

ABSTRACT

BORDEN, NATHAN JOSEPH. Hydrogen metabolism in *Campylobacter jejuni*. (Under the direction of Jonathon Olson.)

Campylobacter jejuni is an important human pathogen. It is the primary cause of acute gastroenteritis in the United States with an estimated 2-10 million cases annually. *C. jejuni* is a commensal colonizer of the avian intestines and contaminated poultry is the primary source of human infection. *C. jejuni* has a unique metabolism and respiratory chain which offer possible targets for antimicrobials. This research project explores the physiological role of hydrogenase in *C. jejuni*. *C. jejuni* contains an uptake hydrogenase that oxidizes H₂ at approximately 40 nmols H₂ oxidized/min/10⁸ cells. H₂ permits growth beyond *C. jejuni*'s microaerophilic limits. In serum bottles containing 25% O₂ and 10% H₂ wild-type *C. jejuni* reaches a terminal OD600 of .695 while cells grown at 25% O₂ are unable to grow and have a terminal OD600 of .0035. The ability to grow at high O₂ atmospheres supplemented with H₂ does not occur in a mutant strain deficient in hydrogenase activity. Hydrogenase could offer respiratory protection to O₂-sensitive enzymes. Growth under H₂-supplemented atmospheres also enhances growth rate. Wild-type *C. jejuni* grown in batch culture without H₂ had a generation time of 1.8 hours while cells grown in the presence of H₂ had a generation time of .96 hours. Mutant *C. jejuni*, unable to oxidize H₂, did not show such a drastic reduction in generation time. The ability of wild-type and mutant *C. jejuni* to colonize the avian intestine were compared and no significant differences resulted. Overall it appears H₂ is an efficient supplemental source of energy that may provide a competitive advantage in the avian intestine.

Hydrogen Metabolism in *Campylobacter jejuni*

by

Nathan J. Borden

A thesis submitted to the Graduate Faculty of the North Carolina State
University in partial fulfillment of the requirements for the Degree of
Master of Science

MICROBIOLOGY

Raleigh

2004

APPROVED BY:

Amy Grunden

Michael Hyman

Chair of Advisory Committee
Jonathan Olson

BIOGRAPHY

Nathan Borden was born February 16, 1978 in Fall River, MA. He grew up in a small town in Southeastern, MA. During his youth he developed a strong passion for science while playing and fishing in the woods near his home. He continued to enjoy and excel in science while enrolled at Bishop Feehan High School where he played on the varsity soccer and baseball teams. In the fall of 1996 he enrolled in the College of Agriculture and Life Science at North Carolina State University majoring in microbiology. Upon graduation in May of 2000, he returned to MA where he became a high school biology and chemistry teacher at Bishop Feehan. Nathan enrolled in the Master's of Science in Microbiology program at North Carolina State in July 2002. Nate plans on pursuing a career in medicine upon graduation.

TABLE OF CONTENTS

LIST OF TABLES.....	iv
LIST OF FIGURES.....	v
INTRODUCTION.....	1
MATERIALS AND METHODS.....	21
RESULTS.....	29
DISCUSSION.....	33
REFERENCES.....	51

LIST OF TABLES

MATERIALS AND METHODS

1.	Volumes of gases added to serum bottles.....	41
----	--	----

RESULTS

2.	Whole-cell hydrogenase activities.....	44
3.	Optical Densities (OD) of <i>C. jejuni</i> grown overnight in serum bottles.....	46
4.	Generation times of <i>C. jejuni</i> grown in batch culture.....	47

LIST OF FIGURES

INTRODUCTION

1. Organization of NDH-1 operon in *C. jejuni* and *E. coli*..... 40

MATERIALS AND METHODS

2. Growth curve experimental set-up..... 42

RESULTS

3. Gel electrophoresis of PCR products from wild-type and mutant DNA..... 43
4. Difference spectra of purified wild-type and mutant membrane..... 45
5. Low O₂ growth curve..... 48
6. High O₂ growth curve..... 49
7. Scatter graph of wild-type and mutant colonization..... 50

Introduction

Campylobacter jejuni infection is the most common cause of acute gastroenteritis worldwide, with between 2 and 10 million cases annually in the United States (15). *C. jejuni* infections, in industrialized nations, are between 2-7 times more common than infections with *Shigella* species, *Salmonella* species, or *Escherichia coli* O157:H7. The large numbers of cases along with the duration of the disease combine to inflict a substantial cost on both personal and national economies. Treatment for individuals with *Campylobacter*-associated Guillan-Barrè is expensive. An individual with *Campylobacter*-associated Guillan-Barrè can expect to pay between \$55,000 and \$329,000, depending on whether mechanical ventilation is necessary, in medical bills (15). Furthermore, the United States economy loses an estimated \$0.2-\$1.8 billion annually from *Campylobacter* and *Campylobacter*-associated illness. Consumption of contaminated poultry, especially chicken, is the primary route of entry into the human gastrointestinal tract. Person-to-person transmission is extremely rare; however, outbreaks of the disease have been traced back to contaminated water. Antibiotics are not typically administered and the duration of illness is between 3 and 10 days. The high cost of campylobacteriosis to individuals in terms of lost wages and personal discomfort combined with the devastating effects of the disease on national economies from lost productivity demands that *C. jejuni* be eradicated from human food supplies. Based on the nature of the organism and the disease the best way to prevent human disease is to eliminate *C. jejuni* from chicken flocks. Currently this is problematic since the exact mechanism of chicken colonization remains unsolved. The best hope for eliminating *C. jejuni* from chicken flocks is through the development of new antibiotics or other

antimicrobials targeting *C. jejuni* gene products. The enzymes of the respiratory chain in *C. jejuni* are ideal targets for antibacterials, as they are unlike eukaryotic mitochondrial respiratory enzymes. Inhibitors, therefore, of *C. jejuni* respiratory enzymes are unlikely to be toxic to the host.

The research presented in this thesis explores the role of hydrogenase in *Campylobacter* physiology. Elucidating the physiological role of this enzyme will provide a greater understanding of *C. jejuni*'s metabolism and perhaps provide a respiratory protein target for the development of novel antimicrobials. A broad understanding of *Campylobacter* physiology, the disease it causes, and the conditions that promote flock colonization, is vital to identifying possible means of eliminating the disease.

Disease Manifestation

Symptoms of campylobacteriosis are typically diarrhea, fever, and abdominal cramps (4). *Campylobacter* infections are indistinguishable from those caused by other intestinal pathogens such as *Salmonella* and *Shigella* (4). Diarrhea is usually loose and watery or grossly bloody with 8-10 bowel movements per day during the peak of illness. Fever is reported by greater than 90% of patients and can be either low-grade or $>40^{\circ}\text{C}$ and can persist for one week (4). The disease is self-limiting and treatment is usually water and electrolytes to prevent dehydration. Antibiotics are reserved for extra-intestinal infections and immunocompromised patients (19).

While there are marked differences in campylobacteriosis in developing and developed nations, the rate of *Campylobacter* infections worldwide has increased,

triggering a response from the World Health Organization (19). Figures for the general population of both developing and developed nations are approximately .09% per year. Infection occurs in both adults and children in developed nations (19). Furthermore, isolation of *Campylobacter* in children without diarrhea occurs in developing nations (14.9%) whereas it is not frequently recovered from asymptomatic people in developed nations (19). It is hypothesized that poor sanitation and early exposure to animals may explain the isolation from healthy children. Perhaps due to constant exposure, *C. jejuni* infections do not show a seasonal pattern in developing nations. Industrialized nations, however, have a marked seasonality that begins in May and peaks in August (4).

Campylobacteriosis in developed countries is typically self-limiting, however, important complications occur. Guillian-Barré syndrome (GBS) is an antibody mediated autoimmune disease that is preceded by an infection. *Campylobacter jejuni* is the most common infectious agent to precede GBS, approximately 1 in 1000 *C. jejuni* infections results in Guillan-Barré syndrome. In the post-polio era, GBS is the most common cause of acute flaccid paralysis (71). GBS is marked by the degradation of the peripheral nervous system. There are two forms of GBS, acute motor axonal neuropathy (AMAN) and acute inflammatory demyelinating polyneuropathy (AIDP) (52, 87). The form of GBS depends upon the type of mimicry, anti-GD_{1a} antibodies are associated with the AMAN form of GBS, whereas anti-GM₁ antibodies are seen in both AMAN and AIDP forms of GBS (52, 87). Regardless of the form of GBS, spontaneous, and in most cases, quick and complete recovery occurs (71). The disease begins as muscle weakness or numbness in the lower extremities (71). Nerve cell degradation is mediated by the immune system, in particular by antibodies that target host gangliosides (6, 7). Immune

cells degrade both the protective myelin sheath and nerve cell axons. This prevents efficient transmission of nerve impulses resulting in varying degrees of muscle weakness or even paralysis. The effective removal of *C. jejuni* from the food chain will severely decrease the number of GBS cases and, therefore, the eradication of *C. jejuni* from poultry flocks should be a priority.

Reservoir and Sources of Flock Contamination

Knowledge regarding sources of flock colonization is essential if *C. jejuni* is to be eliminated from poultry flocks. *C. jejuni* is capable of colonizing the intestinal epithelial of most warm-blooded mammals, including all food-producing animals and humans (72). The preferred environment is the intestines of all avians, including chickens, turkeys, quail, and ducks (43, 74, 77, 85, 91). *C. jejuni* colonizes the avian as a commensal, colonized chickens exhibit no observable clinical symptoms even when chicks are experimentally given high doses. The most common route of entry of *C. jejuni* into humans is through the consumption of contaminated chicken. Poultry meat becomes contaminated with *C. jejuni* during food processing, surviving throughout the food chain supply to constitute a risk to human health. A linear relationship exists between flock prevalence and the probability of human campylobacteriosis (53). The reduction of *Campylobacter*-positive flocks should, therefore, greatly reduce the risk of human disease.

A recent study indicated that approximately 90% of US chicken flocks are colonized and between 18-90% of European flocks are *Campylobacter* positive (75).

Similar to the seasonal trends in human campylobacteriosis, flocks also show a seasonal trend. Chicken flocks have a greater rate of infection in summer than in winter (37).

The sources of poultry colonization continue to be a mystery. Vertical transmission of *C. jejuni* by contaminated eggs remains controversial but it appears vertical transmission is not a source of flock colonization. It appears that horizontal transfer is the most significant cause of *C. jejuni* infection of broiler flocks.

Campylobacters are ubiquitous in the environment in and around broiler houses.

Although these organisms constitute a substantial risk to the flock, no direct relationship between environmental *campylobacters* and flock positivity has been observed (37).

Broiler houses may be contaminated internally by previous *Campylobacter*-positive flocks or associated with resident populations of rodents. External organisms could be transported into the broiler house in water, litter, feed, or by human foot traffic associated with farmers and veterinarians, or by the accidental entrance of wild and domestic animals. Therefore sources of horizontal transmission include feed and litter, water, aerosols, poor broiler room hygiene and human traffic. The literature suggests that standard biosecurity procedures are inadequate for the maintenance of *Campylobacter* negative flocks. Therefore, the developments of novel antimicrobials, such as those that target important metabolic processes, which could eradicate *C. jejuni* in chicken flocks offer the greatest hope for preventing human disease.

