar Typhimurium hydrogenase activity under different growth conditions (53, 112). In addition, the transcriptional regulation of *E. coli* hydrogenases has been examined (102). We used a genetic approach to examine the transcriptional regulation of *S. enterica* serovar Typhimurium hydrogenases and their expression under different growth conditions. The expression of each hydrogenase was measured during fermentation or aerobic and anaerobic respiration. The role of the regulators ArcA/B, FNR, IscR, and NarL was determined by a mutant approach. The methods used and results obtained are described in Chapter 2.

It is known that *S. enterica* serovar Typhimurium uses H2 during mouse infection (73). We used hydrogenase promoter fusions in order to determine where and when the hydrogenases are used during infection. To resolve the role of each hydrogenase in more detail, hydrogenase expression, hydrogenase negative mutant strain entry into and survival in macrophages was determined. The methods and results from this study are presented in Chapter 3. Information gathered from the *in vitro* study of hydrogenase expression under different growth conditions was combined with the *in vivo* results to theorize the role of each hydrogenase during infection.

The hydrogenases in *S. flexneri* have not been studied; however, they are homologous to *E. coli* hydrogenases. We sought to determine whether the role of the *S. flexneri* hydrogenases was similar to that of the *E. coli* hydrogenases. First, hydrogen-uptake activity was measured in *S. flexneri* after growth under different conditions (fermentation, anaerobic and aerobic respiration). Then, a mutant approach was used to analyze the H2 uptake ability of each
hydrogenase. The role of hydrogen in survival under different growth conditions was also examined. The results of this study are presented in Chapter 4.

Sawers et al. theorized that hya is used to recycle Hyc-produced hydrogen (112). We examined hydrogen-production in *S. enterica* serovar Typhimurium *hyb, hyd, and hya* mutants to see if this theory was true. The results from this study are discussed in Chapter 5. Finally, the importance of H₂ during growth with different anaerobic electron acceptors was examined. Growth yield and growth curves were measured and results are shown in Chapter 6. The results of my work have extended our knowledge of the roles and expression conditions of multiple hydrogenases in the family *Enterobacteriaceae*.

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CHAPTER 2

DIFFERENTIAL EXPRESSION OF NIFE UPTAKE-TYPE HYDROGENASE GENES IN SALMONELLA ENTERICA SEROVAR TYPHIMURIUM

ABSTRACT

Salmonella enterica serovar Typhimurium possesses three similar NiFe hydrogenases important to its virulence. Here we show the three hydrogenase operons hyb, hya, and hyd are expressed under different environmental conditions and are subject to control by different regulatory proteins. Hydrogenase promoter-lacZ fusion plasmids were transferred into the wild-type strain or into arcA, fnr, iscR, narL, narP deletion mutants, or into an fnr/arcA double mutant. The hyb promoter had highest β-galactosidase activity under growth conditions promoting anaerobic respiration (glycerol plus fumarate) and may be subject to glucose repression, since cells grown with glucose had about one-half the transcriptional activity of cells grown with mannose. Based on the phenotype of regulatory mutant strains, IscR repressed hyb aerobically, and ArcA plays a role in both hyb and hyd regulation. The hyd promoter had about five times more activity in cells grown under aerobic conditions compared to anaerobic levels, and its activity tripled in an arcA mutant grown anaerobically. The hya promoter had the highest activity when cells were grown anaerobically with glucose and growth yield of the hya mutant was about 25% lower than wild-type cells grown fermentatively, suggesting that Hya may be utilized during fermentation. The hya promoter is repressed by nitrate and this repression was abolished when the NarL binding site was mutated, or in a narL mutant background. FNR is involved in hyb and hya regulation, since β-galactosidase activity decreased significantly in an fnr mutant. These findings suggest

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that the three hydrogenases are used under different conditions, likely enhancing the pathogen’s capacity to survive in a variety of environments.

**INTRODUCTION**

Enteric pathogens are responsible for about 2 million deaths annually and the diarrheal illness they cause results in billions of dollars of treatment costs (de Bruyn, 2000). *Salmonella* species are some of the most common pathogens associated with sporadic diarrhea in adults living in developed countries (de Bruyn, 2000). According to the World Health Organization, the cost associated with *Salmonella* infections alone is estimated at 3 billion dollars annually in the United States.

It was recently shown that hydrogen gas (H\(_2\)) is an important energy source for the survival of pathogens *in vivo* (Mehta *et al.*, 2005; Olson & Maier, 2002). H\(_2\) is produced in the host by colonic bacterial fermentations and diffuses throughout the animal’s body (Maier, 2003). Bacterial NiFe uptake-type hydrogenases catalyze the H\(_2\) oxidizing reaction H\(_2\) → 2e\(^-\) + 2H\(^+\), yielding protons that can contribute to energy production via generation of a proton gradient across the cytoplasmic membrane. In addition, electrons produced in the H\(_2\)-splitting reaction can be passed to other membrane-bound electron carriers, eventually contributing to ATP production (Maier *et al.*, 2004).

Physiological studies indicated that *Salmonella enterica* serovar Typhimurium has at least two Ni-containing hydrogenases that are responsible for hydrogen uptake (Jamieson *et al.*, 1986; Sawers *et al.*, 1986). The genomic sequence (TIGR) indicates that three NiFe membrane-bound hydrogenases exist in the *S. enterica* strain SGSC1412 (McClelland *et al.*, 2001). The results from mouse infection studies suggest that these hydrogenases are individually important for virulence (Maier *et al.*, 2004), and the removal of all three hydrogenases (creating a triple
mutant) resulted in a strain that was avirulent and unable to colonize the mouse liver or spleen. However, the presence of any one hydrogenase on a low copy number plasmid restored some virulence characteristics to the triple mutant and each double-mutant strain with a deletion in two hydrogenases was less virulent than the parent strain (Maier et al., 2004).

The reason that *S. enterica* serovar Typhimurium has three very similar NiFe hydrogenases is unknown, but it may be expected that they are utilized under different environmental conditions. If so, the mechanism of gene expression of these operons is likely to be different. Park *et al.* studied a reporter gene fusion to one of the *S. enterica* serovar Typhimurium NiFe hydrogenase promoters that is homologous to *hya* of a similar bacterium, *Escherichia coli* (Park *et al.*, 1999). They determined that the hydrogenase operon was acid-inducible, repressed by nitrate at acidic pH, but not at neutral or alkaline pH, and induced by anaerobiosis. Cyclic AMP receptor protein (CRP) and tyrosine were required for expression (Park *et al.*, 1999). Several studies that measured hydrogenase protein levels in *S. enterica* serovar Typhimurium under different growth conditions suggested that hydrogenase protein levels are affected by anaerobiosis and catabolite repression (Jamieson *et al.*, 1986; Sawers *et al.*, 1986).

The nomenclature of *Salmonella* serovar Typhimurium hydrogenase genes is somewhat confusing. Therefore, the *Salmonella* hydrogenases were renamed in this manuscript, according to nomenclature used for similar uptake-type hydrogenases in *Escherichia coli*. The *E. coli* hydrogenase genes are located in the *hya* operon (containing the hydrogenase 1 genes) and *hyb* operon (containing the hydrogenase 2 genes). Amino acid sequence alignments using the BLAST program from the NCBI website have shown that the *Salmonella* serovar Typhimurium hydrogenase Hyb large subunit has about 94 percent sequence similarity to the Hyb large subunit.
of *E. coli*. The other two *Salmonella* serovar Typhimurium uptake-type hydrogenases (both annotated as Hyd) are similar to the single *E. coli* Hya (www.tigr.org). The *Salmonella* serovar Typhimurium *hyd* STM1786-STM1787 was called *hya* in this study because the hydrogenase large subunit has a high percent sequence similarity to *E. coli* Hya large subunit (91%). The *Salmonella* serovar Typhimurium *hyd* genes STM1538-1539 were not renamed, since the hydrogenase large subunit has a much lower percent sequence similarity to the *E. coli* Hya large subunit (66%). In a recently published study on decreased virulence in strains lacking the hydrogenases, the *hyb* genes STM3147-STM3150 were called Group I hydrogenase, *hyd* genes STM1538-STM1539 were Group II hydrogenase, and *hya* genes STM1786-STM1787 were called Group III hydrogenase (Maier *et al.*, 2004). Here, the nomenclature was modified for facile comparison with previous work done in *E. coli* and *Salmonella*.

Studies of reporter genes fused to *E. coli* *hya* and *hyb* promoters have shown that their expression is regulated by anaerobiosis, nitrate, catabolite repression, and pH. Several proteins are involved in this regulation, including Fumarate Nitrate Respiration (FNR) protein and Aerobic Respiration Control (Arc) protein. The *hyb* operon in *E. coli* is affected by catabolite repression, mediated by CRP (Richard *et al.*, 1999). Both *hyb* and *hya* operons are regulated by the NarP and NarL proteins, which repress the operons in the presence of nitrate (Richard *et al.*, 1999). Since *E. coli* and *S. enterica* are closely related, these studies led us to test whether the hydrogenases in *S. enterica* serovar Typhimurium were regulated in a similar fashion.

Here we examine the hydrogenase gene expression level in *S. enterica* serovar Typhimurium using reporter gene fusions. We show that the hydrogenases are each expressed under different growth conditions, and are differentially regulated by FNR, ArcA, and NarL. The genes *hyb* and *hya* had the highest expression under anaerobic conditions, were indirectly
regulated by FNR, and hyb may be glucose repressed. Our study shows that hya was nitrate repressed, and it was mediated by NarL, but NarP was not involved. ArcA activated hyb expression. Salmonella serovar Typhimurium hya was not under the control of ArcA. Unlike what has been shown for either hya or hyb in E. coli, the hyd operon was expressed highest aerobically and was repressed by ArcA.

MATERIALS AND METHODS

Strains, growth conditions and reagents.

The S. enterica serovar Typhimurium and E. coli strains, plasmids, and phage used in this study are listed in Table 2.1. Strains were maintained in Luria-Bertani (LB) broth or on plates with appropriate antibiotics: 50 μg ampicillin ml⁻¹, 34 μg chloramphenicol ml⁻¹, 25 μg kanamycin ml⁻¹. CR-HYD media was used where indicated (Sawers et al., 1986). The media was supplemented with glucose (0.4%), mannose (0.4%), glycerol (0.4%), sodium formate (20mM), sodium fumarate (20mM) or sodium nitrate (20mM), where indicated. Cells were grown at 37°C either anaerobically or aerobically. Anaerobic conditions were established by sparging sealed 165mL bottles with N₂ for 15 minutes, then anaerobic mix (10% H₂, 5% CO₂, and 85% N₂) for 20 minutes. o-Nitrophenyl-β-D-galactopyranoside (ONPG) was purchased from Pierce (Rockford, IL). Primers were purchased from Integrated DNA Technologies (Coralville, IA).

Construction of reporter gene fusions.

The entire intergenic region between the gene upstream of each hydrogenase and the start codon of the hydrogenase was amplified by PCR using primer pairs HybHindIII and HybNotI, HydHindIII and HydNotI, or HyaHindIII and HyaNot I (Table 2.2) to generate 300-500bp fragments. Primers were engineered to create a HindIII site on one side of the PCR product and a NotI site on the other end. PCR fragments and a low-copy number vector pNN387, which
contains a promoter-less lacZ gene and the lacY gene, were sequentially digested with HindIII and NotI. Each PCR fragment was ligated into the digested pNN387 and transformed into E. coli TOP10 cells, yielding plasmids pZBL1, pZBL2, and pZBL3, respectively (Table 2.1). To facilitate transformation of plasmids into strain JSG210, plasmids were first introduced into the restriction-deficient strain E218 before being transformed into JSG210. Fusions were confirmed by PCR using a lacZ specific primer, Lac, and HybNot1, HydNot1, or HyaNot1 to generate a 2.7 Kb fragment. The resulting PCR products were sequenced, using the promoter-specific primer (Integrated Biotech Labs, University of Georgia).

A two-step PCR approach was used to make a hya NarL binding-site mutant promoter fusion. In two separate reactions, primers UpSTHyaNarL and HyaHindIII or RevSTHyaNarL and HyaNot I were used to generate two PCR products that were about 250 base pairs each. Both PCR products were mixed together and the final PCR product of about 500 base pairs was obtained using the primers HyaHindIII and HyaNot I. The resulting PCR product had a modified putative NarL binding site, as confirmed by sequencing (DNA Sequencing Core, University of Michigan). The PCR product and the pNN387 plasmid were restriction-digested and ligated into pNN387 as described above to make pZBL4. E. coli and S. enterica serovar Typhimurium cells were then transformed with pZBL4.

**Mutant strain construction.**

Deletion mutants of the arcA, fnr, narL, narP and iscR genes were constructed using the lambda Red system, as described by Datsenko and Wanner (Datsenko & Wanner, 2000). In this system, the target gene is replaced by an antibiotic resistance cassette, which is then removed by site-specific recombination at the FRT (flippase (FLP) recognition target) sites that flank the resistance gene. Gene deletions were confirmed by PCR using primers homologous to regions
flanking the deleted gene (Table 2.2) and by sequencing across the deletion (DNA Sequencing Core, University of Michigan). The mutant strains are listed in Table 2.1.

The double mutant was constructed using the fnr deletion mutant and an arcA mutant in which the kan cassette replaced the arcA gene. The phage P22Htint (J. Gunn, The Ohio State University; Columbus, OH) was used to transduce the antibiotic marker from the ΔarcA:kan strain into the fnr mutant, making an arcA/fnr double mutant. The kanamycin cassette was removed as described above. The double mutant was confirmed by PCR.

Hydrogenase deletion mutants were constructed using the lambda red system as described previously (Maier et al., 2004). Here, deletion of the Group I genes (STM3147-3150) resulted in the hyb mutant, deletion of the Group II genes (STM1538-1539) resulted in the hyd mutant, and deletion of the Group III genes (STM1786-1787) resulted in the hya mutant (Table 2.1).

**Enzyme assay.**

Gene expression was determined using β-galactosidase assays according to Miller (Miller, 1992). *S. enterica* serovar Typhimurium fusion strains were grown overnight anaerobically or aerobically in sealed bottles containing LB broth or CR-HYD media. Cultures were assayed for β-galactosidase activity at 37°C as described previously (Miller, 1992).

**Growth studies.**

Wild-type *S. enterica* serovar Typhimurium JSG210, or hyd, hyb, or hya deletion mutant strains were grown in bottles containing CR-Hyd media supplemented with glucose, or glycerol and sodium fumarate, as indicated. Cells were grown aerobically or anaerobically overnight at 37°C. Growth yield was determined by measuring the optical density of the culture at 600nm.
RESULTS

Effect of growth conditions on hydrogenase gene expression

In order to study the expression of hydrogenase genes, transcriptional reporter fusions were made. A sequence including 300 to 500 bases upstream of the first gene of each hydrogenase operon was PCR amplified from strain JSG210. Each PCR product was then cloned into the vector pNN387 (Elledge & Davis, 1989) directly upstream of a promoterless lacZ gene. The resulting plasmids are listed in Table 2.1. Strain JSG210 was transformed with the plasmids.