Physiology

Insight into *Campylobacter* physiology provides an understanding of how the organism survives in the intestinal tract of avians and also provides insight into possible

targets for new antimicrobials. *C. jejuni* is a gram-negative spiral shaped bacillus. It is a fastidious organism that requires rich media supplemented with blood or serum to grow. A microaerobic atmosphere with between 5-12% O₂ and 5% CO₂ is also required. The microaerobic requirement may be due to physiologically important enzymes being O₂ labile (38). It is unable to metabolize externally supplied sugars and the important glycolytic enzyme phosphofructokinase is absent (59). All other glycolytic enzymes are present and it appears that the pathway operates in reverse for gluconeogenesis (38). The completed genome sequence contains homologues of both pyruvate carboxylase and phosphoenol pyruvate carboxykinase that function in oxaloacetate and phosphoenolpyruvate synthesis respectively. Research indicates that amino acids are the primary carbon and energy sources of *C. jejuni*. *C. jejuni* preferentially utilizes aspartate and serine as carbon and energy sources, and as carbon sources become depleted glutamate and proline are the next preferred amino acids (39). During growth in a chemically defined medium serine is typically the limiting nutrient, indicating its important role as a carbon and energy source. When grown in a complex medium it appears that cystine is the limiting nutrient (39). Overall serine, aspartate, glutamate and proline are important carbon and energy sources for *C. jejuni*.

C. jejuni is able to operate a complete oxidative citric acid cycle. The genome contains all the key TCA enzymes including a succinyl-CoA synthetase and an NAD⁺-linked malate dehydrogenase. *C. jejuni* also encodes a succinate dehydrogenase that plays an important role in the oxidative TCA cycle. A fumarate reductase may allow *C. jejuni* to utilize an anaerobic electron transport chain (38).

C. jejuni contains oxidative stress defense mechanisms such as superoxide dismutase, catalase, and ferritin. Despite these defense mechanisms *C. jejuni* is still O₂-sensitive. According to Hodge and Krieg, O₂ tolerance varies widely depending on media used since *C. jejuni* appears more sensitive to exogenously supplied superoxide and peroxide (32). Exogenous superoxide and peroxide could be produced by the interaction of light striking the media. The media supplement sodium metabisulphite, found in Brucella agar, has been implicated in enhancing O₂ tolerance in *C. jejuni* (32).

Recent evidence illustrates that *C. jejuni* adapts to aerobic metabolism in the environment (36). Growth under aerobic conditions results in a distinct colony morphology. Colonies grown under micro-aerobic conditions are opaque, convex, with a distinct margin. Aerobically grown *C. jejuni* colonies are effuse (36). However, the individual cells from each colony are similar, flagellated and vibroid (36). Aero-tolerant colonies are easily re-converted to micro-aerobic metabolism and colony morphology by a single subculture in a micro-aerobic atmosphere (36). Other adaptive responses also occur under aerobic conditions, including changes in outer-membrane protein composition, however, morphologically altered *C. jejuni* retain the ability to colonize mice (36).

Individual cells undergo a distinct cell morphology change when grown several days *in vitro*. Cells change from a spiral-bacillus to coccoid. This coccoid morphology is deemed viable-non-culturable (VNC) and may represent a starvation survival mechanism (38). This is a controversial concept due to the inability of these coccoid cells to colonize chickens and researcher's inability to revert them to the spiral morphology (38). Evidence supports the theory that these cells are dead. Recent data

indicates that coccoid formation is not an active process but rather a degenerate form resulting from oxidative damage (92).

Respiratory Physiology

Cytochromes are proteins with one or more heme groups bound to a polypeptide chain (26). Those containing heme covalently attached to a thiol group are *c*-type cytochromes. Cytochromes with modifications to their porphyrin ring also exist. These include *a*, *d*, *d*₁ and *o* type cytochromes. They have numerous cellular functions including electron transfer reactions and some act as catalysts in chemical reactions. Catalytic cytochromes, known as terminal oxidases, are involved in the reduction of O₂ to water in the final step of cellular respiration. Difference spectra analysis on *C. jejuni* grown under microaerobic conditions reveals cytochromes of the *b* and *c* type, with the *c* type being the most abundant (33). Although the genome sequence indicates *C. jejuni* contains *d* type cytochromes (59), no evidence of *o*, *a*, or *d*-type cytochromes has been shown under difference spectra analysis (33).

The microaerophilic nature of *C. jejuni* is reflected in the fact that the genome sequence encodes for enzymes found in both aerobic and anaerobic electron transport chains (73). Complex I of aerobic bacterial electron transport is an NADH-quinone oxidoreductase (NDH-1), which is a large enzyme composed of many redox centers and protein subunits. Complex I is a proton translocating and electron transfer enzyme. Four hydrogen ions are translocated across the cytoplasmic membrane for every two electrons transferred (73). The operon encoding Complex I, or NDH-I, of the electron transport chain contains 14 genes, labeled *nuoA-N*. The operon in *C. jejuni* is very similar to the

operon in *E. coli*, except that the *nuoE* and *F* genes of *E. coli* are absent in *C. jejuni* and in their places are two novel genes *nuoX* and *nuoY* (Figure 1). Proteins cj1574c and cj1575c are encoded by *nuoX* and *nuoY*, respectively, and their function remains unknown. The role of *nuoE* and *nuoF* in *E. coli* are well known. NuoF proteins provide the site for NADH binding, a bound FMN and an Fe-S center while NuoE contains cysteinyls that coordinate Fe-S centers (73). An NADH binding site is a requirement of all NADH dehydrogenases, including NDH-1. Since *C. jejuni* does not use NADH as an electron donor it is hypothesized that cj1574c and cj1575c provide docking sites for electron donors other than NADH.

C. jejuni contains genes that encode a quinol:cytochrome *c* oxidoreductase, also known as a cytochrome *bc*₁ complex or complex III (73). This complex possesses one Fe-S cluster and three hemes (21). Three genes in the operon *petABC* that encode for the Rieske Fe-S, cytochrome *b* and *c*₁ subunits, respectively, encode the complex. This complex catalyzes the transfer of two electrons from hydroquinone or hydroquinone to a single one-electron acceptor. This creates an electrochemical gradient across the cytoplasmic membrane (73).

The respiratory chain of *Campylobacter* is branched with two different terminal oxidases, a *cbb*₃ type cytochrome *c* and a *bd* type quinol oxidase (59). The *cbb*₃ type cytochrome is a terminal oxidase found in N₂-fixing rhizobial organisms where O₂ levels are low. While the genome sequence predicts that *C. jejuni* contains a *bd*-type cytochrome, they have never been shown under difference spectra analysis. These cytochromes are quinol oxidases that do not translocate protons and are therefore half as efficient as ubiquinol-oxidizing heme-copper oxidases (58). In *E. coli* the *bd* oxidase has

a very high affinity for O₂ and is maximally expressed under microaerobic conditions (20).

C. jejuni catabolizes amino acids to acetate, formate, lactate, and succinate by fermentative pathways (73). *C. jejuni* utilizes pyruvate, a feature of anaerobic metabolism, as an energy source. Furthermore, they may obtain energy through an electron transport chain that includes fumarate reductase (16).

The unique characteristics of the first and last complexes of the respiratory chains in *C. jejuni* provide targets for future antimicrobial treatments. The prevention of electron transfer of these obligate microaerophiles could have bacteriostatic or bacteriocidal effects. Especially important are the differences between *C. jejuni* respiratory components and eukaryotic respiratory components. Eukaryotic mitochondria lack both *cbb*₃ and *bd* type cytochromes and would, therefore, be ideal targets for antibiotics (73). This research project explores the physiological role of hydrogenase in *C. jejuni* and its potential as a target for antimicrobials designed to eliminate *C. jejuni* from chicken flocks.

Hydrogenase

Functions

Hydrogenases catalyze the reversible reaction $\text{H}_2 \leftrightarrow 2\text{H}^+ + 2\text{e}^-$. Hydrogenases that catalyze the forward reaction are known as uptake hydrogenases while enzymes that catalyze the reverse reaction are known as evolution hydrogenases. They are found in all domains of life and play many physiological roles (78). The diverse cellular functions of hydrogenases are a reflection of the many and diverse organisms that utilize the

metabolic diversity of hydrogenases. In fermentative organisms the reduction of H^+ into H_2 is a way to dispose excess reducing equivalents (1). Both aerobic and anaerobic organisms can use H_2 as an energy source. For example, N_2 -fixing bacteria contain an uptake hydrogenase that recycles H_2 produced by nitrogenase (13, 50, 79, 84). This allows the organism to utilize energy that otherwise would not be used. Sulfate-reducers utilize a transmembrane H_2 redox cycle to create a proton motive force. The PMF can then be used to create energy driven ATP production via ATPase. Similarly, the pathogen *Helicobacter pylori* uses H_2 as an energy source and *H. pylori* strains containing a disrupted hydrogenase gene are unable to colonize mice (57).

Classes

As mentioned previously, enzymes that catalyze the forward reaction are known as uptake hydrogenases while those that catalyze the reverse reaction are known as evolution hydrogenases. These reactions typically occur in different cellular locations. H_2 evolution is typically a cytoplasmic reaction and H_2 uptake usually occurs in the periplasm or is membrane localized. Over 100 hydrogenase sequences are available. These sequences were used to organize known hydrogenases into different classes based on their distinct active site which is conserved within each class of enzyme. These classes are [Ni-Fe], [Fe], and metal-free hydrogenases. The [Ni-Fe] and [Fe] hydrogenases are remarkably similar in structure and chemistry despite different protein folding surrounding the active site (2, 54, 62). Their similarity is a well-documented case of convergent evolution (78). Researchers speculate that the active site of metal-free hydrogenases is unlike either [Ni-Fe] or [Fe] hydrogenases, although not much is known

about these novel enzymes. *C. jejuni* contains a [Ni-Fe] hydrogenase and the following review will focus on those enzymes.

[Ni-Fe] Hydrogenases

These enzymes are subdivided into 4 Groups based upon 2 highly conserved regions surrounding 2 pairs of cysteine ligands of the Ni-Fe active site. These conserved regions are located near the N and C-terminus of the sequence (63, 82, 89) and are labeled L1 and L2 (8). Grouping of [Ni-Fe] hydrogenases based upon differences in these conserved regions agrees with divisions based upon cellular functions. X-ray crystallography data from *Desulfovibrio gigas* (81) and *Desulfovibrio vulgaris* (31) show that the large and small subunits interact through a contact surface and form a globular heterodimer. The [Ni-Fe] active site is located in the large subunit and is buried inside the protein. Three Fe-S clusters conduct electrons between the donor and acceptor hydrogenases. Furthermore, a 4Fe-4S cluster proximal to the active site is required for H₂ activation in [Ni-Fe] hydrogenases (81). H₂ gains access to the active site by traveling through hydrophobic channels. Genes encoding for the proteins involved in electron transfer are located in a polycistronic operon. The catalytic subunits in *C. jejuni* are encoded by *hydA* and *hydB*. The structural unit is an $\alpha\beta$ heterodimer.

Group 1

Group 1 hydrogenases are the most documented and most well known of all hydrogenases. They are trimeric membrane-bound or membrane-associated uptake hydrogenases. The hydrogenase in *C. jejuni* is a member of Group 1, due to the presence

of a cytochrome *b* subunit encoded by *hydC*. These enzymes are capable of supporting growth with H₂ as an energy source. As is the case in *C. jejuni*, respiratory hydrogenases of Group 1 transfer electrons to cytochromes by coupling electron transfer to transmembrane proton translocation. Electrons are transferred from H₂ to the ubiquinone pool via cytochrome *b*. Cytochrome *b* can then transfer electrons to fumarate or, as in *Wolinella succinogenes* to inorganic oxidants (24) or even to O₂ as in *Ralstonia eutropha* (10). H₂ oxidation, therefore, is linked to the reduction of various electron acceptors including O₂, NO₃⁻, SO₄²⁻, fumarate and CO₂. The accompanying proton translocation creates a proton gradient that provides a means for recovering energy (30). Cytochrome *b* also anchors the αβ hydrogenase to the periplasmic side of the membrane and is required for growth on H₂ in *Rhodobacter capsulatus* (18). Heterodimer catalyzed H₂ oxidation in *Azotobacter vinelandii* also requires cytochrome *b* (48). N₂-fixing bacteria also contain a Group 1 hydrogenase. In these organisms hydrogenase production is induced when nitrogenase is synthesized and producing H₂. The hydrogenase recoups energy for the cell by utilizing this H₂ as an electron donor. This reaction has been observed in *Azotobacter chroococcum* (84), *Rhodobacter capsulatus* (50), and all cyanobacteria (13).

Archaeal Group 1 hydrogenases also exist in the methanogens that reduce CO₂ to CH₃. To accomplish this hydrogenase transfers electrons from H₂ to cytochrome *b* which then channels them to a heterodisulfide reductase. This electron transfer system, known as H₂: heterodisulfide oxidoreductase, is energy conserving (34) and resembles respiration-linked oxidative phosphorylation of Proteobacteria containing Group 1 hydrogenases.

Group 2

Cyanobacterial uptake hydrogenases and cytoplasmic hydrogenases involved in H₂-sensing compose Group 2 of the [Ni-Fe] hydrogenases (78). These are cytoplasmic heterodimers that do not provide energy for the cell. Since they are cytoplasmic hydrogenases they all lack the membrane targeting sequence peptide at the N-terminus of the small subunit.

Group 2 H₂-sensing hydrogenases are found in *Bradyrhizobium japonicum* (12), *R. capsulatus* (25), and *R. eutropha* (41). They are involved in hydrogenase gene regulation in response to H₂. The HupUV/HoxBC hydrogenase detects H₂ and transduces a signal to a histidine kinase. The histidine kinase combined with a response regulator, HupR/HoxA, forms a 2-component regulatory system. The histidine kinase exerts negative control in the absence of H₂ (40) while the response regulator exerts positive control in the presence of H₂ (70). In aerobic bacteria such as *B. japonicum* and *R. eutropha*, the HoxBC/HupUV H₂-sensor is required for the synthesis of the uptake hydrogenases. In the presence H₂ HoxBC suppresses the negative effect of HoxJ by preventing the phosphorylation of HoxJ, which allows HoxA to remain non-phosphorylated and active.

The uptake HupSL hydrogenases of Group 2 are found in all cyanobacterial species. They are expressed only under N₂-fixing conditions or in heterocysts (17) and perform the same functions as Group 1 hydrogenases. Genetically they resemble the Group 1 hydrogenases except for deletions throughout the sequences of *hupL*. It is believed that the cyanobacterial enzymes are bound to either the cytoplasmic side of the cytoplasmic membrane or thylakoid membrane.

Group 3

These are cytoplasmic heteromultimeric reversible [Ni-Fe] hydrogenases. They are tetrameric enzymes that are soluble or loosely attached to membranes (78). The tetramer is capable of dissociation into 2 dimeric moieties. One dimer is a diaphorase encoded by *hoxFU* with NAD, FMN, and Fe-S binding sites. The other is a heterodimeric hydrogenase encoded by *hoxYH*. In methanogens Group 3 hydrogenases are F₄₂₀-reducing enzymes. F₄₂₀ is a cytosolic electron carrier in methanogens which does not contain cytochromes. *Methanosarcinales* uses H₂ or F₄₂₀H₂ as electron donors. Electron transfer follows electron donation and involves hydrogenases, *b*-type cytochromes, or F₄₂₀H₂ dehydrogenase. Electron transfer is coupled to proton translocation across the cytoplasmic membrane and the resulting PMF is used in energy formation. The F₄₂₀-reducing hydrogenases are trimeric enzymes encoded by the *fruADGB* operon. The large subunit is FruA while the small subunit is FruG. FruB probably binds F₄₂₀.

Archaeal species also contain Group 3 hydrogenases. The cytoplasmic hydrogenase in *Pyrococcus furiosus* is a tetramer with the α and δ subunits composing the Ni-Fe hydrogenase moiety. The enzyme can utilize S[°] or polysulfide as substrates to produce H₂S. This bifunctional enzyme is able to remove excess reducing elements produced during fermentation using either S[°] or protons as electron acceptors. Since this enzyme has S[°]-H₂ oxidizing bifunctionality it has been deemed a sulfhydrogenase (61). The electron donor to this sulfhydrogenase is NADPH (44).

NAD-linked hydrogenases are also in Group 3. The Group 3 hydrogenases in *R. eutropha* (69) reversibly reduce NAD^+ with H_2 . These enzymes are tetramers composed of 2 dimeric moieties that are capable of functioning independently. One dimer is encoded by *hoxU* and *hoxF* and exhibits diaphorase activity. These two subunits contain NAD^+ , FMN, and Fe-S binding sites and are homologous to two subunits of complex I. In cyanobacteria, these enzymes are linked to complex I (83). Furthermore, they are bidirectional hydrogenases that catalyze H_2 uptake in a light stimulated manner.

Group 4

The Group 4 hydrogenases are membrane-associated H_2 -evolving respiratory [Ni-Fe] hydrogenases. The sequence has been analyzed and illustrates that the small subunit of Group 4 is considerably smaller than the small subunit of other [Ni-Fe] hydrogenases (76). The large subunit is characterized by an arginine and not a histidine at the endopeptidase cleavage site, as in *hycE* (30). Group 4 hydrogenase complexes are very similar to Complex I, especially the subunits involved in proton pumping and energy coupling. These enzymes are capable of coupling the oxidation of carbonyl groups with the reduction of H_2 and in doing so generate a PMF across the membrane.

The most extensively studied Group 4 hydrogenase is hydrogenase 3 from *E. coli*. In *E. coli* hydrogenase 3 is only expressed under strictly anaerobic conditions and is part of a formate- H_2 lyase complex (FHL). FHL couples formate oxidation, by FdhH, to the reduction of protons by hydrogenase 3. This reaction produces H_2 without energy recovery (78).

The *hyc* operon, containing eight genes, encodes for the formate- H_2 lyase complex. *hycGE* encodes for the catalytic subunits. The catalytic subunit proteins are extrinsic membrane proteins while the membrane anchor and the electron carriers, which transfer electrons between formate dehydrogenase and hydrogenase 3, are intrinsic proteins (78). The products of five of the *hyc* genes share similarities with subunits of NADH:ubiquinone oxidoreductase. Sequence similarities between HycE and NuoD exist, while the N-terminus of the small subunit in HycG is homologous to the N-terminus of NuoB's small subunit. Lastly, HycF and HycC are homologous to NuoI and NuoL respectively. Andrews, *et al* (5) have located a 12 gene operon, *hyf*, which encodes a 10 subunit hydrogenase 4 complex. HyfC and hyfF encoded by this operon are related to NuoH and NuoL of Complex I. Since NuoH and NuoL are involved in proton translocation and energy coupling, the author believes that HycF linked to FdhH forms a respiration-linked proton translocating pathway which is unlike the non-energy producing FHL complex.

Rhodospirillum rubrum contains a different Group 4 hydrogenase. It is CO-induced and is encoded by *coolH* (27, 28). This enzyme is part of a CO-oxidizing system that allows the organism to grow in a CO-dependent manner in the dark. This CO oxidation/ H^+ evolution enzyme complex is membrane-bound. It has been proposed that the hydrogenase is the site of energy coupling. In the Archaeal species *Methanosarcina barkeri*, a Group 4 hydrogenase oxidizes the carbonyl group of acetate forming CO_2 and H_2 . *P. furiosus* utilizes a fermentative metabolism that produces acetate, H_2 and CO_2 . This hyperthermophile contains two cytoplasmic H_2 -evolving hydrogenases (I, II) (45, 61) as well as a membrane-bound hydrogenase (76). The Group 4 hydrogenases of *P.*

furiosus are encoded by a 14 gene operon, *mbhA-N* (66). This operon encodes for the catalytic subunit and four proteins that resemble *hyc* (68) and *hyf* (5) operons of *E. coli*. These four proteins have some similarities to Complex I (66).

Hydrogenase Genetics

Genes encoding hydrogenases also reflect the diversity of function and cellular location of these enzymes. The genes encoding [Ni-Fe] hydrogenase structural genes are clustered on the genome and labeled alphabetically according to their order location in the cluster. Hydrogenases are composed of two protein subunits containing a small (28-35 kDa) subunit and a large (45-70 kDa) subunit (78). Downstream of these structural genes are several genes involved in the maturation of the enzyme. The structural genes in *E. coli* hydrogenase 1 are annotated *hyaA-H* (78). The small and large subunits are encoded by *hyaA* and *hyaB*, respectively. A *b*-type cytochrome is encoded by *hyaC* while *hyaD* encodes a maturation protease. A rubredoxin-like protein is encoded by *hyaF* and a response regulator is encoded by *hyaG*. In *C. jejuni* the genes are annotated *hydA-D*: *hydA* encodes the small subunit which is a ligand to the Fe-S cluster, *hydB* encodes the Ni-Fe active site, *hydC* encodes a membrane anchor, *hydD* encodes a protease that plays an important role in enzyme maturation, and the fifth protein remains un-annotated in the *C. jejuni* genome but likely plays a role in H₂ oxidation. Pleiotropic genes involved in Ni, Fe, CO, and CN insertion into the active site are encoded in the *hyp* operon. *C. jejuni* also contains the *hyp* operon (59). There are five genes encoded in the *hyp* operon labeled *hypA-E* with a sixth gene, *hypF*, involved in enzyme maturation but is located outside the operon. The exact role of all of these proteins has not been elucidated

but research is progressing. It appears as though *hypB* is a GDP and GTP-binding protein which exhibits some GTPase (22, 56) activity and binds Ni (22, 55, 64). The insertion of Ni into the protein occurs while pre-HycE is bound to the chaperone-like protein HypC in the absence of the small subunit (23, 46, 47). Following Ni insertion HypC leaves pre-HycE and C-terminal processing then takes place. Sequence similarities between *hypF* and *o*-carbamoyltransferases indicate that *hypF* plays a role in donating CO and CN ligands to the Fe atom in [Ni-Fe] hydrogenases (60). The exact role in hydrogenase maturation of the other *hyp* genes remains unknown. However, *hypB*, *D*, *E*, *F* are involved in the maturation of all three *E. coli* hydrogenases while *hypA*, and *C* are only involved in the maturation of hydrogenase 3 (35). Hydrogenase and urease activity are affected in *H. pylori* if *hypA* and *hypB* are mutated (35). In Proteobacteria other accessory genes are included in the hydrogenase gene cluster. Regulatory genes such as the *hupUV/hoxBC* (41) genes that encode for a H₂-sensing hydrogenase are located in the cluster as are genes for a two-component regulatory system. *C. jejuni* does not contain the *hupUV/hoxBC* regulatory system. The two-component system in *R. capsulatus*, *R. eutropha*, *B. japonicum*, and *E. coli* encodes for a histidine kinase (78). Furthermore, the differences between cytoplasmic and periplasmic or membrane-bound hydrogenases are found at the genetic level. Enzymes bound for the periplasm or membrane are tagged by a signal peptide at the N-terminus of one of the subunits. The tag is typically 30-70 amino acids on the small subunit. Common to all Group I hydrogenases is a tagged sequence that is a highly conserved RRxFxK (89) which is recognized by the twin-arginine translocation pathway (*tat* genes) (67). Only correctly folded and fully active enzymes are able to cross the membrane (9, 82, 88). The twin-arginine translocation

model has been shown to locate hydrogenase 1 and 2 of *E. coli* as well as the membrane-bound enzymes of *W. succinogenes* (29) and *R. eutropha* (11). Based on the reduction of methyl viologen, *C. jejuni*'s hydrogenase is located on the external side of the membrane (33). *C. jejuni* also contains the tat system (59). This model is expected to occur in all Group 1 hydrogenases since organisms containing hydrogenase also contain the *tat* genes. [Ni-Fe] hydrogenases lacking the signal tag are expected to remain in the soluble cytoplasmic compartment. This is, in fact, the case in the H₂-sensing hydrogenase HupUV of *R. capsulatus* (80) and *R. eutropha*. [Fe] hydrogenases, such as the one in *Clostridium pasteurianum*, are cytoplasmic enzymes and lack the conserved sequence tag (49).