To examine the possible roles of these hydrogenases, promoter activity was determined after cells were grown under different environmental conditions in CR-HYD media. The hyb promoter expression was about 13-fold higher when cells were grown under anaerobic respiration conditions (glycerol plus fumarate), compared to fermentation (anaerobic glucose) conditions (Table 2.3), suggesting that the Hyb proteins are used during anaerobic respiration. The hyb mutant had a 16% lower growth yield compared to wild-type when grown anaerobically with glycerol and fumarate, however this reduced growth yield was not significant. The hyd promoter had more than seven times more activity under aerobic conditions, compared to anaerobic glucose levels. The hya promoter had the highest activity when grown under fermentative conditions (anaerobic glucose or mannose), and the hya mutant had about 25% lower growth yield than wild-type when grown fermentatively with glucose, indicating that this hydrogenase is used during fermentation. The presence of nitrate resulted in a 2-fold reduction in β-galactosidase activity for the hya promoter (when grown with glucose), suggesting that hya is repressed by nitrate.

Since some of the E. coli hydrogenases are catabolite repressed when grown with glucose, glucose repression of S. enterica serovar Typhimurium hydrogenase genes was
investigated. Cells were grown in CR-HYD with glucose or mannose as a carbon source. The hyb promoter had about twice as much activity when cells were grown anaerobically on mannose, compared to glucose, suggesting that these genes may be repressed in the presence of glucose (Table 2.3). Such an effect was not observed for the hyd and hya genes, indicating that they are not glucose repressed.

**Anoxic regulation of hydrogenase promoters**

A mutant analysis approach was used to further examine the regulation of *S. enterica* serovar Typhimurium hydrogenase operons in response to oxygen availability. Upon sequence analysis, several putative ArcA binding sites were found in all three hydrogenase promoters. Although no putative FNR binding sites were found in the *S. enterica* serovar Typhimurium hydrogenase promoters, FNR was previously shown to regulate *E. coli* hydrogenase genes (Richard *et al.*, 1999). Therefore, non-polar deletions were made in the *arcA* and *fnr* genes using the lambda red system (Datsenko & Wanner, 2000). Mutant strains that contained the hydrogenase promoter fusion plasmids were grown overnight in LB media and β-galactosidase activity was measured. LB broth was used in this experiment because specific growth conditions were not required, and since overall hydrogen uptake activity is higher when cells are grown in rich media (Maier *et al.*, 2004). β-galactosidase activity was also assayed using wild-type and mutant strains grown anaerobically in CR-Hyd media supplemented with glucose.

β-galactosidase activity from anaerobically-grown cells decreased by at least three-fold for the hyb promoter in the *arcA* mutant background as compared to the wild-type (Table 2.4), suggesting that hyb is upregulated by ArcA. Interestingly, an opposite effect was seen for hyd. β-galactosidase activity tripled for the hyd fusion in the *arcA* mutant background. This result indicates that ArcA represses the hyd genes. ArcA did not appear to regulate hya gene
expression when cells were grown in LB, as β-galactosidase activity remained unchanged in the mutant as compared to the wild-type.

The fnr mutant strain carrying the hyb fusion plasmid showed an over three-fold decrease in β-galactosidase activity when compared to wild-type (Table 2.4). Likewise, β-galactosidase activity decreased by four-fold for the hya fusion in the fnr mutant background, indicating that both hyb and hya are regulated by FNR.

Sequence analysis did not reveal any FNR binding sites in either promoter (using the consensus sequence TTGATNNNATCAA (Zhang & Ebright, 1990)). It has been shown that FNR can upregulate arcA expression in E. coli (Compan & Touati, 1994). In order to examine the possibility that the FNR effect is mediated by ArcA in Salmonella, an arcA/fnr double mutant was constructed using the lambda red method. If FNR activates ArcA, it would be expected that β-galactosidase activity in the double mutant would be the same as in the FNR or ArcA mutant. In contrast, hyb expression decreased about 14-fold in the double mutant, 11-fold lower than in either the arcA or fnr single mutant. This result suggests that both FNR and ArcA are separately involved in hyb expression. Expression was similar between the arcA/fnr double mutant and the arcA mutant for hyd, supporting the conclusion that fnr does not regulate hyd. Likewise, hya expression was similar in the arcA/fnr double mutant compared to the fnr mutant when cells were grown in LB, indicating that arcA does not regulate hya.

A recent study reported that E. coli hya and hyb genes were repressed by IscR (iron-sulphur cluster regulator) under aerobic conditions, but not anaerobically (Giel et al., 2006). A non-polar iscR deletion mutant was made in order to determine whether S. typhimurium hyb, hyd, and hya genes are regulated similarly. Aerobic hyb expression in the iscR mutant was about two fold greater than in wild-type cells, and near the anaerobically achieved levels. These results
indicate that *hyb* is repressed by IscR aerobically; however unlike in *E. coli*, *S. typhimurium hya* genes do not seem to be regulated by IscR.

Similar trends were seen for *hyd* and *hya* when *arcA*, *fnr*, *arcA/fnr*, *iscR* mutant and wild-type cells were grown in CR-Hyd media compared to LB, although the actual values varied. This variation is likely due to the fact that LB is a richer media than CR-Hyd. There were a few differences when cells were grown in CR-Hyd, namely a significant increase in *hyd* expression in the *fnr* mutant and a significant decrease in *hya* expression in the *arcA* mutant (data not shown). This may indicate a role for FNR in the regulation of *hyd* expression and a role for ArcA in the regulation of *hya* expression under some conditions. Overall *hyb* expression was low for both wild-type and mutant strains when cells were grown in CR-Hyd plus glucose, and only slight differences were observed between the mutant and wild-type cells. It seems that LB is a more appropriate medium than CR-Hyd to assay *hyb* expression levels. Clearly, growth conditions play a role in the regulation of these genes, and the ArcA and FNR regulation of these operons may depend on other factors present under certain growth conditions. Gel-shift and DNA binding studies will be useful to address whether this regulation is direct, or dependent on other factors.

**Regulation of *hya* by nitrate**

The *hya* promoter in the wild-type background was shown to be downregulated about 2 fold in the presence of nitrate (Table 2.3). NarX/NarL and NarQ/NarP are common two-component systems that control the nitrate response in *S. enterica* serovar Typhimurium (Rabin & Stewart, 1993). *NarL* and *narP* deletion mutants were constructed using the lambda red system. Wild-type and mutant strains were grown on CR-HYD with glucose and nitrate, and β-galactosidase assays were then performed. Nitrate repression was abolished in the *narL* strain (Table 2.5),
indicating that NarL mediates the nitrate regulation of *hya*. NarP did not appear to play a role in the regulation of this gene, since β-galactosidase activity in the *narP* strain was not significantly different from wild-type levels (Table 2.5).

To further explore the regulation of *hya* by NarL a potential NarL binding site in the *hya* promoter was identified and mutated. The potential binding site, (ATACCCACAC) had six out of seven identical bases as the *E. coli* consensus sequence (NTACCCATN), described by Darwin *et al.* (Darwin *et al.*, 1997). The entire binding site in the *hya* promoter was replaced by a random sequence of nucleotides (AGCAAAAGTC) using a two-step PCR approach. Cells containing *lacZ* driven by the mutant promoter were grown in CR-HYD with glucose and nitrate overnight and β-galactosidase activity was subsequently determined. We found that the strain containing the mutant NarL binding site was not repressed by nitrate (Table 2.5). Expression was likely reduced in this strain because ten base-pairs were replaced in the promoter, which could affect the binding of other regulatory proteins. DNA binding and gel-shift assays will be useful to confirm whether NarL binds to the potential binding site in the *hya* promoter, or whether the nitrate regulation is indirect.

**DISCUSSION**

The *S. enterica* serovar Typhimurium NiFe uptake-type hydrogenases are expressed under different growth conditions, and are differentially regulated at the transcriptional level. Whereas the *hyb* genes were expressed the most when the cells were undergoing anaerobic respiration, the *hyd* genes had the highest expression under aerobic conditions, and the *hya* genes were expressed under conditions favoring fermentation (Table 2.3). The expression of the *hyd* genes was unique in comparison to either *hya* or *hyb* in the similar bacteria, *E. coli*, and it appears that *hyd* is absent from *E. coli*. 

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The results agree with previous work in which researchers analyzed the active hydrogenase enzyme levels of cells grown under different conditions (Jamieson et al., 1986; Sawers et al., 1986). Those studies were based on immunoreaction to antibodies raised against *E. coli* hydrogenases, and it was concluded that there were at least two H₂ oxidizing hydrogenase isoenzymes. Isoenzyme 2 was necessary for anaerobic respiration-dependent hydrogen uptake, since this activity was greatly reduced in a strain lacking that isoenzyme (Sawers et al., 1986). Isoenzyme 2, so named because of its sequence similarity to *E. coli* hydrogenase 2, is called Hyb in this work. Therefore, it is logical that *hyb* would be transcribed maximally when cells are grown with glycerol and fumarate. In light of these results, it seems that *S. enterica* serovar Typhimurium Hyb, like hydrogenase 2 in *E. coli* (Menon et al., 1994), is used for hydrogen oxidation coupled to fumarate respiration. Nevertheless, based on mutant analysis (Maier et al., 2004), this hydrogenase was capable of coupling H₂ oxidation to O₂ as well.

An early report using immunoelectrophoretic methods to study the active hydrogenase activity of wild-type and mutant strains indicated that isoenzyme 2 was catabolite repressed by glucose (Jamieson et al., 1986). This repression was mediated by CRP, since in a *crp* mutant, levels of isoenzyme 2 could not be increased by the addition of cAMP (Jamieson et al., 1986). Our study took a different approach to examine catabolite repression of the uptake-type hydrogenases. We determined the gene expression activity of each hydrogenase grown with different sugars and found that the *hyb* genes may be repressed in the presence of glucose (Table 2.3). Our results agree qualitatively with the early *S. enterica* serovar Typhimurium report, showing that *hyb* is glucose repressed and *hya* is not. In addition, our work shows that *hyd* is not glucose repressed, which was not examined previously.
Sawers et al. reported that *S. enterica* serovar Typhimurium isoenzyme 1 (analogous to both Hya and Hyd) was necessary for hydrogen uptake under fermentative growth in the absence of nitrate (Sawers et al., 1986). Our results support this conclusion, since *hya* had the highest activity under fermentative conditions, and it was repressed by nitrate (Table 2.3). These results indicate that the function of Hya may be to oxidize H$_2$ while the cell is undergoing fermentation. Such hydrogen oxidation would be expected to generate additional energy for metabolism under this condition, and may be necessary for optimal growth, since growth yield was decreased in the *hya* mutant. Previous studies did not distinguish between Hya and Hyd, because it was not known at that time that *S. enterica* serovar Typhimurium had three uptake-type hydrogenases.

Although early work examined hydrogenase production under anaerobic conditions, we report that *hyd* has the highest expression when cells are grown aerobically (Table 2.3). It has been shown that there is significant respiratory hydrogenase activity coupled to oxygen in *S. enterica* serovar Typhimurium cells grown under low oxygen conditions (O$_2$ levels were below 0.2% partial pressure) (Maier et al., 2004). Therefore, we suggest that Hyd is used to completely oxidize hydrogen in a low oxygen environment.

Several transcriptional regulators of each operon were identified in this study. Expression of *hyb* was shown to be downregulated in both the *arcA* and *fnr* mutants (Table 2.4). These results agree with an early study by Jamieson et al., which reports that the presence of isoenzyme 2 was *fnr* dependent, based on immunoreaction to *E. coli* anti-hydrogenase 2 antibodies (Jamieson et al., 1986). In addition, Jamieson et al. showed that protein levels of isoenzyme 1 (analogous to both Hya and Hyd) were dependent on *fnr* (Jamieson et al., 1986). Our work clarifies this result, since we found that *hya* expression was downregulated in an *fnr* mutant, yet *hyd* expression was unaffected by FNR (Table 2.4). Interestingly, ArcA was shown
to repress *hyd* expression under anaerobic conditions (Table 2.4). This is further evidence that Hyd is used under aerobic conditions, since ArcA usually represses genes used for aerobic growth and induces anaerobic genes (Lynch & Lin, 1996). Like in *E. coli*, IscR repressed *hyb* expression aerobically; however *hya* expression was not regulated by IscR under the conditions tested.

Using sequence analysis, we were able to find several potential binding sites for ArcA in the region directly upstream from the first gene in the *hyb* operon, indicating that ArcA may directly regulate *hyb* transcription. DNA-binding studies with purified ArcA will be needed to confirm this result. We were unable to find a binding site for FNR in either the *hyb* or *hya* promoter indicating that FNR regulation is indirect. It was shown that FNR indirectly regulates the hydrogenase genes in *E. coli*, and was speculated that this may occur through altering nickel metabolism (Richard *et al.*, 1999). To test this hypothesis in *S. enterica* serovar Typhimurium, we added up to 1mM nickel to the growth medium, but such nickel supplementation did not significantly change *hyb*, *hyd*, or *hya* promoter activity in *fnr* mutants (data not shown). Therefore, it appears that the FNR effect is not due to nickel availability in *S. enterica* serovar Typhimurium. In order to test the hypothesis that the FNR effect is mediated by ArcA, an *fnr/arcA* double mutant was constructed. Our results demonstrated that both FNR and ArcA are needed for full expression of *hyb* (Table 2.4).

The *hya* operon was shown to be regulated by nitrate (Table 2.3). Nitrate repression of *S. enterica* serovar Typhimurium hydrogenase genes is not well understood. Previous knowledge is limited to the study by Sawers *et al.*, which showed that hydrogen uptake was abolished when cells were grown with nitrate (Sawers *et al.*, 1986). However, the mechanism of nitrate repression of *E. coli* hydrogenases has been studied. Richard *et al.* demonstrated that both *E.
coli hya and hyb operons are repressed by nitrate, mediated by both NarL and NarP (Richard et al., 1999). In contrast, we found that nitrate repression of hya was only mediated by NarL, and not by NarP (Table 2.5). We found a potential NarL heptamer recognition sequence in the promoter region of the hya operon. The heptamer was not in the 7-2-7 arrangement, as described by Darwin et al. (Darwin et al., 1997), which provides further evidence that NarL was involved in the observed repression, but NarP was not. When this site was mutated, gene expression was no longer repressed by nitrate (Table 2.5).

In summary, the three uptake-type hydrogenases in S. enterica serovar Typhimurium are transcribed under different physiological conditions and they are differently regulated. Although some of these results are similar to those described for the homologous enzymes in E. coli, other aspects are unique. For example, the regulation by ArcA and the nitrate repression is different in S. enterica serovar Typhimurium. In addition, the regulation and expression of hyd is unique.

The uptake type hydrogenases are essential for virulence in S. enterica serovar Typhimurium (Maier et al., 2004). Since the cell encounters a variety of environments during infection, it may be beneficial to be able to express a hydrogenase capable of oxidizing H2 at different stages of colonization. Perhaps Hyd is used to conserve energy by oxidizing hydrogen in the initial stages of infection, when the cell encounters a low oxygen environment. As the bacterium progresses through the digestive system into a highly competitive and putatively anaerobic environment, hyb expression may be favored to exploit H2 as an energy source. Maier et al. reported H2 levels to be 43μM in the stomach and 118 to 239 μM in the small intestine of mice, levels much higher than needed to saturate the hydrogenase enzymes (Maier et al., 2004; Olson & Maier, 2002). When the bacterium reaches the bowel, it probably both ferments available substrates and encounters large amounts of H2 produced by the colonic flora. Hya may
be very important in this environment. Future investigation examining the transcriptional level of these hydrogenase operons in the animal model and experiments that examine the H2 affinity of each *S. enterica* serovar Typhimurium enzyme will be useful to test these ideas.