Role of Hydrogenase in Pathogens

H. pylori is a human pathogen implicated in gastric ulcers and stomach cancer. Like *C. jejuni*, *H. pylori* is limited in its ability to utilize carbon substrates as a source of energy. H₂ is present in the gastrointestinal tract of both rodents(14) and humans (42) and studies have shown that colonic H₂ is a significant source of energy for *H. pylori* in the energy deprived environment of the gastric mucosa. Olson and Maier have shown that the average H₂ content of the mucus layer of the murine stomach is 43μM. The hydrogenase in *H. pylori* has a K_m for H₂ that is 20-fold lower than the amount of H₂ in the mouse stomach. Therefore, the H₂ oxidizing system in *H. pylori* is usually saturated. In fact, Olson and Maier reported in *Science* that a mutant strain of *H. pylori* is severely reduced in its ability to colonize the mouse stomach (57). They disrupted the *hydB* gene with a chloramphenicol cassette and compared its ability to colonize the mouse stomach

with the colonization ability of the wild-type strain SS1. The wild-type strain colonized 100% of mice (37/37) while the mutant strain only colonized 24% (9/38) of mice. They also report that although hydrogenase is expressed under all conditions, hydrogenase expression responds to the availability of H₂ (57). Hydrogenase activity increases from a baseline of 0.7 nmoles H₂ oxidized/min/10⁸ cells in cultures grown under microaerobic conditions (12% O₂, 5%CO₂, balance N₂) to 3.1 nmoles H₂ oxidized/min/10⁸ cells when supplemented with 10% H₂. Since *C. jejuni* is also a pathogen and a close relative of *H. pylori* it is hypothesized that hydrogenase plays a similar role in both *C. jejuni* and *H. pylori*. This research project was designed to elucidate whether H₂ could support growth in *C. jejuni* and whether *C. jejuni* deficient in hydrogenase activity were reduced in their ability to colonize the chicken gastrointestinal tract.

Materials and Methods

Strains

Two wild-type strains were used during this research project: the sequence strain, NCTC 11168, a strain isolated from a patient with campylobacteriosis and CVM001, which was isolated from a patient with Guillan-Barré syndrome.

Cloning and Disruption of *hydB*

Primers were designed to PCR amplify a 1300 BP fragment of *hydB* containing an *EcoR*I site. The amplified product was ligated into a *Hind*III cut pBluescript II KS (+/-) Strategene vector using a New England BioLabs Quick Ligation Kit protocol. The ligated products were electroporated at 2500V into electrocompetent DH5α *E. coli* cells. After electroporation 1 mL of LB broth was added to the cells and transferred to a 15mL

test-tube and incubated with shaking for 30 minutes at 37° C. Cells were then plated on LB agar plates containing ampicillin and X-gal (5-bromo-4-chloro-3-indoyl β D-galactopyranose) and incubated overnight. White colonies were inoculated into 5 ml LB broth containing ampicillin. Quiagen miniprep kits were used to perform a plasmid preparation on these cells and plasmids containing the insert were verified by restriction digest analysis. Plasmids containing the correct inserts were labeled pHyd. An 800 base pair chloramphenicol cassette was cut from pJMA001 (90) using *EcoR*I and ligated into *EcoR*I cut pHyd. The ligation product was electroporated into DH5 α *E. coli* cells using the above protocol. Cells were plated on LB agar containing chloramphenicol. Colonies were screened for the proper insert by PCR and restriction digest analysis. Plasmids containing the proper insert were labeled pHyd:Cm.

Transformation of *C. jejuni*

C. jejuni was transformed using three different protocols: 1) plasmid pHyd:Cm electroporated into competent *C. jejuni* NCTC 11168, 2) plasmid pHyd:Cm naturally transformed CVM001, 3) genomic DNA from Hyd:Cm (in the *C. jejuni* NCTC 11168 background).

The plasmid pHyd:Cm was electroporated into electrocompetent *C. jejuni* NCTC 11168. Competent *C. jejuni* were obtained by resuspending 3 overnight blood agar plates incubated at 37°C, 12% O₂, 5% CO₂ by swab into 1.5 mL ice-cold 15% glycerol, 9% sucrose. Cells were then pelleted by centrifugation at 18,000 xg. Cells were resuspended in glycerol/sucrose and pelleted by centrifugation. This was repeated twice. Cells were resuspended in 500 μ L of a 15% glycerol/9% sucrose mixture and aliquoted into 50 μ L

tubes, flash frozen, and stored at -80° C. Competent cells were thawed on ice. While still on ice, 3 µL pHyd:Cm was added. The mixture was added to a pre-chilled 2 mm electrocuvette. The cells were pulsed at 2500V and 50 µL Mueller-Hinton Broth was added immediately following the pulsing of cells. Cells were withdrawn from the cuvette and spotted on a cold blood agar plate. After a 24 hour microaerobic incubation, cells were transferred to a blood agar plate containing 20 µg/ml chloramphenicol. After a 5 day incubation, colonies were picked with a sterile inoculating loop and streaked on a fresh blood agar plate containing 20 µg/ml Chloramphenicol. Genomic DNA was isolated using the Wizard Maxiprep protocol (Promega, Madison WI). PCR using the *hyd* primers verified disruption of *hydB* by the chloramphenicol cassette. Mutants were labeled 11168 hyd:Cm.

pHyd:Cm was naturally transformed into *C. jejuni* CVM001. Half of an overnight plate of CVM001 was resuspended in 1ml PBS at pH 7.0. 20 µL of this was spotted on a blood agar and incubated 3 hours microaerobically. After this incubation, 7 µL of pHyd:Cm was spotted onto the cells and incubated 5 hours microaerobically. After the second incubation the cells were replated onto selective plates, blood agar containing 20 µg/ml chloramphenicol. Colonies were re-streaked on chloramphenicol containing plates. Genomic DNA preparation using the Wizard Maxiprep protocol was performed. PCR was performed on this DNA to verify mutation of hydrogenase gene. Mutants were labeled CVM hyd:Cm.

The above natural transformation protocol was repeated using isolated genomic

DNA from Hyd:Cm in the *C. jejuni* NCTC 11168 background. *C. jejuni* has a restriction modification system which makes transformation difficult. Using genomic DNA improved transformation efficiency.

H₂ Uptake

H₂ uptake was measured amperoterically using a modified Clark-type electrode on whole cells (86). Ten Brucella agar plates supplemented with 10% defibrinated sheep's blood of strains *C. jejuni* CVM001 and CVM:Hyd:Cm were resuspended by swab in 30 ml of phosphate-buffer saline, pH 7.4. Cells were pelleted by centrifugation at 6,084 x g for 5 minutes and washed twice with PBS. Pellets were suspended in 30ml PBS and incubated on ice for 30 minutes to remove endogenous substrates. A baseline was established by adding 100 µL of H₂-sparged deionized water to 5 ml of stirred deionized water (86). The chamber was rinsed twice with deionized water. Five milliliters of the CVM001 suspension was added to the chamber and allowed to equilibrate. Upon equilibration, 100 µL of H₂-sparged water was added. The chamber and electrode were connected to a chart recorder and the H₂-uptake rate was calculated based on the slope of the disappearance of H₂. H₂ concentration was determined using the standard value of H₂ dissolved in water at room temperature. The chamber was again rinsed twice with deionized water and the procedure was repeated for the mutant strain CVM:Hyd:Cm. Activities were expressed as nanomoles of H₂ consumed per minute per 10⁸ cells. Cell counts were obtained by OD 600 using 1 OD = 7.5x10⁸ cells as a conversion factor.

H₂ Dependent O₂ Uptake

O₂ consumption was measured using an O₂ Clarke-electrode inserted into a glass chamber that was also connected to a chart recorder. Cells were harvested by swab into 30 ml of PBS collected by centrifugation and washed twice with PBS. Pellets were resuspended in 30 ml PBS and incubated on ice for 30 minutes to remove any endogenous substrates. Five milliliters of the cell suspension was added to the chamber and allowed to equilibrate. No change in O₂ concentration for several minutes confirmed the depletion of respiratory substrates. The rate of O₂ consumption was measured upon the addition of 500 µL H₂-sparged water to the chamber (33) and measuring the steady-state O₂ consumption which followed a rapid decrease in the partial pressure of O₂ resulting from the addition of H₂ (33). Respiration rates were measured in nanomoles of O₂ consumed per minute per 10⁸ cells. Cell counts were obtained by OD 600 using 1 OD = 7.5x10⁸ cells as a conversion factor.

Membrane Preparation

Ten brucella agar plates supplemented with 10% defibrinated sheep's blood were incubated overnight at 37° C, 12% O₂, 5% CO₂. Cells were resuspended in 30 ml cold PBS (pH 7.4) and centrifuged cold for 10 minutes at 6,084 x g in a Sorvall RC5C centrifuge. The pellet was resuspended in 20 ml cold 50 mM phosphate buffer (pH 7.0) and centrifuged for 10 minutes at 6,084 x g. The pellet was again resuspended in 12 ml phosphate buffer with 1 mM PMSF. This suspension was passed 2X through a French Pressure Cell operated at 20, 000 lb/in². Lysed cells were centrifuged at 11,950 x g for 15 minutes in a Sorvall RC5C centrifuge. The supernatant was loaded into 11.5 ml crimp

top tubes and centrifuged at 100,000 x *g* for 90 minutes in a Beckman L8-55 Ultracentrifuge. The pellet was resuspended by dounce homogenizer in of cold K₂PO₄ buffer with 1 mM PMSF.

Difference Spectra

Membranes were resuspended in 6 ml of a phosphate/MgCl₂ buffer (51). The suspension was added to a quartz cuvette and sealed with a rubber stopper. The quartz cuvette was then sparged with 100% H₂ for 10 minutes and H₂-reduced spectra was recorded (12). Air-oxidized membranes were used as a reference. Dithionite-reduced spectra were obtained by adding several grains sodium dithionite to the membranes. Difference spectra from 700-450 nm were performed on a Shimadzu UV160U spectrophotometer on both H₂ and air-oxidized membranes.