**ACKNOWLEDGEMENTS**

We thank John Gunn for supplying bacterial strain JSG210, phage, and plasmids pCP20, pKD46, and pKD4. We thank Timothy Hoover for supplying bacterial strain E218, and Anna Karls for plasmid pNN387. This work was supported by the University of Georgia Foundation.

**REFERENCES**


## Table 2.1 Strains and plasmids

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<th>Genotype or description</th>
<th>Source or reference</th>
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<td></td>
<td></td>
</tr>
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<td>ATCC</td>
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<td>T. Hoover, University of Georgia</td>
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<td>(Elledge &amp; Davis, 1989)</td>
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<td>Cm(^\prime); pNN387 with mutant hya-lacZ fusion</td>
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*FRT, FLP recognition target*
Table 2.2. Primers.*

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<tr>
<td>HybNotI</td>
<td>ACTGCA<strong>AGGC</strong>GCCTGCAACAGGGGTTTATTC</td>
<td>hyb fusion plasmid</td>
</tr>
<tr>
<td>HydHindIII</td>
<td>GCAC<strong>AGCT</strong>TAGTTAGTGTTGGGCTCA</td>
<td>hyd fusion plasmid</td>
</tr>
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<td>HydNotI</td>
<td>ACTGCA<strong>AGGC</strong>GCCTGCAACAGGGGTTTATTC</td>
<td>hyd fusion plasmid</td>
</tr>
<tr>
<td>HyaHindIII</td>
<td>GCAC<strong>AGCT</strong>TGGGACCTGCAACAGGGGTTTATTC</td>
<td>hya fusion plasmid</td>
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<td>Lac</td>
<td>GACCATTTTACATCCGCA</td>
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* Restriction enzyme sites are in bold, and the mutated putative NarL binding site is in lowercase.
Table 2.3 β-galactosidase activities of wild-type *S. typhimurium* containing hydrogenase promoter-*lacZ* fusions.

<table>
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<tr>
<th>Condition</th>
<th>β-galactosidase activity (Miller units)*</th>
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<tr>
<td></td>
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<tr>
<td>Aerobic glucose</td>
<td>2.5 +/- 0.8</td>
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<tr>
<td>Anaerobic glucose</td>
<td>2.8 +/- 0.7</td>
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<tr>
<td>Anaerobic mannose</td>
<td>3.4 +/- 1.3</td>
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<tr>
<td>Anaerobic glucose + formate</td>
<td>3.7 +/- 0.3</td>
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<tr>
<td>Anaerobic glycerol + fumarate</td>
<td>3.9 +/- 0.6</td>
</tr>
<tr>
<td>Anaerobic glucose + nitrate</td>
<td>4.0 +/- 0.7</td>
</tr>
</tbody>
</table>

* Cells were grown overnight in bottles containing CR-HYD media with various growth substrates added as indicated. Results are an average of six replicates from three or more independent experiments. Like superscript numbers after the standard deviation values indicate a significant difference in mean between two values, based on students t-test (P<0.01).

# Vector-only cells are wild-type cells containing the vector pNN387 with a promoter-less *lacZ*. 
### Table 2.4 Anaerobic regulation of hydrogenase expression.

<table>
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<th>Strain</th>
<th>β-galactosidase activity (Miller units)*</th>
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<td></td>
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<tr>
<td>JSG210 (wt) aerobic</td>
<td>7.1 +/- 0.8</td>
</tr>
<tr>
<td>JSG210 (wt) anaerobic</td>
<td>3.3 +/- 3.5</td>
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<tr>
<td>ALZ10 (arcA)</td>
<td>4.1 +/- 0.9</td>
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<tr>
<td>ALZ11 (fnr)</td>
<td>2.8 +/- 2.1</td>
</tr>
<tr>
<td>ALZ14 (arcA fnr)</td>
<td>0.1 +/- 4.4</td>
</tr>
<tr>
<td>ALZ25 (iscR) aerobic</td>
<td>6.9 +/- 1.1</td>
</tr>
<tr>
<td>ALZ25 (iscR) anaerobic</td>
<td>5.8 +/- 0.9</td>
</tr>
</tbody>
</table>

* Wild-type (wt) and iscR mutant cells containing hydrogenase promoter fusions were grown aerobically or anaerobically overnight in LB media and β-galactosidase activity was measured. The arcA, fnr, and arcAfnr mutant strains were grown anaerobically. Results are an average of six to eight replicates from three or more independent experiments. Like superscript numbers after the standard deviation values indicate a significant difference in mean between two values, based on students t-test (P<0.01).
Table 2.5 Nitrate regulation of hydrogenase expression.

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</thead>
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<td></td>
<td>Glucose</td>
<td>Glucose + nitrate</td>
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<tr>
<td>JSG210 (wild-type) hya fusion</td>
<td>37.2 +/- 8.8 (1)</td>
<td>23.4 +/- 2.4 (1,2)</td>
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<tr>
<td>JSG210 pZBL4 (hya mutant promoter fusion)</td>
<td>9.6 +/- 3.8</td>
<td>13.0 +/- 1.4</td>
<td></td>
</tr>
<tr>
<td>ALZ12 (narL) hya fusion</td>
<td>36.3 +/- 5.5</td>
<td>38.5 +/- 4.5 (2)</td>
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<tr>
<td>ALZ11 (narP) hya fusion</td>
<td>33.4 +/- 13.6</td>
<td>25.7 +/- 2.1</td>
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</table>

* Mutant and wild-type cells containing hydrogenase promoter fusions were grown anaerobically overnight in CR-HYD media. For strain JSG210 pZBL4, results are an average of six replicates; for the other strains the data is the mean and standard deviation of at least 14 replicates. Like superscript numbers after the standard deviation values indicate a significant difference in mean between the two values, based on students t-test (P<0.01).
CHAPTER 3

SALMONELLA ENTERICA SEROVAR TYPHIMURIUM NiFe UPTAKE-TYPE HYDROGENASES ARE DIFFERENTIALLY EXPRESSED IN VIVO

ABSTRACT

Salmonella enterica serovar Typhimurium, a common enteric pathogen, possesses three NiFe uptake-type hydrogenases. The results from mouse infection studies suggest that the H₂ oxidation capacity provided by these hydrogenases is important for virulence. Since the three enzymes are similar in structure and function, it may be expected that they are utilized under different locations and times during an infection. A recombination-based method to examine promoter activity in vivo (RIVET) was used to determine hydrogenase gene expression in macrophages, polymorphonuclear leukocyte (PMN)-like cells, and in a mouse model of salmonellosis. The hyd and hya promoters showed increased expression in both murine macrophages and in human PMN-like cells, compared to the media-only control. qRT-PCR results suggested that hyb is also expressed in phagocytes. A non-polar hya mutant was compromised for survival in macrophages, compared to the wild-type. This may be due to lower tolerance to acid stress, since the hya mutant was much more acid sensitive than the wild-type. In addition, hya mutant cells were internalized by macrophages the same as wild-type cells. Mouse studies (RIVET) indicate that hyd is highly expressed in the liver and spleen early during infection, but poorly in the ileum of infected animals. Late in the infection, the hyd genes were expressed at high levels in the ileum as well as in liver and spleen. The hya genes were

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expressed at low levels in all locations tested. These results suggest that the hydrogenases are used to oxidize hydrogen in different stages of an infection.

**INTRODUCTION**

There are an estimated 13.8 million food-borne illnesses per year in the United States that result in about 5,020 deaths annually (17). *Salmonella* species are some of the most common pathogens associated with food-borne illness in adults living in developed countries (5) and are responsible for about 30% of food-related deaths in the United States (17). *Salmonella enterica* serovar Typhimurium causes gastroenteritis in humans and a typhoid fever-like disease in mice (25).

*S. enterica* serovar Typhimurium possesses three uptake-type NiFe hydrogenases that are important for virulence (16). H₂ produced by colonic bacterial fermentation can be split by these membrane-bound hydrogenases. This process yields energy in the form of protons that contribute to the proton gradient and electrons that can be passed down the electron transport chain (27). H₂ use may be common among bacterial pathogens, since the ability to use H₂ is also important for *Helicobacter pylori* to efficiently colonize the mouse stomach (21).

It was recently shown that the three uptake-type hydrogenases in *S. enterica* serovar Typhimurium are expressed under different conditions *in vitro*. The *hyb* promoter had the most expression under anaerobic respiration conditions with fumarate as the terminal electron acceptor. The *hyd* promoter was expressed highest under aerobic conditions, while the *hya* promoter had the best expression during fermentation (28). In addition, Park *et al.* reported that *hya* expression may be acid-induced (23). Since the three hydrogenases are differentially expressed *in vitro*, it may be predicted that they are used in different locations and times during an infection.
Here we examine hydrogenase expression in vivo. Hydrogenase promoter fusions were made using resolvase-in vivo expression technology (RIVET). This system was originally developed to identify Vibrio genes induced during infection (3) and was later adapted for use in S. enterica serovar Typhimurium (18). In this study, expression of hydrogenase promoter fusions was assayed in murine and human phagocytes. Then, expression of the hya and hyb promoters was assessed in the ileum, liver, and spleens of mice. Hydrogenase mutant survival was determined in murine macrophages. In order to examine the role of hydrogenases in macrophage survival in more depth, hydrogenase mutant survival was determined in acid stress conditions. Internalization assays were also performed in order to determine if there are differences in internalization of wild-type and mutant strains during macrophage infection.

**MATERIALS AND METHODS**

**Bacterial strains, growth conditions and reagents.**

The S. enterica serovar Typhimurium and E. coli strains, plasmids, and phage used in this study are listed in Table 3.1. Strains were maintained in Luria-Bertani (LB) broth or on plates with appropriate antibiotics at the following concentrations: ampicillin, 100 μg/ml; chloramphenicol, 34 μg/ml; kanamycin, 25 μg/ml; tetracycline, 12.5 μg/ml. CR-HYD medium was used where indicated (26). The medium was supplemented with glucose (0.4%), glycerol (0.4%), or sodium fumarate (20 mM) where indicated. Anaerobic culture conditions were established by sparging sealed 165 ml bottles with N₂ for 15 minutes, then anaerobic mix (10% H₂, 5% CO₂, and 85% N₂) for 20 minutes. Bismuth-sulfite agar, a selective and differential medium for S. enterica serovar Typhimurium, was used in mouse experiments. M9 minimal medium with 20% mannose was used where indicated. O-Nitrophenyl-β-D-galactopyranoside (ONPG) was
purchased from Pierce (Rockford, IL). Primers were purchased from Integrated DNA Technologies (Coralville, IA).

**Construction of reporter gene fusions.**

Resolvase-*in vivo* Expression Technology (RIVET) fusions were made as described previously (3, 18). In brief, three genes arranged in tandem: a transposon resolvase gene (*tnpR*), *lacZ* and *lacY* were put under the control of each hydrogenase promoter. When the promoter was active, TnpR was made and catalyzed the excision of a chromosomal tetracycline resistance cassette (*tetRA*) located at the *yjeP* locus, (*yjeP8103::resI-tetRA-resI*), resulting in the heritable loss of tetracycline resistance.

Primers Hyb1BamHI (5’ CGGGATCCTGGCTTTTATTTTGCAG 3’) and REVHyb1BamHI (5’ CGGGATCCAACAGGGGTATTATC 3’) were used to generate a 200 bp fragment containing the putative *hyb* promoter with *BamHI* sites engineered into both ends of the PCR product. Primers Hyb2BamHI (5’ CGGGATCCTAGTTAGTGGGCATC 3’) and REVHyb2BamHI (5’ CGGGATCCGCTCTCCTTTGTTGTA 3’) were used to generate a 500 bp PCR product containing the putative *hyd* promoter while primers Hyb3BamHI (5’ CGGGATCCTTGACACCACGCTCCGC 3’) and REVHyb3BamHI (5’ CGGGATCCGCCCTCCTTTTCAAACAGTC 3’) were used to generate a 550 bp product containing the putative *hya* promoter. Each PCR product was digested with *BamHI* and ligated into three vectors containing different ribosome binding sites that allow varied levels of TnpR translation (pGOA1193, pGOA1194, and pGOA1195). The resulting nine plasmids are listed in Table 3.1. Plasmids were transferred into *E. coli* DH5αpir and then into *E. coli* SM10λpir. Finally, each of the nine fusion constructs was then transferred into *S. enterica* serovar Typhimurium JS246 by conjugation, yielding nine strains (Table 3.1). The fusion constructs
integrated into the JS246 chromosome by homologous recombination, since the plasmids cannot replicate in JS246. The three strains constructed for each hydrogenase promoter confer different levels of expression of the transposon resolvase genes. This occurs because each strain contains a unique ribosome binding site mutation or no ribosome binding site mutation in front of the transposon resolvase genes.

**RT-PCR**

In order to confirm the non-polar nature of the *hya* mutation, RT-PCR was performed on wild-type and *hya* mutant strains. Bacteria were grown overnight in LB media at 37°C. RNA was extracted using an Aurum Total RNA Mini kit (Biorad) and was digested with RQ1 DNase (Promega) to remove contaminating genomic DNA. This digested RNA was the template to generate cDNA, using an iScript select cDNA synthesis kit (Biorad) and primers UPST1788 (5’ ATGTTGGTACTGATGGTAAC 3’) and REVST1788 (5’ AATAGCGGGTTGCCGACACAG 3’) that amplified 178 bp from the gene directly downstream (STM 1788) of the deleted *hya* genes. These same primers were used to amplify the fragment by PCR with 30 amplification cycles.

**In vitro RIVET transcription assays.**

RIVET fusion cells were grown overnight at 37°C under conditions previously shown to induce hydrogenase promoter activity, or under uninducing conditions (28). After overnight growth at these conditions, β-galactosidase activity was assayed according to Miller (19). Cells were diluted and plated on M9 minimal media agar plates supplemented with ampicillin. After overnight growth, between 50 and 100 colonies were patched on LB plates containing tetracycline. Percent resolution, a measurement of promoter activity, was determined by dividing the number of tetracycline sensitive cells by the total number of colonies patched. One
strain for each promoter (ALZ 18, ALZ 16, or ALZ 17 for hyb, hyd, or hya, respectively) was chosen for further study based on detectable and reliable gene expression.

**Tissue culture conditions and cell invasion assays.**

RAW 264.7 murine macrophages (ATCC) or HL-60 human polymorphonuclear leukocyte (PMN)-like cells (D. Evans, University of Georgia) were grown at 37°C, 5% CO₂ in Dulbecco’s modified Eagle medium (DMEM) with 10% heat-inactivated fetal bovine serum. Cells were passaged every 3-5 days as required. HL-60 cells were incubated with 1 μM retinoic acid (Sigma, St. Louis, MO) for 4 days immediately prior to cell invasion assay to stimulate differentiation and maturation of the PMNs. Tissue culture cells were added to 24-well plates at a concentration of 1 x 10⁵ cells per well one day prior to infection with *S. enterica* serovar Typhimurium. Cells were infected with RIVET fusion strains or hydrogenase deletion mutants in a multiplicity of infection (MOI) of 10 (1 x 10⁶ bacteria per well). This was considered time zero for the assay, in order to determine the survival of each strain at early time points, before gentamicin was added. The plate was then centrifuged at 100 X g and incubated at 4°C for 30 min, then incubated at 37°C for 1 hr. Extracellular bacteria were killed by incubating with gentamicin (100 μg/ml) at 37°C for 2 hrs, and then incubating for an additional 16 hrs with 10 μg/ml gentamicin. Tissue culture cells were lysed with 1% Triton X-100, and the lysate was diluted and plated on LB. Expression of each of the three hydrogenase promoters was monitored by determining the ratio of tetracycline sensitive colonies to ampicillin resistant colonies, as described above. Bacterial survival in wild-type or hydrogenase deletion mutant strains was determined by diluting and plating lysates from RAW 264.7 macrophages on LB plates at different time points during the assay. The resulting colonies were then counted and the number of colony-forming units per ml was determined.
Real-Time PCR

5 x 10^5 RAW 264.7 murine macrophages were grown overnight in 12-well plates and then infected with 5 x 10^7 wild-type *S. enterica* serovar Typhimurium cells. Total RNA from bacteria and macrophages was isolated using TRIzol (Invitrogen) or Aurum Total RNA Mini Kit (Biorad) immediately after infection, and at 2 hrs, 4 hrs, 12 hrs, and 24 hrs post-infection. RNA was digested with RQ1 DNase (Promega) to remove contaminating genomic DNA. cDNA was then synthesized using 0.5 – 1 μg RNA and iScript Select cDNA synthesis kit (Biorad). Quantitative PCR was performed with Platinum SYBR Green qPCR SuperMix UDG (Invitrogen) and iCycler iQ PCR Detection System (Biorad). Primers were designed to amplify a 100-200 bp product, and were homologous to the small subunit of the *S. enterica* serovar Typhimurium hyb (STM 3150), hyd (STM 1539), or hya (STM 1786) structural genes, or to the rpoA gene encoding the α subunit of the RNA polymerase (STM 3415), used for sample normalization. Transcript abundance for each gene in DMEM-only was standardized to 1 and transcript levels in macrophages were relative to levels in DMEM-only. Relative quantification was used to calculate differences between samples, using the ΔΔCt method. Standard deviations and the range of ΔΔCt values was calculated for each sample, as described in the Applied Biosystems User Bulletin #2.

**Internalization assays.**

The ability of the hya mutant and wild-type cells to enter RAW 264.7 murine macrophages was assessed by using a fluorescence quenching assay, as described by Fallman *et al.* (8). Wild-type and hya mutant *S. enterica* serovar Typhimurium cells were grown overnight in 2 ml LB broth upright, without shaking. Bacterial cells (1 x 10^9 cells/ml) were washed in PBS and labeled with 0.1 mg/ml 5-(6)-carboxyfluorescein-succinylester, or FITC, (Molecular Probes) in PBS for 30
minutes at 4°C. Cells were washed in sterile PBS, resuspended in DMEM + 10% FBS, and 1x 10^8 cells were added to 1x 10^5 RAW 264.7 cells that had been grown overnight in 24-well tissue culture plates. FITC-conjugated 0.5-μm diameter polystyrene beads (1x 10^8) or FITC-labeled E. coli (5 x 10^6) were added to the macrophages as controls. Plates were centrifuged at 100 X g for 10 minutes at 4°C and then were incubated at 37°C for 1.5 hours, to allow for phagocytosis. The number of macrophages in ¼ of a random frame of view was counted by using a Leica TCS inverted epifluorescence microscope. Then, 0.1 ml trypan blue (1 mg/ml in citrate buffer; pH 4.4) was added to quench the fluorescence of extracellular bacteria or beads. The number of macrophages containing intracellular bacteria or beads was then counted.

**Acid survival assays.**

Hydrogenase deletion mutants and wild-type cells were grown anaerobically overnight in sealed 165 ml bottles containing 20 ml LB broth. Cells were harvested by centrifugation and resuspended in McIlvaine’s citric acid-phosphate buffer (7). Sealed, anaerobic 165 ml bottles containing 5 ml McIlvaine’s buffer at pH 4 or pH 7 were inoculated with 1 x 10^8 cells. Samples were taken at various time points up to 24 hours post inoculation, diluted in PBS and plated on LB agar. The resulting colonies were counted and the number of colony-forming units per ml was determined.

**Animal studies.**

Female 4.5 week-old BALB/c mice (National Cancer Institute, Frederick, MD) were orally inoculated with 1 x 10^6 RIVET fusion cells that had been grown overnight in LB media with 100 μg/mL ampicillin and 12.5 μg/mL tetracycline, washed in PBS, and then resuspended in PBS. The inoculant strains were thus not induced. Mice were sacrificed at various time points as indicated in Figure 3.8, and ileum, liver, and spleens were mechanically homogenized in PBS
with 10-ml Dounce hand homogenizers. The homogenate was plated on LB or Bismuth-sulfite agar containing 100 μg/ml ampicillin, and resulting colonies were patched onto LB plates containing 12.5 μg/ml tetracycline to determine percent resolution.

**RESULTS**

**Hydrogenase expression *in vitro***.

In order to study the expression of hydrogenase genes, transcriptional reporter fusions were made using the RIVET technique. The resulting constructs (Table 3.1) have a *lacZ* gene and a transposon resolvase gene under the control of the hydrogenase promoter. Three strains were made for every promoter, each containing a different ribosome-binding site mutation that would result in differential translation of the TnpR resolvase.

In order to determine which fusion expressed detectable levels of TnpR, strains were grown under conditions known to induce or repress promoter activity (28). The *hyb* promoter fusion strains were grown in CR-Hyd media with glycerol and fumarate (anaerobic respiration to fumarate) for inducing conditions, and grown in CR-Hyd with added glucose (fermentation) for uninducing conditions. The *hyd* fusion strains were grown aerobically (under atmospheric conditions) in LB broth (inducing) or anaerobically in CR-Hyd with glucose (uninducing). The *hya* fusion strains were grown on blood agar (BA) plates in anaerobic jars (inducing), or on LB plates aerobically (uninducing). β-galactosidase assays were used to determine *lacZ* expression. Transposon resolvase expression was determined by finding the percentage of tetracycline sensitive colonies/ total colonies (percent resolution). The fusions that showed higher *lacZ* and *tnpR* expression under inducing conditions compared to uninducing conditions were chosen for further study (ALZ 18, ALZ 16, or ALZ 17 for *hyb*, *hyd*, or *hya*, respectively). The *in vitro* expression of these three fusions is shown in Figure 3.1.
**Hydrogenase expression in phagocytes.**

It was recently shown that uptake-type hydrogenases are important for *S. enterica* serovar Typhimurium virulence in mice (16). We wanted to examine whether hydrogenase gene expression is upregulated intracellularly, or in contrast, only expressed outside of phagocytes, in order to predict where each gene might be expressed during a mouse infection. RAW 264.7 murine macrophages were infected with each of the three hydrogenase RIVET fusion strains, and gene expression was determined at about 20 hours post infection (post inoculation). The hyd genes were upregulated inside the macrophages, compared to media-only control (Fig. 3.2). Expression of *hyd* was then determined at various time-points during a tissue culture survival assay. The results suggest that *hyd* has highest expression between 2 to 12 hours post inoculation (Fig. 3.3). Although expression levels were different between *hyd* fusion cells in Fig. 3.2 and Fig. 3.3, relative expression levels between intracellular bacteria and DMEM-only controls were similar. Expression was about 14% to 23% higher intracellularly as compared to the media-only control (data not shown). qRT-PCR assays at 2 hrs, 4 hrs, 12 hrs, and 24 hrs post inoculation indicate that the *hyd* genes are expressed in macrophages starting at the 12 hr timepoint with an approximately 3-fold higher (range of 1.8 to 4.3) expression compared to the DMEM-only control (data not shown). Levels of *hyd* expression were normalized to α subunit of RNA polymerase transcript levels.

According to the student’s T-test, *hya* expression was significantly upregulated in cells isolated from RAW 264.7 macrophages compared to media-only control (P< 0.005). Overall expression for *hya* seems to be low in macrophages and relative *hya* transcript abundance was too low to make significant conclusions from qRT-PCR experiments.
The results from RIVET experiments indicated that the *hyb* genes are not upregulated in RAW 264.7 murine macrophages, since expression was similar in the cell line and controls (Fig. 3.2); however, background expression was high in these experiments. qRT-PCR results suggested that *hyb* was indeed expressed in RAW 264.7 macrophages starting at 4 hrs post inoculation. Expression in one sample was about 3-fold higher (range of 2.3 – 3.4 among three replicates) at 4 hours post inoculation and 43-fold higher (range of 28 – 66 among six replicates) at 12 hours post inoculation, compared to DMEM-only controls.

PMNs are among the first cells recruited to the intestine to combat a *S. enterica* serovar Typhimurium infection in mice and humans (9). In order to determine whether hydrogenases would be expressed in the human PMN-like cell line HL-60, cells were infected with hydrogenase fusion strains. The *hyd* fusion was upregulated in this cell line compared to media-only controls and the *hyb* was not upregulated, just as in RAW 264.7 cells (Fig. 3.2). The *hya* fusion was slightly but significantly upregulated in cells isolated from HL-60 PMNs, compared to media-only control, according to the student’s T-test (P< 0.005).

**Hydrogenase mutant survival in macrophages and internalization assays.**

Since hydrogenase expression was upregulated for the three hydrogenases during intracellular growth and since hydrogenases are important for virulence, we wanted to determine whether hydrogenase expression was important for survival and growth in macrophages. RAW 264.7 macrophages were infected with hydrogenase deletion mutants, and bacterial numbers were counted at various time points post inoculation. Only the *hya* genes were necessary for survival inside macrophages, since there was about 22 times less *hya* mutant cells as compared to wild-type as early as four hours post inoculation, and *hya* cell survival remained lower than wild-type
levels at all time points tested. The hyb and hyd mutants were similar to wild-type at all time points (Fig. 3.4).

In order to confirm that the survival defect was due to the hya gene deletion and not some polar effect, reverse-transcriptase PCR was performed on wild-type and hya mutant cells. Cells were grown overnight in LB media, RNA was isolated from both strains, and cDNA was synthesized. Primers specific to the gene directly downstream the hya mutation, STM 1788, were used to generate a PCR product from the cDNA. Both the wild-type and hya strain cDNA yielded PCR product in greater quantities than in the no reverse-transcriptase control (Fig. 3.5), demonstrating that the gene directly downstream the hya deletion is transcribed in the hya mutant, and the mutation is non-polar.

In order to determine whether the observed PCR products were within the linear range of the amplification reaction, PCR was performed with 20, 25, 27, and 30 amplification cycles. After 20 amplification cycles, no product was observed for the cDNA for either strain, or for the no reverse-transcriptase controls, although product was observed for the positive control (genomic DNA). After 25 and 27 amplification cycles, faint bands of approximately the same intensity were observed for both wild-type and hya mutant, while no product was observed for the no reverse-transcriptase control samples (results not shown). The PCR products shown in Fig. 3.5 are the result of 30 amplification cycles. Since faint bands were observed at 27 cycles, it can be assumed that the reactions were within the linear range after 25 or 27 cycles, and are not saturating at 30 cycles.

Interestingly, in macrophage survival assays, the hya mutant had similar cell survival compared to wild-type as late as two hours post inoculation, suggesting that these cells were able to combat reactive oxygen and nitrogen species and enzymes released by the macrophages.
However, cell numbers decreased dramatically after extracellular bacteria were killed with gentamicin, compared to wild-type cell numbers. This may indicate that there are less hya mutant cells internalized by the macrophage, as compared to wild-type or that the hya mutant does not survive the early Salmonella-containing vacuole (SCV) as well as wild-type. The hya mutant was able to grow inside macrophages, since cell numbers increased at a logarithmic rate as early as four hours post inoculation (Fig. 3.4).

Internalization assays using fluorescence quenching method were performed in order to determine whether the decreased survival of the hya mutant in macrophages was due to reduced survival once cells are inside macrophages (as was hypothesized), or due to decreased entry into macrophages. The results (Fig. 3.6) demonstrated that the hya mutant entered RAW 264.7 macrophages just as well as wild-type cells. The macrophages were also able to internalize inert polystyrene beads or killed E. coli, which demonstrates that they were capable of effective phagocytosis.

The hya mutant is compromised for survival in acidic conditions.

Salmonella must withstand hostile conditions in order to survive and replicate inside macrophages. The Salmonella-containing vacuole acidifies soon after bacteria enter the cells and macrophages release reactive oxygen and nitrogen species (10, 13). If the hya mutant was more sensitive to acid stress, compared to wild-type, it might explain why this mutant did not survive as well inside macrophages.

In order to test resistance to acid stress, the hya, hyb, and hyd mutant cells, as well as wild-type were exposed to citric acid-phosphate buffer at pH 4 or 7, and cell numbers were determined at various time points. The hya mutant had lower tolerance to acid stress, with about 90% fewer cells than wild-type after 24 hours at pH 4 (Fig. 3.7). The hyb and hyd mutants had
similar cell numbers as compared to wild-type after exposure to pH 4 (results not shown). Hya gene expression is also upregulated by acid. Hya genes had about 4 fold more expression after overnight growth in anaerobic bottles containing LB buffered with 2-(N-Morpholino)ethanesulfonic acid (MES) at pH 5.8 compared to LB at pH 7 (results not shown), according to β-galactosidase assays using a lacZ fusion to the hya promoter. Park et al. also suggested that hya is acid-induced (23). The results above suggest that the hya mutant is compromised for survival in macrophages at least in part because it cannot survive acid stress as well as the wild-type.

**Hydrogenase expression in BALB/c mice.**

In order to determine hydrogenase expression during an infection, female BALB/c mice were orally inoculated with 1 x 10^6 hyb, hyd, or hya RIVET fusion strains. The hyb RIVET fusion strain ALZ18 had similar levels of resolution in the mouse as in media-only background (around 80% resolution), and therefore was not further studied. The hyb RIVET fusion strains ALZ 15 and ALZ 21 were not used to determine hyb expression in the mouse because they did not produce significant TnpR when tested *in vitro*. Mice were inoculated with hyd and hya strains at a young age (30-34 days old) and began to achieve morbidity four days post inoculation. Therefore, at day one post inoculation the infection was considered to be in the early stages, while day 3-4 is considered late in the infection. Mice were euthanized at various time points and bacteria were recovered from the ileum, liver, and spleen. Bacteria were plated on Bismuth-sulfite agar or LB agar containing ampicillin to select for *S. enterica* serovar Typhimurium colonies that contained RIVET fusions. Percent resolution of each promoter was determined by subtracting the level of resolution in the inoculum from the percent resolution in cells isolated from the mouse. There was significant contamination in ileum samples when cells were plated
on LB plates containing ampicillin. Further investigation revealed that the contaminant was naturally ampicillin resistant *Enterobacter cloacae*. The experiments were then repeated, plating ileum samples on Bismuth-sulfite agar, which is a selective and differential media for *S. enterica* serovar Typhimurium. Liver and spleen samples were not significantly contaminated and percent resolution from colonies plated on LB or Bismuth sulfite agar were similar.