Growth Experiments

Serum bottles were used to determine the ability of *C. jejuni* to grow under different atmospheric conditions. 20 ml of Mueller-Hinton Broth was added to 150 ml serum bottle. Bottles were then capped using a rubber stopper and aluminum cap that was crimp sealed. Bottles were sparged for 15 minutes using a needle through the rubber stopper with a gas cylinder containing 5% CO₂ and balance N₂. Another needle, serving as an outlet, was placed into the rubber stopper. Atmospheres were obtained according to Table 1 using a 60 ml syringe and a 20 gauge needle. Cells were swabbed off an overnight plate of Brucella agar supplemented with 10% sheep's blood and suspended in 2 ml of phosphate-buffer saline (pH 7.4). 100 µL of the cell suspension was added

through the rubber stopper using a 20 gauge needle and a 1 ml syringe. Optical densities at 600 nm were taken after a 14 hour overnight incubation in a 42° C floor shaker.

Growth Curves

Growth curves were performed on both wild-type *C. jejuni* strain CVM001 and CVM001 Hyd:Cm in a 42° C incubator. Growth experiments were performed at 42° C to mimic the internal temperature of the avian intestines. Growth curves were conducted in batch culture using a specially designed glass vessel which could be continuously sparged with a specified gas mixture (Figure 2). A stir bar was added to 80 ml of Mueller-Hinton broth and gas was continually sparged through a glass dispersion tube. Growth curves were performed under the following atmospheric conditions: 12% O₂, 23% O₂ and 10% H₂, 23% O₂, and 23% O₂ plus 10% H₂ balance was N₂ in each case. Gas flow was controlled by flowmeters. 12% O₂ conditions were obtained by continuously sparging cultures with a cylinder containing 12% O₂, 5% CO₂, and balance N₂ (Blood gas). Under these conditions the flowmeter read 100. Mueller-Hinton Broth was sparged with 90% blood gas and 10% H₂, as read by the flowmeters, to achieve 12% O₂ and 10% H₂ conditions. Similarly, 23% O₂ conditions were achieved by 77% gas from cylinder containing 5% CO₂, balance N₂ and 23% O₂ from an O₂ cylinder. Anaerobic mix (5% CO₂, 10% H₂, balance N₂) was sparged at 77% along with 23% from an O₂ cylinder to obtain 23% O₂ plus 10% H₂ conditions. Mueller-Hinton broth was allowed to equilibrate to both temperature (42°C) and gas conditions for 10 minutes prior to inoculation. 100 µL of an overnight culture of either *C. jejuni* CVM001 or Hyd:Cm was used to inoculate 80 ml of Mueller-Hinton broth. Optical densities at 600 nm were taken every hour.

Every 2 hours the culture was serially diluted and 100 µL was plated on Tryptic Soy Agar supplemented with 10% sheep's blood. Colonies were counted after a 48 hour incubation at 37° C, 12% O₂, 5% CO₂.

Chicken Colonization

Colonization studies were performed on wild-type strain *C. jejuni* CVM001 and mutant strain CVM001 Hyd: Cm. Eggs were donated by a commercial hatchery (GoldKist Hatchery, Siler City, NC) and washed with 70% ethanol 4 days prior to hatching. Each colonization study (negative control, wild-type, mutant) was conducted in a separate room using brooder batteries containing 10 chicks per cage. Each strain was used to inoculate 20 birds by oral gavage. Negative control chicks were inoculated by oral gavage with 1 ml sterile PBS. *Campylobacter* strains were grown on Tryptic Soy Agar supplemented with 10% sheep's blood for 24 hours at 37° C, 12% O₂, 5% CO₂. Plates were resuspended in sterile PBS and serially diluted to attain a suspension containing approximately 3x10⁷ CFU/ml. Concentration was verified by optical density at 600 nm and colony counts. Day of hatch chicks were inoculated by oral gavage with 1 ml of the cell suspension. Twenty un-inoculated birds were humanely killed and cecal contents collected to identify if the birds were *campylobacter*-free. Cecal contents were serially diluted and plated on campylobacter selective plates (Tryptic Soy Agar supplemented with 10% sheep's blood and 4 µg/ml cefoperazone, 4 µg/ml vancomycin, 4 µg/ml trimethoprin, 10 µg/ml cycloheximide). Chickens were fed (Purina Chick Start & Grow) and watered *ad libidum* for 21 days post-inoculation. After 21 days chickens were humanely sacrificed and cecal contents harvested. 1 g of cecal contents was serially

diluted and plated on *Campylobacter*-selective plates. Plates were incubated at 37° C, 12% O₂, 5% CO₂ for five days. Colonies were counted and CFU/g cecal content was calculated.

Results

Mutagenesis

The goal was to clone the *hydB* gene into the vector pBluescript II KS (+/-) to make a gene-directed mutant for use in physiological studies. The cloned gene was then disrupted with an 800 bp chloramphenicol cassette from pJMA001 (90). Restriction digest verification of the ligation of *hydB* into pBluescript II KS (+/-) and the subsequent disruption of *hydB* with the Cm^R cassette was performed. Once the construct was verified, it was transformed *via* electroporation into *C. jejuni* strain NCTC 11168 and plated on selective media (brucella agar plates supplemented with 10% sheep's blood and 20 µg/mL chloramphenicol). Colonies from these plates were sub-cultured on selective plates and genomic DNA isolated following an overnight incubation. PCR amplification of the *hyd* gene was used to verify that allelic exchange had occurred. A noticeable 800 bp increase in the size of the PCR product from mutated colonies verified the construction of a *C. jejuni* mutant (Figure 3).

Hydrogen Uptake

A common method used to verify the presence of uptake-hydrogenase is by H₂ uptake. We performed H₂ uptake assays on whole-cells of both wild-type and mutant *C. jejuni*. Dissolved H₂ was measured amperoterically using a modified Clarke-type

electrode. A chart recorder monitored the disappearance of H₂; and the slope of the line determined the rate of H₂ oxidation. Hydrogenase activity was then expressed as nmoles of H₂ oxidized/min/10⁸ cells. We calculated the rate of H₂ uptake in wild-type strains NCTC 11168 and CVM001 to be 19.7 and 47.7 nmoles H₂ oxidized/min/10⁸ cells, respectively (Table 2). The mutant was unable to oxidize H₂. Thereby further verifying the disruption of the *hydB* gene in our mutant.

Difference Spectra

Room temperature reduced minus oxidized difference spectra of purified membranes revealed cytochromes of the *b*- and *c*-type (Figure 4). In keeping with the published data of Hoffman and Goodman, no spectral evidence of *o*-, *a*-, or *d*- type cytochromes was found (33), despite the presence of a *bd*-type oxidase in the genome sequence (59). Cytochrome *c* predominated in *C. jejuni* membranes as shown by dithionite-reduced minus air-oxidized spectra. Cytochrome *c* absorbs at a maximum of approximately 550 nm while cytochrome *b* was seen at 595 nm. Although dithionite was able to reduce the mutant's cytochromes, H₂ was unable to reduce Hyd: Cm cytochromes. This provides further proof that hydrogenase is not functional in the mutant. H₂ and dithionite both reduced wild-type cytochromes. The ability of H₂ to reduce wild-type cytochromes provides evidence that the electrons released by the oxidation of H₂ and are shuttled through the respiratory cytochromes.

H₂ Dependent O₂ Uptake

To test whether H₂ uptake was coupled to O₂ uptake H₂ dependent O₂ uptake was performed using a Clarke-type electrode connected to a chart recorder. The assay was performed on 5 ml of whole cells. Wild-type cells respired at a rate of 5.0 nmoles O₂/min/10⁸ cells and mutant cells displayed no activity. The difference spectra combined with the H₂ dependent O₂ uptake seems to indicate that H₂ oxidation is linked to O₂ reduction in *C. jejuni*.

Growth Data

To test whether H₂ could indeed support growth of *C. jejuni*, overnight growth in serum bottles with atmospheres containing different concentrations of O₂ and H₂ were performed. Growth of *C. jejuni* in serum bottles incubated overnight at 42°C was measured by optical densities (OD) at 600 nm. Growth was enhanced when bottles were supplemented with H₂ under low O₂ conditions (Table 3). Furthermore, wild-type *C. jejuni* was able to grow under high O₂ atmospheres (25% O₂) when bottles were supplemented with H₂ while little or no growth was seen under high O₂ atmospheres without H₂. This phenomenon could be the result respiratory protection. Respiratory protection could be a mechanism where the cells rapidly oxidize H₂ and transport the electrons through the electron transport chain terminating in the reduction of O₂ to H₂O. The ensuing depletion of O₂ lowers O₂ to levels supporting the growth of *C. jejuni*. Under both high and low O₂ conditions, Hyd: Cm cells did not show an increase in growth between bottles supplemented with H₂ and those without H₂. Unlike the wild-type cells, Hyd: Cm is unable to grow at 25% O₂ even when H₂ is present. Since the mutant is

attenuated for hydrogenase activity it is unable to utilize H₂ and therefore unable to lower O₂ to levels that support growth.

Growth Curve Data

Growth curves were performed in the device shown in Figure 2. Growth curves on wild-type and mutant cells were performed to calculate generation times of *C. jejuni* under different atmospheric conditions. Optical density values were plotted against time on a logarithmic scale. Generation times were calculated using the equation $g = t/n$ with $n = 3.3(\log N - \log N_0)$. Values in Table 4 are the average of at least 2 growth curves. Wild-type generation times decreased when H₂ was sparged into the culture medium. Sparging 10% H₂ gas into the culture medium had no effect on the growth of Hyd:cm. Furthermore, *C. jejuni* had a generation time approximately 2 times longer under microaerobic conditions without H₂ as compared to cells grown microaerobically with H₂. Interestingly, the generation time of wild-type *C. jejuni* grown under extra-aerobic conditions supplemented with 10% H₂ was approximately 2 times less than cells grown under extra-aerobic atmospheres without H₂. This supports the data shown for cells grown overnight in serum bottles (Table 3). Generation times for mutant hyd:cm cells were only slightly shorter when grown with or H₂ under microaerobic atmospheres. Similarly, generation times for hyd:cm grown under extra-aerobic conditions showed a small decrease in generation time when grown with H₂.

Wild-type *C. jejuni* grown with H₂ had a significantly reduced lag phase both under low and high O₂ atmospheres (Figures 5 and 6). Under low O₂ conditions cells grown with H₂ started log phase approximately 2 hours earlier than cells growing without

H₂ (Figure 5). This effect was more dramatic under high O₂ conditions. Wild-type *C. jejuni* started log phase approximately 3 hours earlier than wild-type cells grown without H₂. This effect was not seen with Hyd:Cm.

Chicken Colonization

Three weeks post-inoculation the chickens were humanely sacrificed by CO₂ and cecal contents collected. Cecal contents were diluted and plated on *Campylobacter* selective plates. After a 72 hr incubation (37° C, 12% O₂, 5%CO₂, balance N₂) colonies were counted. Wild-type strain CVM001 had an average colonization level of 4.4×10^7 CFU/g cecal contents while the mutant strain, Hyd:Cm, had an average colonization level of 3.6×10^7 CFU/g cecal contents, the difference not being significant as determined by the Mann-Whitney analysis. However, there was a significant range of colonization levels under each condition (Figure 7). CVM001 colonization levels ranged from 7.5×10^5 to 2.5×10^8 while Hyd:Cm had colonization levels ranging from 4.0×10^2 to 2.1×10^8 . Furthermore, CVM001 had a median colonization level of 1.1×10^7 CFU/g and Hyd:Cm had a median colonization level of 5.65×10^6 CFU/g.