At day one post-infection, the *hyd* promoter was expressed poorly in the ileum, but highly in the liver and spleen (Fig. 3.8). During mouse infection, *S. enterica* serovar Typhimurium resides primarily within macrophages and other phagocytic cells, especially in the liver and spleen (24, 25). Since *hyd* was highly expressed in macrophages, it is reasonable that it would be expressed in the liver and spleen early in the infection, since *S. enterica* serovar Typhimurium resides within phagocytes in those organs. After 2.5 days post-inoculation *hyd* was highly expressed in all locations (Fig. 3.8). This increase of expression in the ileum is probably due to the accumulation of low levels of resolution over time, not due to bacterial reinfection of the ileum from the bile duct. A recent study examined the expression of several *S. enterica* serovar Typhimurium genes in mice by the RIVET system and showed that bacterial reinfection of the ileum can occur, but at such a low level that it does not affect the apparent gene expression of bacteria isolated from the ileum (18).

The *hya* promoter was expressed to some extent in all locations tested in the mouse (Fig. 3.8), albeit with considerable variability among all tissues. Hya gene expression was low at both initial (one day, data not shown, or at 2 days, Fig. 3.8C) and late stages in the infection (4 days, Fig. 3.8D) the point at which mice began to succumb to the infection. These levels of resolution are in agreement with the relatively the low levels of *hya* expression *in vitro* and in macrophages.
However, as *hya* is important for survival both in macrophages and in mice, it appears that it is expressed at high enough levels to be important during infection.

**DISCUSSION**

In this study, we used the RIVET reporter system to demonstrate that *hyd* and *hya* are expressed in phagocytic cells and in the mouse during *Salmonella* infection. Previously, it was shown that the ability to use H$_2$ was necessary for *S. enterica* serovar Typhimurium to cause morbidity in the mouse model (16). The results from the RIVET expression studies in this report suggest where and when two of the hydrogenases are expressed inside the animal, and therefore at which point each hydrogenase may be used during infection.

The *hyd* genes were initially expressed in the liver and spleen of mice, probably within phagocytes. Later in the infection, *hyd* was highly expressed in the ileum as well. The *hyd* genes were previously shown to be upregulated under aerobic conditions (28). After activation, phagocytes typically undergo a respiratory burst in order to generate toxic oxidants; therefore we can assume that the environment inside phagocytes is somewhat oxic. A recent study reported that an activated RAW 264.7 murine macrophage had a respiratory increase of 1.5-2 nmol O$_2$/10$^6$ cells-min after activation with *E. coli* lipopolysaccharide (22). Another study used a microelectrode to measure the amount of superoxide (O$_2^-$) present within the PMN-like cell line, HL-60 (12). They reported that there was about 15 ± 5 fmol O$_2^-$ present per cell. The oxygen present in these cell lines could cause *hyd* genes to be upregulated. It is possible that Hyd provides reductant used for oxidative stress-combating enzymes.

This same hydrogenase (Hyd) may be used to oxidize hydrogen with oxygen as the terminal electron acceptor in order to conserve energy while the bacterium resides in the host. Comparisons between the *hyd* large subunit and the gene database on the NCBI website revealed
that *S. enterica* serovar Typhimurium *hyd* is more similar to the oxygen-tolerant hydrogen oxidizing enzymes from *Methylococcus capsulatus* (*hup*) and *Azotobacter vinelandii* (*hox*) than it is like the anaerobic hydrogen oxidizing hydrogenases from other enteric bacteria (www.ncbi.nlm.nih.gov). In addition, it has been shown that H₂ oxidation can be coupled to O₂ reduction in *S. enterica* serovar Typhimurium (16). Hyd may oxidize hydrogen with oxygen as the terminal electron acceptor while cells are residing in macrophages. This could either conserve energy in the form of ATP production and/or decrease the amount of O₂ present in macrophages, which would reduce the toxic oxidation of molecules inside the bacterium.

The *hya* genes were expressed at low levels both in macrophages and in all locations tested within the mouse. Although it was expressed at low levels, *hya* was important for survival within macrophages. This may be due to reduced acid resistance in the SCV; since the *hya* mutant strain entered macrophages just as well as wild-type, yet did not survive acidic conditions as well as wild-type in acid survival assays. It has been shown that the macrophage SCV pH decreases to between 4.5 and 6 one hour post inoculation with *S. enterica* serovar Typhimurium (4, 6). The Hyc proteins in *E. coli* are known to produce H₂ when the growth media is maintained at a low pH during mixed-acid fermentation (1, 20). Hya may be used to recycle the H₂ produced by Hyc under low pH, thereby conserving energy, as was suggested by Sawers *et al.* (26). Hydrogenase activity has been shown to affect acid resistance in *E. coli*, since a recent study demonstrated that complete loss of hydrogenase activity (by a *hyp* mutation) reduced acid resistance to 3% of the wild-type levels (11). Alternatively, previous *S. enterica* serovar Typhimurium mutant strain analysis supports the hypothesis that Hya can operate in an H₂-uptake manner coupled to respiration as well (16).
We have shown that the *S. enterica* serovar Typhimurium uptake-type hydrogenases are differentially expressed in the mouse and in murine macrophages. Although *hyb* was important for survival in the mouse, we were unable to determine where this gene was expressed *in vivo*. qRT-PCR results suggested that *hyb* is expressed in macrophages, which supports the likelihood that it is expressed in the animal as well. Further studies with promoter fusions to other reporter proteins, such as green fluorescent protein, would be useful to examine *hyb* expression in mice. Several studies have used green fluorescent protein fusions to determine bacterial or host promoter activity in animal models (2, 14). This report shows that *hya* plays a role both for acid tolerance and for survival in macrophages. Additional studies that examine the mechanism behind *hya* acid resistance will be useful to explore this relationship further.

**ACKNOWLEDGEMENTS**

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**REFERENCES**


**Table 3.1 Strains and plasmids used in this study**

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| SM10*pir          | thi-1 thr leu tonA lacy supE  
                   | recA::RP4-2-Tc::Mu (Kan') λ::pir | John Gunn |
| DH5α*pir          | F' lacU169 (φ80lacZΔM15) recA1  
                   | endA1 hsdR17 supE44 thi-1 gyrA96  
                   | (Nal') relA1 λ::pir | John Gunn |
| **S. enterica serovar Typhimurium** | | |
| JSG210            | 14028s (wild-type) | ATCC |
| ALZ7              | JSG210 with Δhyb::FRT* | (28) |
| ALZ8              | JSG210 with Δhyd::FRT | (28) |
| ALZ9              | JSG210 with Δhya::FRT | (28) |
| JS246             | 14028 yjeP8103::resl-tetAR-resl | (18) |
| ALZ15             | JS246 hyb':::Φ(hyb'-tnpR-lacZY) | This study |
| ALZ16             | JS246 hyd':::Φ(hyd'-tnpR-lacZY') | This study |
| ALZ17             | JS246 hya':::Φ(hya'-tnpR-lacZY') | This study |
| ALZ18             | JS246 hyb':::Φ(hyb'-tnpRmut135-lacZY') | This study |
| ALZ19             | JS246 hyd':::Φ(hyd'-tnpRmut135-lacZY') | This study |
| ALZ20             | JS246 hya':::Φ(hya'-tnpRmut135-lacZY') | This study |
| ALZ21             | JS246 hyb':::Φ(hyb'-tnpRmut168-lacZY') | This study |
| ALZ22             | JS246 hyd':::Φ(hyd'-tnpRmut168-lacZY') | This study |
| ALZ23             | JS246 hya':::Φ(hya'-tnpRmut168-lacZY') | This study |
| **Plasmids**      |                         |                     |
| pGOA1193          | pIVET5n with promoterless tnpR-lacZY (Ap') | (18) |
| pGOA1194          | pIVET5n with promoterless tnpRmut135-lacZY (Ap') | (18) |
| pGOA1195          | pIVET5n with promoterless tnpRmut168-lacZY (Ap') | (18) |
| pZBL6             | pGOA1193 with P_hyb | This study |
| pZBL7             | pGOA1193 with P_hyd | This study |
| pZBL8             | pGOA1193 with P_hya | This study |
| pZBL9             | pGOA1194 with P_hyb | This study |
| pZBL10            | pGOA1194 with P_hyd | This study |
| pZBL11            | pGOA1194 with P_hya | This study |
| pZBL12            | pGOA1195 with P_hyb | This study |
| pZBL13            | pGOA1195 with P_hyd | This study |
| pZBL14            | pGOA1195 with P_hya | This study |

FRT, FLP recognition target
Figure 3.1 Expression of RIVET fusions in vitro. RIVET fusion strains ALZ 18, ALZ 16, or ALZ 17 for hyb, hyd, or hya, respectively, were grown overnight under conditions previously shown to induce the promoter or under uninducing conditions (aerobically or anaerobically on CR-Hyd, LB, or BA media). β-galactosidase activities were measured (A), and tetracycline
sensitivity was determined (B). N = 3-7. According to the Mann-Whitney-Wilcoxon test, β-galactosidase activities were significantly higher for ALZ17 cells grown anaerobically on blood agar (BA) than for cells grown aerobically on LB agar (α = 0.01).

A.
Figure 3.2 Expression of RIVET fusions in murine macrophages and human PMN-like cells. 1 x 10^5 RAW 264.7 murine macrophages (A) or HL-60 human PMN-like cells (B) were infected with 1 x 10^6 RIVET fusion strains ALZ18 (hyb), ALZ16 (hyd), or ALZ17 (hya) in a multiplicity of infection (MOI) of 10 in 24-well plates. Extracellular bacteria were killed with 100μg/ml gentamicin and macrophages were lysed with 1% TritonX-100 16 hours later. Hydrogenase expression was monitored by determining the ratio of tetracycline sensitive colonies/ ampicillin resistant colonies, as described in materials and methods. Results are an average of three replicates. Hya gene expression was significantly higher in bacterial cells isolated from RAW 264.7 or HL-60 tissue culture cells compared to media-only control, based on student’s T-test (P<0.005).
Figure 3.3 Expression of hyd genes in RAW 264.7 murine macrophages. 1 x 10^5 RAW 264.7 murine macrophages were infected with 1 x 10^6 RIVET fusion strain ALZ16 (hyd) in a multiplicity of infection (MOI) of 10 in 24-well plates. Tissue culture cells were lysed with Triton X-100 at various time points as indicated, and bacterial cells were diluted in PBS and plated. Hydrogenase expression was monitored by determining the ratio of tetracycline sensitive colonies/ ampicillin resistant colonies, as described in materials and methods. Results are an average of four replicates.
Figure 3.4 Hydrogenase mutant survival in RAW 264.7 murine macrophages. $1 \times 10^5$ RAW 264.7 murine macrophages were infected with an MOI of 10 ($1 \times 10^6$) wild-type or hyb, hyd, or hya mutant cells (strains ALZ7, ALZ8, or ALZ9, respectively). Extracellular bacteria were killed with 100 $\mu$g/ml gentamicin at 2 hours post inoculation. Macrophages were lysed with 1% Triton-X 100 at various time points and bacteria were enumerated by dilution plating on LB. Results are an average of four replicates.
**Figure 3.5 STM1788 expression in wild-type and hya mutant cells.** RNA was extracted from wt and hya mutant cell cultures grown aerobically overnight in Luria-Bertani broth, using an Aurum Total RNA Mini kit (Biorad). RNA was digested with RQ1 DNase (Promega) to remove contaminating genomic DNA. This was used as the template to generate cDNA, by an iScript select cDNA synthesis kit (Biorad) and primers homologous to the gene directly downstream of the hya mutation (STM1788). PCR was performed with the same primers used to generate cDNA with 30 amplification cycles. Lane 1 contains the Promega 1kb DNA ladder. Lane 2
contains the positive control, PCR product from wild-type genomic DNA. Lanes 3 and 4 contain PCR product from wild-type and hya cDNA, respectively. Lanes 5 and 6 contain the negative controls, PCR product from wild-type and hya cDNA reactions where reverse-transcriptase was not added.

Figure 3.6 Phagocytosis of wild-type and hya mutant S. enterica serovar Typhimurium by RAW 264.7 murine macrophages. 1x 10^5 macrophages were infected with 1 x 10^8 FITC-conjugated live wild-type or hya cells, beads, or 5 x 10^6 FITC-conjugated killed E. coli cells. Macrophages were examined 1.5 hours later for adherence or phagocytosis by fluorescence quenching and using a Leica TCS inverted epifluorescence microscope. The number of macrophages containing one or more bacterial cells or beads per 100-200 macrophages was counted and expressed as percent internalization. The results are an average of three trials.
Figure 3.7 Hya mutant survival in acid stress conditions. Anaerobic bottles containing citric acid-phosphate buffer at pH 4 were inoculated with $10^8$ wild-type or hya mutant cells that were grown anaerobically in LB overnight. Bottles were incubated for various amounts of time, as indicated, and samples were taken. Cells were enumerated by dilution plating on LB agar. Results are an average of at least 4 replicates.
Figure 3.8 Hydrogenase gene expression in mouse tissues. Female BALB/c mice were orally inoculated with $1 \times 10^6$ ALZ 16 (hyd fusion) or ALZ 17 (hya fusion) cells. Mice were sacrificed at one day (A) and 2.5 days (B) post inoculation for hyd or 2 days (C) and 4 days (D) post inoculation for hya. Ileum, livers, and spleens were taken. The organs were mechanically homogenized in PBS and plated on Bismuth-sulfite or LB agar containing ampicillin. The resulting colonies were patched on LB containing tetracycline and the percent of Tet$^+$ colonies/total colonies patched per mouse organ was calculated. Cells isolated from the liver and spleen had significantly more hyd expression at day one (A) compared to cells isolated from the ileum on the same day, according to the Mann-Whitney Wilcoxon test ($\alpha = 0.01$).
CHAPTER 4
RESPIRATORY HYDROGEN USE IN SHIGELLA FLEXNERI

ABSTRACT

Shigella flexneri is an enteric pathogen commonly associated with food-borne illness. It causes serious and debilitating bacillary dysentery in both the industrialized and the developing world. S. flexneri contains two uptake-type hydrogenases, Hya and Hyb, which may be important in virulence. In order to examine the role of these hydrogenases in the free-living bacterium, we assayed hydrogenase activity in wild-type and hydrogenase mutant cells grown under various conditions. Our results showed that whole-cell hydrogenase activity was higher in wild-type cells grown anaerobically with glycerol and fumarate (anaerobic respiration), than in cells grown on blood agar plates. The presence of hydrogen also augmented growth yield in media containing fumarate. Hydrogenase activity was higher in cells grown anaerobically or in low oxygen, compared to cells grown at atmospheric conditions. In addition, hya was necessary for hydrogenase activity in cells grown on blood agar, while hyb was important in cells grown under anaerobic respiration.

INTRODUCTION

Enteric pathogens are responsible for about 2 million deaths annually, resulting in billions of dollars of treatment costs (6). Shigella species are enteric pathogens that are a common cause of food-borne illness. According to the Center for Disease Control and Prevention (www.cdc.gov), an estimated 448,240 Shigella sp. infections in the United States occur annually. Shigella

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1 Zbell, A. L., McNorton, M. M. and R. J. Maier
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*S. flexneri* causes shigellosis, a disease characterized by bloody diarrhea. In addition, the closely related organism *Shigella dysenteriae* type 1 causes deadly epidemics in the developing world (www.cdc.gov).