Discussion

The respiratory physiology of the human intestinal pathogen *C. jejuni* is uniquely suited for low O₂ environments. *C. jejuni* contains high concentrations of *b* and *c*-type cytochromes. *C. jejuni* has a *cbb*₃ terminal oxidase that is in keeping with its micro-aerophilic nature. *cbb*₃ oxidases have a high affinity for O₂ which is important under low O₂ conditions such as the intestines of avians or humans. These cytochromes are reduced

by H_2 when H_2 is present in the environment. *C. jejuni* contains an uptake hydrogenase which can efficiently oxidize H_2 . Hoffman and Goodman showed that *C. jejuni* respire H_2 at a rate of 219 nmol of O_2 /min/mg protein. This respiration rate was 50-100 times greater than all other substrates (malate, succinate, lactate, NADH) tested except formate (33). They obtained similar enzyme activities in whole-cells and cell extracts. H^+/O ratios are values that indicate the stoichiometry of proton translocation per O_2 consumed. Hoffman and Goodman obtained a maximum H^+/O quotient of 3.7 with H_2 ; poor substrates such as NADH were not oxidized and H^+/O ratios could not be calculated. Their experimental data supports the hypothesis that *C. jejuni* cannot utilize NADH as an electron donor. In our experiments wild-type strains CVM001, a strain isolated from a Guillan-Barré syndrome patient oxidizes H_2 at a rate of 47 nmoles H_2 oxidized/min/ 10^8 cells while NCTC 11168 oxidizes H_2 at a rate of 19 nmoles H_2 /min/ 10^8 cells (Table 2). Strain Hyd:Cm displayed no hydrogenase activity (Table 2) and therefore provided an excellent comparison for physiological studies. Hydrogenase activity is linked to the reduction of O_2 . H_2 -dependent O_2 uptake assays illustrated that wild-type cells channel the electrons liberated from H_2 through the electron transport chain culminating in the reduction of O_2 to H_2O . This provides evidence that *C. jejuni* can utilize H_2 as an energy source since the reduction of O_2 is coupled to the production of ATP. The above data indicates hydrogenase plays an important physiological role in *C. jejuni*. The organism clearly evolved the ability to efficiently utilize H_2 . It appears the organism gains a physiological edge by growing on H_2 since H_2 provides the greatest proton gradient. The proton motive force is then used to drive the production of ATP.

To test whether H₂ supported growth, *C. jejuni* was grown under atmospheres both containing and lacking H₂. Growth experiments in serum bottles illustrate enhanced growth when H₂ is present (Table 3). This enhanced growth occurred under both micro-aerobic and extra-aerobic conditions. Growth in the presence of H₂ under high O₂ concentrations is especially interesting considering that *C. jejuni* has long been designated a microaerophile. The ability to grow at 25% O₂ argues for the role of hydrogenase in a respiratory protection mechanism. Respiratory protection is a mechanism whereby organisms undergo rapid metabolic activity to decrease the concentration of toxic O₂. Respiratory protection occurs in the N₂-fixing bacterium *Azotobacter vinelandii*. In *A. vinelandii*, rapid O₂ consumption by the electron transport chain results in the maintenance of the low intracellular O₂ levels required for N₂-fixation. It is possible that a similar mechanism occurs in *C. jejuni*. The genome of *C. jejuni* contains known O₂-labile enzymes, such as pyruvate-ferrodoxin oxidoreductase (59). In fact, Kelly hypothesizes that the microaerophilic nature of *C. jejuni* could be due to the presence of these O₂-sensitive enzymes (38).

In growth experiments, serum bottles are gased to attain a specific atmosphere. During incubation in serum bottles containing 25% O₂ and 10% H₂ *C. jejuni* may be rapidly oxidizing H₂ leading to the reduction of O₂ to H₂O. This process would occur rapidly until the O₂ concentration is low enough to support growth. Once the O₂ concentration decreases to levels that are non-toxic to important O₂ sensitive enzymes, *C. jejuni* is able to grow and divide. When growth phase begins *C. jejuni* can then utilize H₂ as a supplemental source of energy. This is, in fact, the case when *C. jejuni* is grown under micro-aerobic conditions in the presence of H₂. The presence of this supplemental

energy source allows the organism to grow and divide at a faster rate. The ability to use H_2 in such a manner may provide a selective advantage during colonization since both the avian and mammalian gut contains saturating levels of H_2 .

To further test whether hydrogenase provides a means of respiratory protection, *C. jejuni* was grown in Mueller-Hinton media that was constantly sparged with specified gas mixtures. Under these conditions *C. jejuni*'s hydrogenase will be unable to lower the O_2 concentration since the specified gas composition is constantly replenished. These experiments were used to calculate generation times of wild-type and mutant strains grown at low and high O_2 with and without H_2 . Under these conditions hydrogenase is unable to reduce the concentration of O_2 to levels supporting growth since gas is constantly pumped into the system. During experiments at high O_2 wild-type *C. jejuni* had a generation time twice as long when grown without H_2 as compared to when grown in the presence of H_2 (Table 4). Similarly, the mutant (hyd:Cm) had a two-thirds longer generation time when grown under high O_2 compared to when grown at high O_2 in the presence of H_2 (Table 4). However, these growth experiments illustrate that cells begin logarithmic growth earlier when H_2 is present than when H_2 is not present (Figure 6). This phenomenon does not occur in the mutant strain since it cannot oxidize H_2 . Typically wild-type *C. jejuni* grown at high O_2 in the presence of H_2 begins logarithmic growth 3 hours earlier than cells grown without H_2 . This provides evidence that H_2 is an efficient supplemental energy source, perhaps even providing *C. jejuni* an advantage over other intestinal colonizers. The ability of the mutant to grow under high O_2 conditions both with and without H_2 is somewhat confusing. *C. jejuni* is a microaerophile and therefore should not be able to grow at such high O_2 levels. Furthermore, the fact that the

generation time is shorter when grown in the presence of H₂ is puzzling. Perhaps the media is providing some defense against oxidative stress. Krieg and Hodge report that O₂ tolerance in *C. jejuni* varies depending on the media used. They argue that sodium metabisulphite, a common component of brucella agar, enhances O₂ tolerance (32). However, our experiments are carried out in Mueller-Hinton broth which does not contain sodium metabisulphite. A more plausible reason why our mutant cells have different generation times under high O₂ with and without H₂ is that H₂ helps keep media components, such as cysteine, reduced.

As predicted by growth in serum bottles, wild-type *C. jejuni* had a faster generation time when grown microaerophilically in the presence of H₂ (Table 4). This supports the theory that *C. jejuni* utilizes H₂ as a supplemental energy source. The generation times of the mutant when grown microaerobically with and without H₂ are approximately the same further indicating their inability to use H₂ as an energy source. As expected this generation time is also similar to the microaerobic generation time of wild-type *C. jejuni*.

Both the serum bottle experiments and the growth curve experiments indicate that H₂ greatly enhances the growth of *C. jejuni*. To test whether the ability to oxidize H₂ was required for chicken colonization an animal model study was performed. Published reports indicate that some *C. jejuni* strains, including the sequence strain *C. jejuni* NCTC 11168, are poor colonizers (3) while other reports claim that strain NCTC 11168 is unable to colonize chickens (65). For this reason we also made a *hydB* mutant in *C. jejuni* strain CVM001, a clinical isolate from a patient suffering from Guillan-Barré syndrome. To our knowledge this strain had not been characterized for colonization

potential. Unsure if our wild-type strain would effectively colonize chickens, we chose to inoculate the birds at a high level, 3×10^7 CFU/ml, with both our wild-type and mutant strains. This inoculum was significantly higher than other researchers who inoculate with between 1×10^3 CFU/ml and 1×10^6 CFU/ml (65). Our data indicates that both CVM and Hyd:cm are effective colonizers when inoculated at high levels (Figure 7). Under these experimental conditions hydrogenase is not required for colonization. However, further studies are required to elucidate what role hydrogenase may play in colonization. Experiments using a significantly lower inoculum would differentiate between whether the mutant colonizes at a reduced level or if it in fact colonizes equally as well as the wild-type strain. Furthermore, colonization competition experiments would elucidate whether the ability to use H_2 as an energy source provides a competitive advantage to *C. jejuni* over microflora unable to utilize the abundant H_2 in the avian intestine.

This research project clearly illustrates H_2 is an efficient source of energy in *C. jejuni*. We have shown that *C. jejuni* contains an uptake hydrogenase capable of oxidizing H_2 at a rate of approximately 40 nmoles H_2 oxidized/min/ 10^8 cells. The electrons liberated from the oxidation of H_2 are passed through the electron transport chain terminating with the reduction of O_2 to H_2O . Growth experiments illustrate that the ability to utilize H_2 as an energy source allows wild-type *C. jejuni* to divide in half the time as *C. jejuni* grown in the absence of H_2 . Cells deficient in hydrogenase activity did not show as drastic a difference in generation time when grown in the presence of H_2 . It appears that H_2 provides an efficient source of supplemental energy for the organism and may be an important component of a respiratory protection mechanism designed to protect O_2 -sensitive enzymes from oxidative damage. Further characterization of

hydrogenase will determine the exact role of hydrogenase in *C. jejuni* metabolism.

Under our experimental conditions hydrogenase was not required for chicken colonization. Further studies are required to elucidate the role of hydrogenase in chicken colonization. Determining the role of hydrogenase in chicken colonization will provide invaluable knowledge that may lead to the development of novel antimicrobials designed to eliminate *C. jejuni* from chicken flocks. Eliminating *C. jejuni* from the food chain would remove an important risk to human health.

Campylobacter jejuni NCTC 11168

A	B	C	D	X	Y	G	H	I	J	K	L	M	N
---	---	---	---	---	---	---	---	---	---	---	---	---	---

Escherichia coli K12

A	B	C D	E	F	G	H	I	J	K	L	M	N
---	---	-----	---	---	---	---	---	---	---	---	---	---

Figure 1. Organization of NDH-1 genes in bacterial genomes. The letters *A-N* represent the *nuo* genes. *X* and *Y* denote the *Campylobacter jejuni* genes corresponding to *nuoE* and *nuoF*, respectively.

Table 1. Volumes added to serum bottles to attain specific atmospheres.

Atmosphere	ml O₂	ml H₂
12% O₂	18	
12% O₂ + H₂	18	15
25% O₂	37.5	
25% O₂ + H₂	37.5	15



Figure 2. Experimental set-up of growth experiments used to calculate generation times. Inside the glass vessel rests a stir bar. Suspended within the device is the glass dispersion tube.

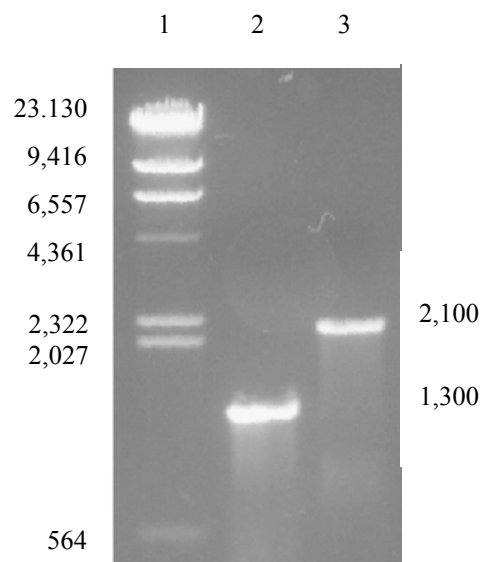


Figure 3. 1% agarose gel of *hydB* PCR products from DNA isolated from wild-type (lane 2) and *Hyd: Cm C. jejuni* (Lane 3). Lane 1 contains lambda *Hind*III markers.

Table 2. Whole-cell hydrogenase activities of wild-type and mutant *C. jejuni*.

Strain	H ₂ Uptake Rate [°]
NCTC 11168	19.67
Hyd:Cm	0
CVM001	47.47
CVM001 Hyd:Cm	0

[°] Units are nmoles of H₂ oxidized/min/10⁸ cells

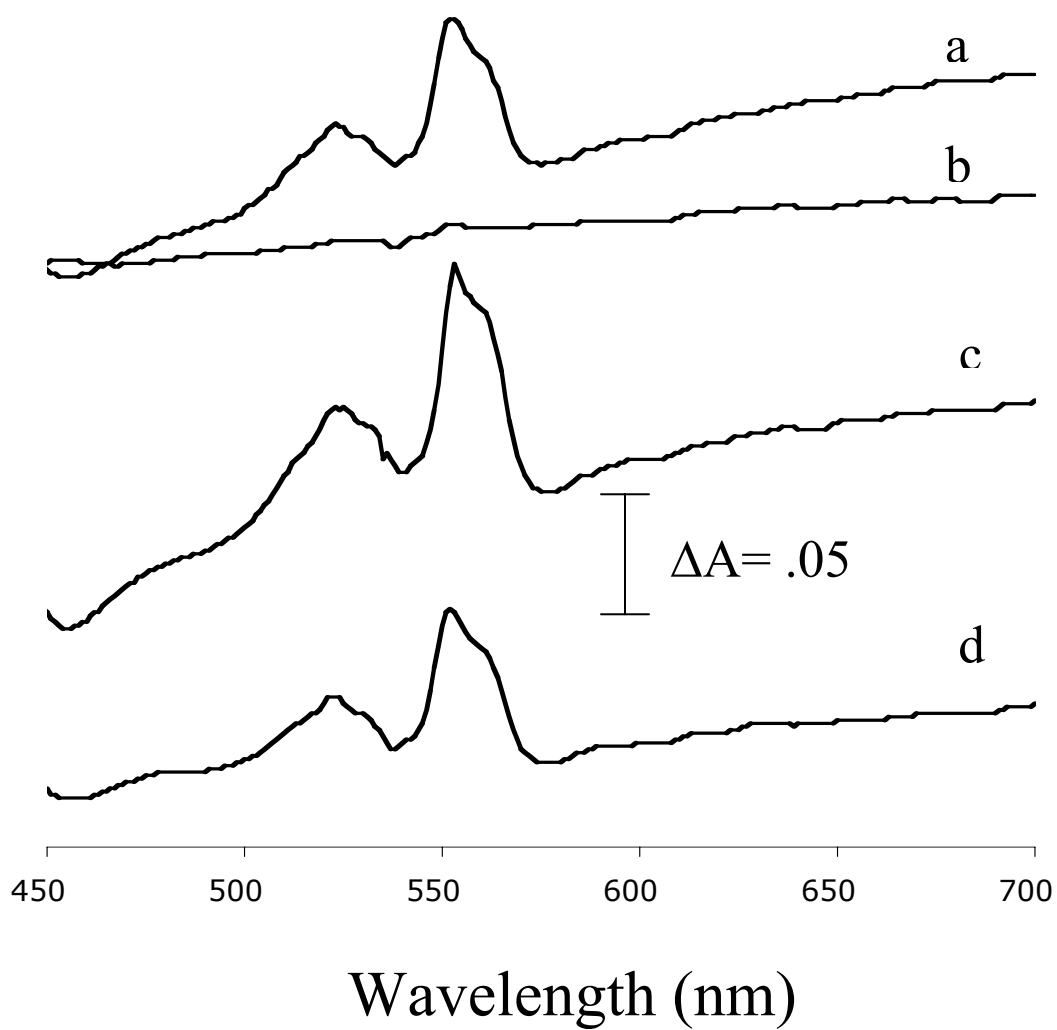


Figure 4. Difference spectra of wild-type and mutant membranes. (a) dithionite reduced hyd:Cm membranes, (b) H₂ reduced hyd:Cm membranes, (c) wild-type dithionite reduced membranes, (d) wild-type H₂ reduced membranes.

Table 3. Optical densities of *C. jejuni* strains grown overnight in serum bottles*.

Strain	Condition			
	12% O ₂	12% O ₂ + 10% H ₂	25% O ₂	25% O ₂ + 10% H ₂
CVM001	.4460	.5963	.0035	.6950
CVM:Hyd:Cm	.5195	.5214	.0015	.004

*Values are the average of 2 independent experiments.

Table 4. Generation times of both wild-type and mutant *C. jejuni*.

Conditions	Generation Time (hrs)	
	CVM001	Hyd:Cm
12% O₂	1.78 ^ϕ +/- 0.09	1.77 [°] +/- 0.38
12% O₂ + H₂	.96 ^ψ +/- 0.12	1.38 [°] +/- 0.10
25% O₂	2.38 [*] +/- 0.62	2.61 [*] +/- 0.01
25% O₂ + H₂	1.16 [*] +/- 0.04	1.58 [*] +/- 0.02

^ϕ Generation time is the average of four independent experiments.

^ψ Generation time is the average of five independent experiments.

[°] Generation times are the average of two independent experiments.

^{*} Generation times are the average of three independent experiments.

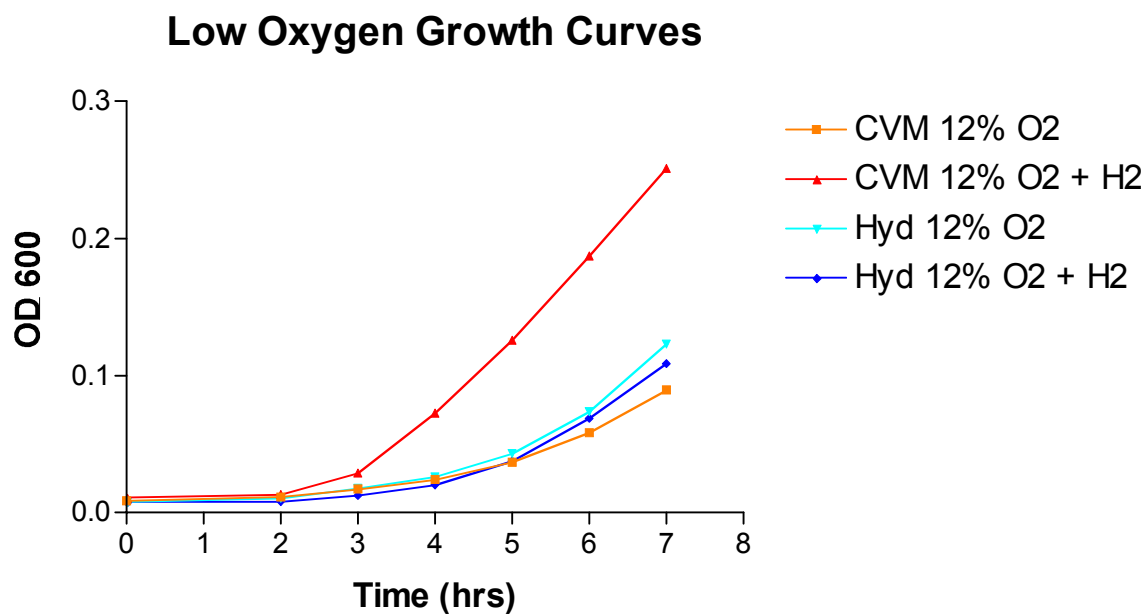


Figure 5. Representative low O₂ growth curves used to calculate generation times for wild-type and mutant *C. jejuni* grown at 12% O₂ with and without H₂.

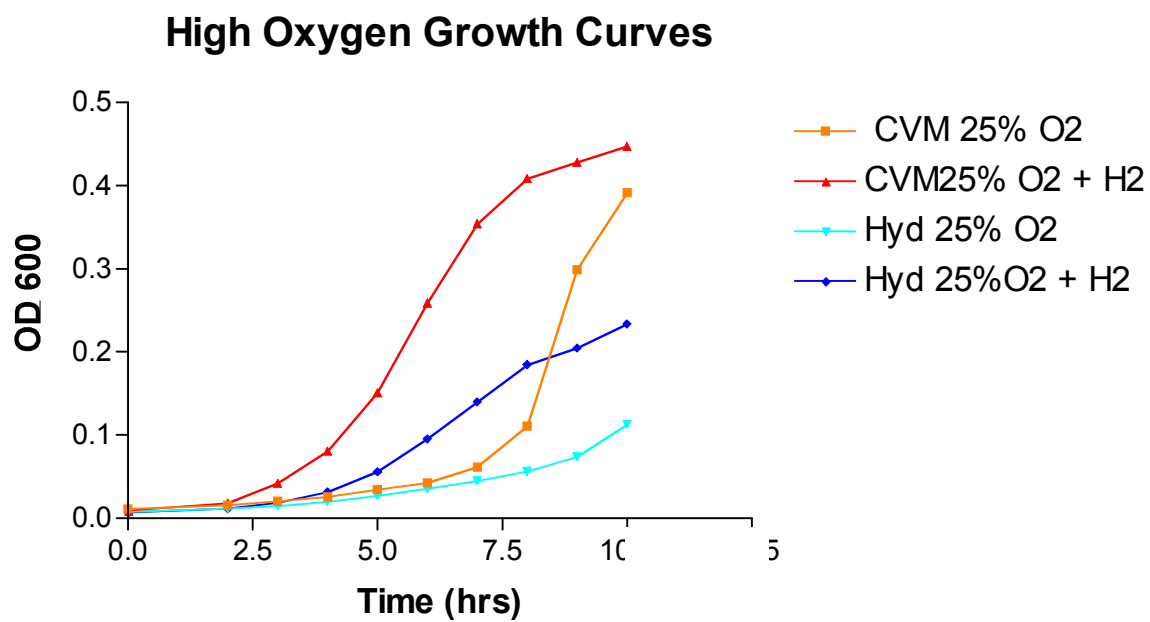


Figure 6. Representative high oxygen growth curves used to calculate generation time for wild-type and mutant *C. jejuni* grown at high O₂ with and without H₂.