It was recently shown that hydrogen gas (H₂) is an important energy source for enteric pathogens in vivo (4, 11). H₂ is produced in the host by colonic bacterial fermentations and diffuses throughout the body (8). Bacterial NiFe uptake-type hydrogenases catalyze the H₂ oxidizing reaction H₂ → 2e⁻ + 2H⁺, yielding protons that contribute to energy production via the proton gradient across the cytoplasmic membrane. In addition, electrons generated in the reaction can be passed to other membrane-bound electron carriers, eventually contributing to ATP production (9).

*S. flexneri* contains two uptake-type NiFe hydrogenases that are homologous to *Salmonella enterica* serovar Typhimurium and *Escherichia coli* uptake-type hydrogenases, annotated as *hya* (which encodes hydrogenase 1) and *hyb* (which encodes hydrogenase 2). *S. enterica* serovar Typhimurium also contains another uptake-type hydrogenase encoded by the *hyd* genes (18). Gene sequence alignments from the J. Craig Venter Institute have shown that *S. flexneri* *hya* genes are homologous to *E. coli* and *S. enterica* serovar Typhimurium *hya* genes. Likewise, the *S. flexneri* *hyb* genes are homologous to *E. coli* and *Salmonella hyb* genes (www.tigr.org).

The *S. flexneri* uptake-type hydrogenases have not been studied. However, hydrogenases in *E. coli* have been well characterized. Hya is present under fermentative conditions and is expected to recycle the H₂ produced by formate hydrogen lyase during fermentation (15). The *hya* operon is also upregulated under acidic conditions and may be involved in the acid stress response (12). There is evidence that Hyb is used during anaerobic respiration, coupling H₂
oxidation to fumarate reduction (1, 14). Hyb may also be used to oxidize H$_2$ with O$_2$ as the
terminal electron acceptor, as suggested by Maier et al in their study on S. enterica serovar
Typhimurium hydrogenases (9). Since S. flexneri hydrogenases are homologous to E. coli
hydrogenases, they may have similar functions. In addition, one or both S. flexneri hydrogenases
may function like the S. enterica serovar Typhimurium hyb, which is upregulated under aerobic
conditions, and may be used to oxidize H$_2$ with O$_2$ as the terminal electron acceptor (18).

The results from mouse infection studies suggest that the uptake-type hydrogenases in S.
enterica serovar Typhimurium, may be important for virulence (9). The removal of all three
hydrogenases in a triple mutant resulted in a strain that was avirulent and unable to colonize the
mouse liver or spleen. In addition, hydrogen is an energy source for Helicobacter pylori during
stomach infections and may be an important energy source for another enteric pathogen,
Bilophila wadsworthia (4, 7). Since S. flexneri is very similar to S. typhimurium and colonizes a
similar environment, it can be predicted that S. flexneri uptake-type hydrogenases will be
important for host colonization and infection.

**MATERIALS AND METHODS**

**Strains, growth conditions and reagents.**

The S. flexneri and E. coli strains and plasmids used in this study are listed in Table 4.1. Strains
were maintained in Luria-Bertani (LB) broth or on LB plates with appropriate antibiotics: 50 μg
ampicillin ml$^{-1}$, 25 μg kanamycin ml$^{-1}$. Brucella agar plates containing 10% sheep blood (blood
agar) (Atlanta Biologicals, GA) were used when indicated. CR-Hyd media with glycerol (0.4%)
and sodium fumarate (20 mM) was used where indicated (16). Cells were grown at 37°C either
anaerobically or aerobically. Anaerobic conditions were established by sparging anaerobic jars
or sealed 165 ml bottles with N$_2$ for 15 minutes, then anaerobic mix (10% H$_2$, 5% CO$_2$, and 85%
N₂) for 20 minutes. Primers were purchased from Integrated DNA Technologies (Coralville, IA).

**Mutant strain construction.**

Non-polar deletion mutants of the *hya* and *hyb* genes were constructed using the lambda Red system, as described by Datsenko and Wanner (5). The *hya* or *hyb* structural genes (*hyaA* and *hyaB* together or *hybA*, *hybB*, and *hybC* together, respectively) were separately replaced by a kanamycin resistance cassette, which was then removed by site-specific recombination at the FRT (flippase (FLP) recognition target) sites that flank the resistance gene.

The primers UPSFHyADel (5’CCGGTGTTGTAGGAGAAGAGACGTGGTGTGTAGGCTGG AGCTGCTTC 3’) and REVSFHyADel (5’ TTTCTGTTGATGATTCTCCTTCGC TGCATATGAATATCCTCCTTA 3’) were used to generate a PCR product from plasmid pKD4. The resulting PCR product contained a kanamycin resistance cassette with flanking sequences that were homologous to parts of the *hya* operon. The PCR product was then transformed into *S. flexneri*, where it replaced the wild type copy of *hyaA* and *hyaB* (S1040 - S1041), resulting in a 2,900 bp deletion. The kanamycin cassette was removed by addition of pCP20, resulting in a *hya* deletion strain called ALZ44 (Table 4.1). The *hya* deletion was confirmed by PCR using primers homologous to regions flanking the deleted gene UPSFHyACheck (5’ TTTCTGGCGA CGTGTGCCAGTG 3’) and REVSFHyACheck (5’ ATGCATAACACCCTAGCCAGCCAG 3’).

In order to construct the *hyb* deletion mutant, the primers UPSFHybDel (5’ AGATAACGCTGACTCAGGGGAGAAATACCTGTGAGGCTGGAGCTGGCTTC 3’) and REVSFHybDel (5’ TATTGGCCAGCTCATAAGACTAAAATACGCACATATG AATATCCTCCTTA 3’) were used to generate a PCR product using pKD4 as the template. The
resulting PCR product was transformed into *S. flexneri*, where the kanamycin resistance cassette replaced the *hybA*, *hybB*, and *hybC* genes (S3244, S3243, and S3242, respectively), resulting in a 3,850 bp deletion. The kanamycin cassette was removed by addition of pCP20, resulting in a *hyb* deletion strain called ALZ47 (Table 4.1). The *hyb* deletion was confirmed by PCR with UPSFHybCheck (5’ AACGTCTCTGCAG GCGCTAATG 3’) and REVSFHybCheck (5’ AGGATCTCAACATAATCCGGCAG 3’).

**Amperometric hydrogenase assays.**

Wild-type or *hya*, *hyb*, or double mutant cells were grown overnight on LB or BA (blood agar). Plates were placed in an anaerobic jar which was flushed with N₂ for 15 minutes and then anaerobic mix for 20 minutes to generate anaerobic conditions. Oxygen was added at 2% or 20% where indicated. Cells were harvested and suspended in phosphate-buffered saline (PBS). Cells were placed in a stirred and sealed chamber containing a Clark type probe, 100μL of H₂-saturated PBS was added, and hydrogen uptake was recorded by amperometry (10).

**End-point growth assays**

In order to determine the effect of hydrogen on growth yield, end-point growth assays were performed. Sealed 165 mL bottles containing CR-Hyd media with various electron acceptors (sodium fumarate (20 mM), sodium nitrate (20 mM), trimethylamine N-oxide (TMAO) (20mM) or dimethyl sulfoxide DMSO (0.25%)) were inoculated with 1.0 x 10⁶ wild-type *S. flexneri* cells. Cells were grown anaerobically with or without 10% H₂ for 18 hours at 37°C with shaking. OD₆₀₀ was measured after growth in order to determine cell number.
RESULTS AND DISCUSSION

H₂-oxidation activity with cells grown under different growth conditions.

*S. flexneri* has two putative membrane-bound hydrogenases that could be used to oxidize H₂ anaerobically with fumarate or alternatively, some other terminal electron acceptor, or even aerobically with O₂ as the electron acceptor. Past studies have typically measured hydrogen uptake anaerobically in enteric bacteria (16). However, H₂ uptake can also be coupled to O₂ reduction, which is common in aerobic hydrogen-oxidizing bacteria (2). A recent study on the enteric bacterium *S. enterica* serovar Typhimurium found that H₂ oxidation could be coupled to O₂ reduction (9). In order to test *S. flexneri*’s ability to oxidize H₂ in the presence of oxygen, cells were grown on rich media under different concentrations of oxygen. Hydrogen oxidation then was measured aerobically by use of a Clark-type electrode.

When wild-type *S. flexneri* was grown on LB or BA plates incubated under atmospheric conditions, hydrogen uptake was almost completely absent (Fig. 4.1). In contrast, when cells were grown anaerobically on plates incubated with H₂, CO₂, and N₂, hydrogen uptake was about 20 nmol H₂/min/10⁹ cells. Hydrogen uptake levels were similar between cells grown anaerobically and cells grown under 2% O₂. However, when cells were grown under 20% O₂ (but in sealed vials with CO₂ and N₂), hydrogenase activity decreased to levels comparable to cells grown in atmospheric conditions (Fig. 4.1). These results suggest that hydrogen uptake activity is efficiently expressed either anaerobically or microaerobically. High levels of O₂ may repress hydrogenase expression indirectly by regulators such as FNR or ArcA, as shown in *E. coli* and *S. enterica* serovar Typhimurium (13, 18). In addition, the low levels of H₂ under atmospheric conditions may be insufficient to induce hydrogenase genes.
The growth studies revealed that hydrogen is important for growth in media containing exogenous fumarate. Growth yield was on average $1.75 \times 10^8$ cells/mL ($\pm 1.7 \times 10^6$) in bottles with $H_2$ and $5.2 \times 10^7$ cells/mL ($\pm 9.8 \times 10^6$) in bottles without added $H_2$ ($n = 3$). This 3.3 fold-increase indicates that cells are able to couple hydrogen oxidation to fumarate reduction under this condition. Growth was not augmented by $H_2$ in media containing nitrate, DMSO, or TMAO (results not shown).

**H$_2$-oxidation by the uptake-type hydrogenase mutants.**

*S. flexneri* has two uptake-type hydrogenases, Hya and Hyb, which have sequence similarity to each other and to *E. coli* and *S. enterica* hydrogenases. Hya in *E. coli* and in *S. enterica* serovar Typhimurium is expressed anaerobically and may be used to recycle hydrogen generated during fermentative growth (1, 3, 15, 18). The *hyb* operon in *E. coli* and *S. enterica* serovar Typhimurium has the highest expression during anaerobic growth on glycerol and fumarate (anaerobic respiration) (13, 18). Hyb could be used to conserve energy by coupling hydrogen oxidation to fumarate reduction or to $O_2$ reduction if cells were grown with oxygen (9, 14). *S. enterica* serovar Typhimurium contains another uptake-type hydrogenase, Hyd, which has the highest expression aerobically (18). The *S. flexneri* Hya and Hyb may have similar roles as one or both *E. coli* and *S. enterica* serovar Typhimurium uptake-type hydrogenases.

In order to determine possible roles for each *S. flexneri* uptake type hydrogenase, wild-type, *hya*, and *hyb* mutant cells were grown under different conditions and whole-cell hydrogen uptake was measured. When cells were grown anaerobically on BA plates, hydrogen uptake in the *hyb* mutant was similar to that of wild-type. Hydrogen uptake in the *hya* mutant was almost completely abolished, suggesting that Hya, and not Hyb, is important under this condition (Fig. 4.2).
When cells were grown anaerobically in liquid CR-Hyd media containing glycerol and fumarate, hydrogen uptake in the wild-type was higher than when cells were grown on blood agar plates. This may indicate that hydrogenase is especially important under this growth condition. A recent study suggested that *S. flexneri* cannot use glycerol as a growth substrate (17). Therefore, under these conditions, H₂ may be the most important electron donor for energy conservation by anaerobic respiration to fumarate. Under this growth condition, hydrogen uptake was undetectable in the *hyb* mutant (Fig. 4.2), so Hya may not play a role in H₂/fumarate respiration. Hydrogen uptake was similar in the wild-type and *hya* mutant cells. Like in *E. coli*, the *S. flexneri* Hyb appears to be used for hydrogen oxidation during anaerobic respiration.

**ACKNOWLEDGEMENTS**

We thank John Gunn for supplying plasmids pKD4, pKD46, and pCP20. This work was supported by the University of Georgia Foundation.

**REFERENCES**


Table 4.1. Strains and plasmids

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* FRT, FLP recognition target
Figure 4.1. Effect of oxygen on hydrogen uptake activity. Wild-type *S. flexneri* cells were grown overnight aerobically on LB or blood agar plates or in anaerobic jars sparged with 5% CO₂, 10% H₂ and 85% N₂. Hydrogen uptake was assayed aerobically and measured with a Clark-type electrode. The results are an average of six replicates.
Figure 4.2. Hydrogenase activity in wild-type and mutant cells grown under different conditions. Wild-type, hya, or hyb mutants (ALZ 44, or ALZ 47 respectively), were grown overnight on blood agar plates in anaerobic jars or in sealed 165 ml bottles containing CR-Hyd media with 0.4% glycerol and 20mM sodium fumarate. Anaerobic conditions were obtained by sparging jars or bottles with N₂ for 15 minutes, then anaerobic mix (5% CO₂, 10% H₂, and 85% N₂) for 20 minutes. Hydrogen uptake was assayed aerobically using a Clark-type electrode. Results are an average of four to nine replicates.
CHAPTER 5

ROLE OF HYA IN RECYCLING ANAEROBICALLY-PRODUCED H₂ IN SALMONELLA ENTERICA SEROVAR TYPHIMURIUM¹

ABSTRACT

Double and triple uptake-type hydrogenase mutants were used to determine which hydrogenase recycles fermentatively-produced hydrogen. The double mutants ΔhybΔhya and ΔhydΔhya evolved H₂ at rates similar to the triple-mutant strain, so Hya alone oxidizes the bulk of H₂ produced during fermentation. When only Hya was present, no hydrogen production was observed. H₂-uptake assays show that Hya can also oxidize exogenously-added H₂.

RESULTS AND DISCUSSION

Due to the anticipated scarcity of fossil fuels, there has been a surge of interest in H₂ production as an alternative energy source. Numerous studies have attempted to engineer H₂-producing organisms such as photosynthetic bacteria, cyanobacteria, and Escherichia coli to produce maximal amounts of H₂ while minimizing the organism’s H₂-oxidizing capability (4, 5, 9, 10, 17). Hydrogenase expression and activity is controlled by multiple regulatory pathways and responds to fluctuations in pH, oxygen levels, and availability of metabolites and metal cofactors (16). In addition, the presence of hydrogen uptake-hydrogenases decreases the net H₂ yield even in conditions that favor H₂ production. It is therefore important to understand the interactions between H₂-oxidizing enzymes (i.e. “respiratory hydrogenases”) and H₂-producing enzymes.

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Gene sequence analysis has revealed that many enteric bacteria contain the genes necessary for hydrogen production and oxidation. The *E. coli* hydrogenases have been studied extensively, while *Salmonella enterica* serovar Typhimurium hydrogenases have been studied to a lesser extent. Both *E. coli* and *S. enterica* serovar Typhimurium contain the hydrogen-oxidizing hydrogenases Hya and Hyb. *S. enterica* serovar Typhimurium also contains Hyd, which is another hydrogen-oxidizing hydrogenase (2, 13, 15). Hyc and Hyf are hydrogen-evolving hydrogenases that are present in both *E. coli* and *S. enterica* serovar Typhimurium, although it is unknown whether Hyf is functional (1).

The *S. enterica* serovar Typhimurium hydrogenases are important for cellular metabolism. Hyc produces H₂ in order to remove excess reductant generated during mixed-acid fermentation. Hyc, together with formate dehydrogenase constitute the formate hydrogen lyase (FHL) complex that oxidizes formate to produce CO₂ and H₂ (12). The *hyb* genes in *E. coli* and *S. enterica* serovar Typhimurium are highly expressed under anaerobic respiration conditions, and Hyb probably contributes to energy conservation (11, 15, 18). Hyb splits H₂ and generates electrons, which are passed through the electron transport chain to terminal acceptors such as fumarate. The protons generated contribute to the proton-motive force. The role of Hya is not as well characterized. It may be used to recycle Hyc-produced H₂, since the *hya* operon is highly expressed during fermentative growth, or it may play a role in acid stress resistance (6, 14, 18, 19). The *hyb* genes are highly expressed under aerobic conditions in *S. enterica* serovar Typhimurium and Hyb may couple H₂ oxidation to O₂ reduction (18).

Redwood *et al.* recently examined the roles of uptake-type hydrogenases on net hydrogen production in *E. coli* (10). Cells were pre-grown aerobically or anaerobically with formate and then allowed to ferment in anaerobic bottles. H₂ gas was collected, and other fermentation
products were measured. They found that H₂ production increased by 37% in a hya/hyb double mutant (compared to wild-type) that was grown overnight aerobically with formate. This increase in production was due to the loss of hyb, not hya. Therefore, in E. coli, Hyb is responsible for recycling fermentatively-produced H₂.

In this study, we measured the effect of uptake-type hydrogenase mutations on H₂ production in S. enterica serovar Typhimurium. We found that the majority of H₂-recycling activity in fermenting cells was dependent on the presence of hya. These results demonstrate another difference between H₂ metabolism in E. coli and S. enterica serovar Typhimurium.

**Bacterial strains, growth conditions and reagents.**

The strains and plasmids used in this study are listed in Table 5.1. Strains were maintained in Luria-Bertani (LB) broth or on LB plates with appropriate antibiotics: 50 µg ampicillin ml⁻¹, 25 µg kanamycin ml⁻¹. CR-Hyd media with glucose (0.4%) and sodium formate (20 mM) was used where indicated (15). Cells were grown at 37°C either anaerobically or aerobically. Anaerobic conditions were established by sparging sealed 165 ml bottles with N₂ for 15 minutes, then anaerobic mix (10% H₂, 5% CO₂, and 85% N₂) for 20 minutes.

**Mutant strain construction.**

Deletion mutants of the hyb, hyd, and hya genes were constructed using the lambda Red system, as reported previously (7, 18). These deletions are non-polar and strains do not contain any antibiotic resistance markers. The double mutants and triple mutant were constructed using each hydrogenase single deletion mutant and a hydrogenase mutant in which the kanamycin cassette replaced the hydrogenase gene (7). The phage P22Htint (J. Gunn, The Ohio State University; Columbus, OH) was used to transduce the antibiotic marker from the Δhyb:kan, Δhyd:kan, or Δhya:kan strain into the hya, hyb, or hyd single mutant, making double mutant strains. The
kanamycin cassette was removed by transforming with pCP20 and then growing at 37°C. The triple mutant was constructed similarly by transducing the antibiotic resistance cassette from a hydrogenase single mutant strain into a hydrogenase double mutant. The double mutants and triple mutant were confirmed by PCR. The resulting strains are listed in Table 5.1.

**Amperometric hydrogenase assays.**

Wild-type, *hya/hyb*, *hyb/hyd*, or *hya/hyd* double mutant or *hya/hyb/hyd* triple mutant cells were grown overnight under anaerobic conditions in bottles containing CR-Hyd with glucose and formate. Cells were removed from the closed bottle with a syringe after overnight growth. The cell suspension was injected into a stirred and sealed chamber containing a Clark type probe. Hydrogen evolution was recorded by amperometry (8). In order to measure hydrogen uptake, 100µL of H2-saturated PBS was added to the chamber and the disappearance of H2 was recorded.

**Effect of hydrogenase deletions on H2 production.**

Redwood *et al.* found that in *E. coli*, Hyb is important for recycling Hyc-produced H2 during fermentative growth (10). Whether Hyb had capacity to recycle additional (exogenous) H2 was not addressed. However, it has also been hypothesized that *E. coli* Hya may function to oxidize H2 during fermentation (14). Our previous study revealed that in *S. enterica* serovar Typhimurium, *hya* gene expression was maximal during fermentative growth (18). We constructed hydrogenase double and triple mutants in *S. enterica* serovar Typhimurium to determine which hydrogenase was important in recycling fermentatively produced H2 in this organism.

**Wild-type S. enterica serovar Typhimurium, hydrogenase double and triple mutants were grown anaerobically overnight in sealed bottles containing CR-Hyd with glucose and formate.** This media was designed for optimal expression and maturation of the FHL complex. Hydrogen
evolution was measured by injecting cells into a sealed chamber containing a Clark-type electrode. H₂ production was observed after about a 5 minute lag time, during which the cells respired any O₂ which may have entered the chamber. The presence of O₂ would inhibit Hyc function. When sodium dithionite (50 µM) was added to the chamber to make it anaerobic, the lag time to reach maximal activities was only about 10 seconds (the likely time for complete dithionite mixing/oxygen removal).

H₂ production was not observed for wild-type cells (Table 5.2). This is undoubtedly due to the activity of the H₂-oxidizing enzymes. As Hyc produces molecular hydrogen, the uptake-type hydrogenases oxidize it, resulting in no observable H₂ uptake or evolution. Interestingly, H₂ production was not observed in the Δhyb Δhyd mutant, either. This result suggests that Hya has sufficient H₂-uptake activity to use all of the hydrogen produced by Hyc. Hydrogen uptake assays indicated that Hya also has some limited capacity to use exogenous H₂ above what is evolved from the FHL system (Table 5.2). The Δhyb Δhya and Δhyd Δhya double mutants produced hydrogen at comparable rates to the triple mutant (Table 5.2). Whenever hya was absent, hydrogen production was observed. This result indicates that Hyb and Hyd are not important for anaerobic recycling of H₂ during fermentative growth.

Our previous study showed that hyb and hyd are expressed at low levels during fermentative growth with formate (6 times and 3 times less than at their optimal growth condition, anaerobic with glycerol and fumarate and aerobic with glucose, respectively) (18). However, the high rates of hydrogen production in double mutants (that only contain Hyb or Hyd) indicate that these protein levels are either too low to oxidize FHL-produced H₂ or these hydrogenases are not properly localized to couple H₂ oxidation to the FHL system. Curiously, in our previous study, hya expression was repressed when cells were grown with formate, as
compared to glucose alone. Hya gene expression levels were 45.7 +/- 3.2 Miller units when cells were grown in CR-Hyd with glucose and 9.9 +/- 1.6 when cells were grown with glucose and sodium formate (18). This level of hya expression must be sufficient for H₂ oxidation under this condition. A previous study by Sawers et. al used rocket immunoelectrophoresis with E. coli immunoglobulins to measure levels of S. enterica serovar Typhimurium hydrogenase isoenzyme under different growth conditions. They found that the cellular content of isoenzyme 1 (presumably Hya) was higher in cells growth with formate as compared to glucose alone (15).

It has been shown that Hyb but not Hya is important for hydrogen uptake during fermentation in E. coli (10). In contrast, Hya is responsible for hydrogen recycling under these conditions in S. enterica serovar Typhimurium . This study further defines the roles of the S. enterica serovar Typhimurium uptake-type hydrogenases. Hya is used during fermentation, and has been shown to be important in acid-resistance (19). Nevertheless, it has been shown that hyd is highly expressed during aerobic growth and while S. enterica serovar Typhimurium resides in phagocytes (18, 19). Hyd is probably used for efficient O₂-coupled energy conservation/ respiration at a time of severe external stress. The hyb genes are maximally expressed during anaerobic fermentation (18). The multiple hydrogenases appear to play important roles in increasing the fitness of the bacterium in order to survive a variety of growth environments.

ACKNOWLEDGEMENTS

We thank John Gunn for supplying plasmids pKD4, pKD46, and pCP20 and phage P22Htint.

This work was supported by the University of Georgia Foundation.

REFERENCES


Table 5.1. Strains and plasmids

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Genotype or description</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Strains</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S. enterica serovar Typhimurium</td>
<td></td>
<td></td>
</tr>
<tr>
<td>JSG210</td>
<td>14028s (wild-type)</td>
<td>ATCC</td>
</tr>
<tr>
<td>ALZ7</td>
<td>JSG210 with Δhyb::FRT*</td>
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</tr>
<tr>
<td>ALZ8</td>
<td>JSG210 with Δhyd::FRT</td>
<td>(18)</td>
</tr>
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<td>ALZ9</td>
<td>JSG210 with Δhya::FRT</td>
<td>(18)</td>
</tr>
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<td>Kan'; Contains kan cassette</td>
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* FRT, FLP recognition target
Table 5.2. Hydrogenase activity in wild-type *S. enterica* serovar Typhimurium or in hydrogenase mutant cells.

<table>
<thead>
<tr>
<th>Strain</th>
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<th>Hydrogenase activity (nmol H₂/min/10⁹ cells)*</th>
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<td>H₂ evolution</td>
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<tr>
<td>Wild-type</td>
<td>Hya, Hyb, Hyd</td>
<td>&lt; 0.02ᵃ</td>
</tr>
<tr>
<td>Δhyb Δhyd</td>
<td>Hya</td>
<td>&lt; 0.02ᵃ</td>
</tr>
<tr>
<td>Δhyb Δhya</td>
<td>Hyd</td>
<td>18.3 ± 8.9</td>
</tr>
<tr>
<td>Δhyd Δhya</td>
<td>Hyb</td>
<td>17.2 ± 7.2</td>
</tr>
<tr>
<td>Triple mutant</td>
<td>None</td>
<td>14.4 ± 5.7</td>
</tr>
</tbody>
</table>

* Cells were grown overnight anaerobically in bottles containing CR-HYD media with 0.4% glucose and 20mM sodium formate. Results are the mean ± standard deviation for at least three independent experiments. ᵃThis was deemed to be the minimum detectable level by the amperometric assay. ¹H₂ uptake was assayed anaerobically with overnight cultures in media, with no exogenous electron acceptor added. 75.4 nM H₂ was injected into the chamber for H₂ uptake assays.
CHAPTER 6
HYDROGEN-DEPENDENT GROWTH OF SALMONELLA ENTERICA SEROVAR TYPHIMURIUM DURING ANAEROBIC RESPIRATION

ABSTRACT

Salmonella enterica serovar Typhimurium is an enteric pathogen that has the capacity to use H₂ in the animal host. The three uptake-type hydrogenases Hya, Hyd, and Hyb can oxidize H₂ to generate a proton motive force across the plasma membrane. This can either occur during respiration or fermentation. Hyc produces H₂ during fermentative growth. The hydrogenases are important for virulence in a mouse model, but it is unknown whether they increase growth yield in nutrient-poor media. Wild-type cells were grown anaerobically with or without added H₂ in CR-Hyd media with 20mM sodium fumarate, sodium nitrate, trimethylamine N-oxide (TMAO), or 0.25% dimethyl sulfoxide. Growth curves were obtained for each condition and growth yield was determined. The results indicate that, like in Escherichia coli, the presence of H₂ increases growth yield in media containing fumarate, nitrate, or TMAO as the terminal respiratory acceptor. This growth yield enhancement may be due to the delayed onset of stationary phase when H₂ is provided. The use of H₂ inside the animal host likely allows the bacterium to achieve greater cell numbers and launch a more successful invasion.

INTRODUCTION

Molecular hydrogen (H₂) can be used as an energy source by a variety of prokaryotes including aerobic hydrogen oxidizers, cyanobacteria, photosynthetic bacteria, rhizobia, sulfate reducers,

1 A. L. Zbell and R. J. Maier
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anaerobic fermenters, and extremophiles (11). These organisms contain hydrogenase enzymes, which either catalyze the oxidation of H$_2$ to protons and electrons, or produce H$_2$ in order to dispose of excess reductant (12). The uptake-type (H$_2$-oxidizing) hydrogenases often conserve energy by contributing to the proton gradient across the cell membrane. In addition, electrons are passed down the electron transport chain to a variety of acceptors, including fumarate, nitrate, sulfate, CO$_2$, or O$_2$ (12).

There are five hydrogenases in _S. enterica_ serovar Typhimurium. These membrane-bound hydrogenases all contain [NiFe]. Three oxidize H$_2$ (called Hyb, Hyd, and Hya), while Hyc (and possibly Hyf) produce H$_2$ (1, 9, 15). The expression of the uptake-type hydrogenase genes varies under different growth conditions, which indicates that they are used in different conditions. The _hyb_ genes are preferentially expressed during anaerobic respiration with fumarate as the electron acceptor. The _hyd_ genes are expressed the most in aerobically-grown cells, while _hya_ is preferentially expressed during fermentative growth with glucose (9, 15).

Since H$_2$ can be used to conserve energy, it may be assumed that its presence would affect growth rate in nutrient-poor media. The effect of H$_2$ on growth has not been studied in _S. enterica_ serovar Typhimurium, but has been examined in the closely related bacterium _Escherichia coli_. _E. coli_ contains four hydrogenases that are homologous to those in _S. enterica_ serovar Typhimurium, called Hyb, Hya, Hyc and Hyf (1, 2, 8). Yamamoto and Ishimoto found that H$_2$ was important for _E. coli_ growth during anaerobic respiration with fumarate, nitrate, or trimethylamine N-oxide (TMAO). Growth yield was increased three to eight times in cells grown in a media containing peptone, H$_2$ and the electron acceptor as compared to cells in the same media without H$_2$ (14). H$_2$ enhances growth of other organisms as well. For example, the methanogen _Methanothermobacter thermautotrophicus_ had higher growth yield in the presence
of H₂ and there is an enrichment of unidentified Acinetobacter-like spp. in the rhizosphere of soybean root nodules (which contains high concentrations of H₂) (4, 13).

In this study, we investigated the affect of H₂ on S. enterica serovar Typhimurium growth with various electron acceptors. Growth under anaerobic respiration with fumarate and TMAO was significantly enhanced by the addition of H₂, while growth with H₂ and nitrate was also enhanced, but to a lesser extent. These results indicate that hydrogen can be used as an important energy source when cells are exposed to limiting nutrients. Such an environment is expected to occur during an animal infection when invading pathogens are competing with resident flora and the host for nutrients.

**MATERIALS AND METHODS**

**Strains, growth conditions and reagents.**

Wild-type Salmonella enterica serovar Typhimurium14028s was used in this study (ATCC). Strains were maintained in Luria-Bertani (LB) broth or on LB plates. CR-Hyd media with sodium fumarate (20 mM), sodium nitrate (20 mM), trimethylamine N-oxide (TMAO) (20mM) or dimethyl sulfoxide DMSO (0.25%) was used where indicated (2, 3). Cells were grown at 37°C anaerobically with or without H₂. Anaerobic conditions with H₂ were established by sparging sealed 165 ml bottles with N₂ for 15 minutes, then anaerobic mix (10% H₂, 5% CO₂, and 85% N₂) for 20 minutes. Cells were grown anaerobically without H₂ in bottles sparged with N₂ for 15 minutes and then injected with 5% CO₂.

**End-point growth assays and growth curves**

In order to determine the effect of hydrogen on growth yield, end-point growth assays were performed. Sealed 165 mL bottles containing CR-Hyd media with various electron acceptors (as described above) were inoculated with 1.0 x 10⁶ wild-type S. enterica serovar Typhimurium
cells. Cells were grown anaerobically with or without 10% H₂ for 18 hours at 37°C with shaking. OD₆₀₀ was measured after growth in order to determine cell number.

Growth curves were used to measure the effect of H₂ on growth rate. 1 x 10⁷ wild-type *S. enterica* serovar Typhimurium cells were injected into sealed anaerobic bottles as described above. Cells were grown for 8 hours at 37°C and growth was monitored by measuring OD₆₀₀ at various time points. OD₆₀₀ was experimentally correlated with cell number. Growth curves were performed with cells grown anaerobically in LB broth. OD₆₀₀ was measured throughout growth and cells were plated on LB plates. The relationship between OD₆₀₀ and cell number was calculated and used throughout this study.

**RESULTS AND DISCUSSION**

The ability to use hydrogen is important for *S. enterica* serovar Typhimurium survival within the animal host (6). However, it is unknown whether the presence of H₂ enhances *S. enterica* serovar Typhimurium growth. Yamamoto and Ishimoto demonstrated that *E. coli* growth yield increased when cells were grown with H₂ and fumarate, TMAO, or nitrate, as compared to growth without added H₂. In this study, we investigated whether this would also be true for *S. enterica* serovar Typhimurium.

Wild-type *S. enterica* serovar Typhimurium cells (1 x 10⁶ cells/ mL) were inoculated into sealed anaerobic bottles containing 10 mL CR-Hyd growth media with fumarate, nitrate, TMAO, or DMSO. Cells were grown with or without 10% H₂. The OD₆₀₀ was taken after 18 hours growth, and cell numbers were calculated. Growth curves with wild-type cells demonstrated that OD₆₀₀ = 6.74 x 10⁸ CFU. This numbers was used to convert OD units to CFU. Results were similar to what was observed in *E. coli* (14). Cell yield increased by 2.7 fold for cells grown with fumarate and H₂, compared to fumarate alone. In addition, growth yield was increased by
1.4 fold and 2.2 fold for cells grown with nitrate and TMAO with H₂, respectively (Fig. 6.1). Growth was not significantly higher in cells grown with DMSO and H₂, compared to DMSO alone.

In order to study the affect of H₂ in more depth, growth curves were performed in cells grown in CR-Hyd containing fumarate or TMAO with or without added H₂. Bottles were prepared the same as for the growth yield studies and were inoculated with 1 x 10⁷ wild-type S. enterica serovar Typhimurium cells/mL. Growth was monitored for 8 hours. The results indicate that hydrogen affects growth by delaying the onset of stationary phase. Cells grown in the presence of H₂ began stationary phase at about 6 hours post inoculation, as compared to 3 hours post inoculation for cells grown without H₂ (Fig. 6.2). Since this media does not contain glucose or glycerol, cells are likely using respiratory dehydrogenases such as NADH dehydrogenase I or II, succinate dehydrogenase, pyruvate oxidase, or D-amino acid dehydrogenase to generate electrons for the reduction of electron acceptors (10). When H₂ is available, cells can also use the uptake-type hydrogenases. This probably extends the amount of time cells can grow exponentially. Cells have an additional electron donor (H₂) available for anaerobic respiration, while alternative electron donor substrates are probably limiting at an earlier time in cells grown without H₂.

Sawers et al. demonstrated that H₂ evolution is low (between 0.016 – 0.001 μmol of H₂ evolved per minute) when S. enterica serovar Typhimurium cells are grown under anaerobic respiration with fumarate (9). We wanted to determine whether cells are producing H₂ (which might affect growth yield) under the growth conditions used in this study. One mL of headspace gas from 8 hour stationary-phase cultures grown with fumarate was assayed for the presence of H₂ using a Clark-type electrode (7). There was no detectable H₂ present in headspace gas from
bottles sparged only with N₂ and CO₂. This result indicates that the cells are not producing H₂ under these conditions. There was 50.3 ± 6.9 nmol H₂ present in bottles sparged with N₂ and anaerobic mix, as was expected.

Collectively, our results indicate that the presence of exogenous hydrogen increases growth yield of *S. enterica* serovar Typhimurium cells by delaying the onset of stationary phase. This may be important for virulence in the animal. Even if *S. enterica* serovar Typhimurium does not produce H₂ in the animal, there are sufficient levels of H₂ present from natural flora in the gut to saturate uptake-type hydrogenases (6). A recent study showed that nitrate and fumarate reductase were necessary for *E. coli* survival in the mouse intestine (as was the low-affinity cytochrome bo₃ oxidase) (5). *S. enterica* serovar Typhimurium colonizes a similar environment as *E. coli*, and probably uses both aerobic and anaerobic respiration during infection. It is likely that H₂ oxidation coupled to anaerobic respiration augments Enterobacteriaceae growth and therefore their survival in the animal host.

**ACKNOWLEDGEMENTS**

This work was supported by the University of Georgia Foundation.

**REFERENCES**


Figure 6.1. *S. enterica* serovar Typhimurium hydrogen-dependent growth yield. 1 x 10⁶ wild-type cells were grown for 18 hours anaerobically in bottles containing CR-Hyd media with 20 mM sodium fumarate, sodium nitrate, trimethylamine N-oxide (TMAO) or 0.25% dimethyl sulfoxide (DMSO). Colony-forming units (CFU) per mL culture were determined by measuring OD₆₀₀. Results are an average of three or more independent experiments.
Figure 6.2. Effect of hydrogen on growth over time in *S. enterica* serovar Typhimurium grown anaerobically with fumarate or TMAO. Sealed, anaerobic bottles containing CR-Hyd with 20mM sodium fumarate (A) or 20mM trimethylamine N-oxide (TMAO) (B) were inoculated with 1 x 10⁷ wild-type *S. enterica* serovar Typhimurium cells per mL. Cells were grown at 37°C for 8 hours, and cell numbers were measured by reading OD₆₀₀ at various time points. Results are an average of three independent experiments.
CHAPTER 7
DISSERTATION SUMMARY AND FUTURE DIRECTIONS

*Salmonella enterica* serovar Typhimurium and *Shigella flexneri* are common food-borne pathogens that are responsible for illnesses and deaths in both the industrialized world and in emerging nations (4). When these pathogens enter the animal host, they encounter competition from indigenous species living in the intestinal tract (2). In addition to competing with resident flora, they must combat the host immune response in order to mount a successful infection. Acid and toxin resistance and the ability to survive in or escape from macrophages are among the characteristics needed to successfully colonize the host (3, 8, 16).

It has recently been shown that uptake-type hydrogenases are important for *Helicobacter* spp. and *S. enterica* serovar Typhimurium pathogenesis (9, 11). These hydrogenases split the molecular hydrogen that is produced by the natural gut flora in an energy-conserving manner (12, 20). Therefore, hydrogenases provide an alternative means for energy conservation, one that is not used by the host itself (10). In addition, it has been shown that hydrogenases are important for acid resistance in *Escherichia coli* (by using a hypF mutant, which removes all hydrogenase activity) (6).

The results from my studies have shown that the three uptake-type hydrogenases in *S. enterica* serovar Typhimurium are used under different growth conditions and are regulated differently. First, in order to gain insight on the function of the hydrogenases, their expression and transcriptional regulation was monitored *in vitro*. Then, hydrogenase expression was analyzed in an animal model (mouse infection) and while *S. enterica* serovar Typhimurium resided in phagocytes. The interactions between the hydrogen-producing Hyc and the uptake-
type hydrogenases were analyzed. Finally, I determined the contribution of the uptake-type hydrogenases to growth yield during anaerobic fermentation. In addition, the roles of the S. flexneri hydrogenases were preliminarily characterized. The combined results from these studies yields much information about the roles of each hydrogenase in the free-living bacteria and during an infection or organ colonization. It has become increasingly apparent that H₂ use is an important energy source for pathogens (10). My studies have revealed the complex regulation and roles of enterobacterial hydrogenases and highlighted the importance of H₂ use in these two enteric bacteria.

**EXPRESSION AND REGULATION OF S. ENTERICA SEROVAR TYPHIMURIUM UPTAKE-TYPE HYDROGENASES**

**Expression of hydrogenases under different growth conditions**

Reporter fusion plasmids were made with each hydrogenase promoter cloned upstream of a promoterless lacZ gene. Cells were grown aerobically or anaerobically in CR-Hyd (a growth media designed for optimal hydrogenase activity) with glucose, mannose, glucose plus formate (fermentation), glucose plus nitrate, or glycerol plus fumarate (anaerobic respiration). The results demonstrated that the three hydrogenase genes are differentially expressed. The hyb mutant had the most expression when cells were grown under anaerobic respiration to fumarate. The hya genes were optimally expressed in fermentative cells (anaerobic glucose or mannose), and interestingly, the hyd genes were optimally expressed under aerobic growth conditions (23). These results were similar to what has been reported for E. coli for hyb and hya, whereas the hyd gene studies are new, as E. coli does not contain a hyd homolog (15).
Transcriptional regulation of the uptake-type hydrogenases

A mutant approach was used to determine what proteins were responsible for regulating hydrogenase expression. Common regulators of the anaerobic/aerobic switch, regulators that respond to nitrate availability, and IscR, which responds to iron availability, were deleted and hydrogenase expression was subsequently monitored. The hydrogenases were regulated differently, as might be expected from their expression profiles under various growth conditions. The *hya* genes were nitrate repressed by NarL, but not NarP. This is different than *E. coli*, where *hya* is repressed by both nitrate regulators (15). The *hya* genes were activated by FNR. The *hyb* genes were repressed by IscR aerobically, which is also different than what happens in *E. coli* (where both *hyb* and *hya* are repressed) (5). Our results indicated that FNR and ArcA activate *hyb* expression. We also found that *hyb* is glucose-repressed, as was shown previously (7). The *hyd* genes were repressed by ArcA.

Collectively, these results augment the data collected in the growth experiments and, together with the results from previous experiments, allowed us to assign putative roles for each hydrogenase. Hya is probably important during anaerobic growth when cells are fermenting (and nitrate is not available), Hyd is used aerobically, and Hyb is important during anaerobic respiration. It would be interesting to see whether these regulators bind directly to hydrogenase promoters, or whether the regulation is indirect. In addition, some reports suggest that hydrogenases are regulated by the nickel concentration and pH (13, 17). We have seen that *hya* is upregulated under acidic conditions, but it would be interesting to know how this regulation occurs and if the hydrogenases are also regulated by nickel availability (24).
IN VIVO ROLE OF S. ENTERICA SEROVAR TYPHIMURIUM HYDROGENASES

My in vitro work has increased our understanding of the gene expression and putative roles of each hydrogenase when cells are free-living. In this study, I examined how each hydrogenase was expressed during an infection. The \textit{hyd} genes were highly expressed in bacteria cells residing in phagocytes and in the livers and spleens of mice (24). The expression of \textit{hyd} in the liver and spleen was expected, since \textit{S. enterica} serovar Typhimurium resides within macrophages in these organs (21). The \textit{hya} genes were also expressed while \textit{S. enterica} serovar Typhimurium was in phagocytes, and were expressed at low levels in all mouse organs tested. Hya was important for survival in macrophages and for survival at low pH and these two conditions are probably physiologically related. qPCR results indicated that \textit{hyb} may be expressed in macrophages, although more work needs to be done to confirm this (24).

Since \textit{hyd} is highly expressed in phagocytes and during infection, it is probably important in these environments. It would be interesting to know the exact role of Hyd under these conditions. It may oxidize H$_2$ simply for the cell to use as an alternate energy source, or may be important in scavenging O$_2$ in the macrophage. The latter would reduce the anti-bacterial oxidative stress response mounted by the host. However, a \textit{hyd} mutant survived just as well as wild-type when cells were exposed to various oxidative and nitrosative stress conditions. Nevertheless, more stress conditions could be tested. It would also be interesting to know how Hya contributes to acid resistance. Hya could interact with some of the known acid-tolerance response mechanisms, or could simply be another means to conserve energy under these stress conditions. Finally, another promoter fusion method could be used to determine if, when, and where \textit{hyb} is expressed in vivo.
**ROLE OF Hya IN RECYCLING Hyc-PRODUCED H$_2$ IN *S. ENTERICA SEROVAR TYPHIMURIUM***

Hydrogen evolution activity was assayed in hydrogenase double mutant cells (which only contain one uptake-type hydrogenase). This study demonstrated that Hya is the hydrogenase responsible for recycling H$_2$ produced during fermentative growth, since hydrogen uptake was only observed when Hya was absent (when cells are grown in CR-Hyd with glucose and formate). This is different than what has been shown in *E. coli*, where Hyb recycles fermentatively-produced H$_2$ (14). This study allows us to assign another role for Hya. It seems that Hya is involved in acid resistance and in energy-conservation by recycling fermentatively-produced H$_2$. It would be interesting to know exactly how the interaction between Hya and Hyc occurs, and whether Hya is localized near Hyc in the cell membrane or sub-membrane particles.

**H$_2$ AUGMENTATION OF *S. ENTERICA SEROVAR TYPHIMURIUM* GROWTH**

In this study, I examined the affect of H$_2$ on growth yield during anaerobic respiration. Like in *E. coli*, *S. enterica* serovar Typhimurium had from 1.5 to 3 fold increase in growth yield when H$_2$ was present in cells grown in nutrient-poor media (CR-Hyd) with sodium fumarate, sodium nitrate, or trimethylamine N-oxide (TMAO) (22). In this study, growth curves revealed that cells grown with H$_2$ remained in stationary phase for a longer time period than cells grown without H$_2$. This may account for the higher cell yield. It is likely that this growth augmentation is due to the action of Hyb, since this is the hydrogenase that has historically been associated with hydrogen oxidation during anaerobic respiration, and it has increased gene expression in cells grown with glycerol and fumarate (7, 19, 23). Experiments with hydrogenase deletion mutants could be done to confirm the role of Hyb under these conditions.
**S. FLEXNERI UPTAKE-TYPE HYDROGENASES**

*S. flexneri*, like *E. coli*, contains two uptake-type hydrogenases called Hya and Hyb. The hydrogenases in *S. flexneri* have not been characterized. In this study, we first analyzed hydrogen-uptake activity in cells grown under various conditions. Activity was higher in cells grown anaerobically or under low oxygen concentration, as compared to cells grown under atmospheric conditions. In addition, activity was highest and growth yield was increased in cells grown under anaerobic respiration with fumarate and exogenous H₂, indicating that hydrogenase is especially important under this condition. Hydrogenase activity with deletion mutants revealed that Hya is important for cells grown anaerobically on blood-agar. Hyb was important for cells grown under anaerobic respiration, like in *E. coli* (1, 18). It would be interesting to further characterize these hydrogenases by observing hydrogenase activity under more growth conditions (such as during fermentative growth). In addition, mutants could be used in animal studies and macrophage experiments to see if the hydrogenases play a role in *S. flexneri* virulence.

**REFERENCES**