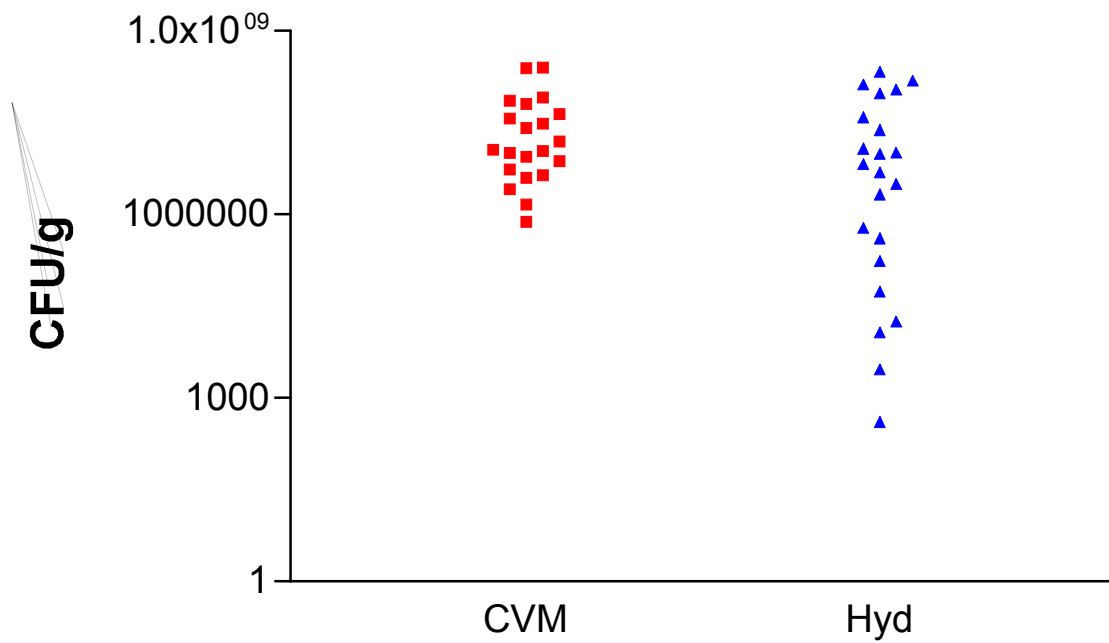


Figure 7. Scatter graph of colonization levels of wild-type and mutant *C. jejuni*. The wild-type strain had an average colonization level of 4.4×10^7 while the mutant had an average colonization level of 3.6×10^7 .

REFERENCES

1. **Adams, M. W., L. E. Mortenson, and J. S. Chen.** 1980. Hydrogenase. *Biochim Biophys Acta* **594**:105-76.
2. **Adams, M. W., and E. I. Stiefel.** 2000. Organometallic iron: the key to biological hydrogen metabolism. *Curr Opin Chem Biol* **4**:214-20.
3. **Ahmed, I. H., G. Manning, T. M. Wassenaar, S. Cawthraw, and D. G. Newell.** 2002. Identification of genetic differences between two *Campylobacter jejuni* strains with different colonization potentials. *Microbiology* **148**:1203-12.
4. **Allos, B. M.** 2001. *Campylobacter jejuni* Infections: update on emerging issues and trends. *Clin Infect Dis* **32**:1201-6.
5. **Andrews, S. C., B. C. Berks, J. McClay, A. Ambler, M. A. Quail, P. Golby, and J. R. Guest.** 1997. A 12-cistron *Escherichia coli* operon (hyf) encoding a putative proton-translocating formate hydrogenlyase system. *Microbiology* **143** (Pt 11):3633-47.
6. **Ang, C. W., H. P. Endtz, B. C. Jacobs, J. D. Laman, M. A. de Klerk, F. G. van der Meche, and P. A. van Doorn.** 2000. *Campylobacter jejuni* lipopolysaccharides from Guillain-Barre syndrome patients induce IgG anti-GM1 antibodies in rabbits. *J Neuroimmunol* **104**:133-8.
7. **Ang, C. W., P. G. Noordzij, M. A. de Klerk, H. P. Endtz, P. A. van Doorn, and J. D. Laman.** 2002. Ganglioside mimicry of *Campylobacter jejuni* lipopolysaccharides determines antiganglioside specificity in rabbits. *Infect Immun* **70**:5081-5.
8. **Bairoch, A., and P. Bucher.** 1994. PROSITE: recent developments. *Nucleic Acids Res* **22**:3583-9.
9. **Berks, B. C., F. Sargent, and T. Palmer.** 2000. The Tat protein export pathway. *Mol Microbiol* **35**:260-74.
10. **Bernhard, M., B. Benelli, A. Hochkoeppler, D. Zannoni, and B. Friedrich.** 1997. Functional and structural role of the cytochrome b subunit of the membrane-bound hydrogenase complex of *Alcaligenes eutrophus* H16. *Eur J Biochem* **248**:179-86.
11. **Bernhard, M., B. Friedrich, and R. A. Siddiqui.** 2000. *Ralstonia eutropha* TF93 is blocked in tat-mediated protein export. *J Bacteriol* **182**:581-8.
12. **Black, L. K., C. Fu, and R. J. Maier.** 1994. Sequences and characterization of hupU and hupV genes of *Bradyrhizobium japonicum* encoding a possible nickel-sensing complex involved in hydrogenase expression. *J Bacteriol* **176**:7102-6.
13. **Bothe, H., E. Distler, and G. Eisbrenner.** 1978. Hydrogen metabolism in blue-green algae. *Biochimie* **60**:277-89.
14. **Brown, N. J., R. D. Rumsey, and N. W. Read.** 1987. Adaptation of hydrogen analysis to measure stomach to caecum transit time in the rat. *Gut* **28**:849-54.
15. **Buzby, J. C., T. Roberts, B. M. Allos, and United States. Dept. of Agriculture. Economic Research Service.** 1997. Estimated annual costs of *Campylobacter*-associated Guillain-Barré syndrome. U.S. Dept. of Agriculture, ERS,, [Washington, DC].

16. **Carlone, G. M., and J. Lascelles.** 1982. Aerobic and anaerobic respiratory systems in *Campylobacter fetus* subsp. *jejuni* grown in atmospheres containing hydrogen. *J Bacteriol* **152**:306-14.
17. **Carrasco, C. D., J. A. Buettner, and J. W. Golden.** 1995. Programmed DNA rearrangement of a cyanobacterial hupL gene in heterocysts. *Proc Natl Acad Sci U S A* **92**:791-5.
18. **Cauvin, B., A. Colbeau, and P. M. Vignais.** 1991. The hydrogenase structural operon in *Rhodobacter capsulatus* contains a third gene, hupM, necessary for the formation of a physiologically competent hydrogenase. *Mol Microbiol* **5**:2519-27.
19. **Coker, A. O., R. D. Isokpehi, B. N. Thomas, K. O. Amisu, and C. L. Obi.** 2002. Human campylobacteriosis in developing countries. *Emerg Infect Dis* **8**:237-44.
20. **D'Mello, R., S. Hill, and R. K. Poole.** 1996. The cytochrome bd quinol oxidase in *Escherichia coli* has an extremely high oxygen affinity and two oxygen-binding haems: implications for regulation of activity in vivo by oxygen inhibition. *Microbiology* **142** (Pt 4):755-63.
21. **Darrouzet, E., M. Valkova-Valchanova, T. Ohnishi, and F. Daldal.** 1999. Structure and function of the bacterial bc1 complex: domain movement, subunit interactions, and emerging rationale engineering attempts. *J Bioenerg Biomembr* **31**:275-88.
22. **Dernedde, J., T. Eitinger, N. Patenge, and B. Friedrich.** 1996. hyp gene products in *Alcaligenes eutrophus* are part of a hydrogenase-maturation system. *Eur J Biochem* **235**:351-8.
23. **Drapal, N., and A. Bock.** 1998. Interaction of the hydrogenase accessory protein HypC with HycE, the large subunit of *Escherichia coli* hydrogenase 3 during enzyme maturation. *Biochemistry* **37**:2941-8.
24. **Dross, F., V. Geisler, R. Lenger, F. Theis, T. Krafft, F. Fahrenholz, E. Kojro, A. Duchene, D. Tripier, K. Juvenal, and et al.** 1992. The quinone-reactive Ni/Fe-hydrogenase of *Wolinella succinogenes*. *Eur J Biochem* **206**:93-102.
25. **Elsen, S., A. Colbeau, J. Chabert, and P. M. Vignais.** 1996. The hupTUV operon is involved in negative control of hydrogenase synthesis in *Rhodobacter capsulatus*. *J Bacteriol* **178**:5174-81.
26. **Ferguson, S. J.** 2001. Keilin's cytochromes: how bacteria use them, vary them and make them. *Biochem Soc Trans* **29**:629-40.
27. **Fox, J. D., Y. He, D. Shelver, G. P. Roberts, and P. W. Ludden.** 1996. Characterization of the region encoding the CO-induced hydrogenase of *Rhodospirillum rubrum*. *J Bacteriol* **178**:6200-8.
28. **Fox, J. D., R. L. Kerby, G. P. Roberts, and P. W. Ludden.** 1996. Characterization of the CO-induced, CO-tolerant hydrogenase from *Rhodospirillum rubrum* and the gene encoding the large subunit of the enzyme. *J Bacteriol* **178**:1515-24.
29. **Gross, R., J. Simon, and A. Kroger.** 1999. The role of the twin-arginine motif in the signal peptide encoded by the hydA gene of the hydrogenase from *Wolinella succinogenes*. *Arch Microbiol* **172**:227-32.

30. **Gross, R., J. Simon, F. Theis, and A. Kroger.** 1998. Two membrane anchors of *Wolinella succinogenes* hydrogenase and their function in fumarate and polysulfide respiration. *Arch Microbiol* **170**:50-8.
31. **Higuchi, Y., T. Yagi, and N. Yasuoka.** 1997. Unusual ligand structure in Ni-Fe active center and an additional Mg site in hydrogenase revealed by high resolution X-ray structure analysis. *Structure* **5**:1671-80.
32. **Hodge, J. P., and N. R. Krieg.** 1994. Oxygen tolerance estimates in *Campylobacter* species depend on the testing medium. *J Appl Bacteriol* **77**:666-73.
33. **Hoffman, P. S., and T. G. Goodman.** 1982. Respiratory physiology and energy conservation efficiency of *Campylobacter jejuni*. *J Bacteriol* **150**:319-26.
34. **Ide, T., S. Baumer, and U. Deppenmeier.** 1999. Energy conservation by the H₂:heterodisulfide oxidoreductase from *Methanosarcina mazei* Go1: identification of two proton-translocating segments. *J Bacteriol* **181**:4076-80.
35. **Jacobi, A., R. Rossmann, and A. Bock.** 1992. The hyp operon gene products are required for the maturation of catalytically active hydrogenase isoenzymes in *Escherichia coli*. *Arch Microbiol* **158**:444-51.
36. **Jones, D. M., E. M. Sutcliffe, R. Rios, A. J. Fox, and A. Curry.** 1993. *Campylobacter jejuni* adapts to aerobic metabolism in the environment. *J Med Microbiol* **38**:145-50.
37. **Kapperud, G., E. Skjerve, L. Vik, K. Hauge, A. Lysaker, I. Aalmen, S. M. Ostroff, and M. Potter.** 1993. Epidemiological investigation of risk factors for *campylobacter* colonization in Norwegian broiler flocks. *Epidemiol Infect* **111**:245-55.
38. **Kelly, D. J.** 2001. The physiology and metabolism of *Campylobacter jejuni* and *Helicobacter pylori*. *Symp Ser Soc Appl Microbiol*:16S-24S.
39. **Leach, S., P. Harvey, and R. Wali.** 1997. Changes with growth rate in the membrane lipid composition of and amino acid utilization by continuous cultures of *Campylobacter jejuni*. *J Appl Microbiol* **82**:631-40.
40. **Lenz, O., and B. Friedrich.** 1998. A novel multicomponent regulatory system mediates H₂ sensing in *Alcaligenes eutrophus*. *Proc Natl Acad Sci U S A* **95**:12474-9.
41. **Lenz, O., A. Strack, A. Tran-Betcke, and B. Friedrich.** 1997. A hydrogen-sensing system in transcriptional regulation of hydrogenase gene expression in *Alcaligenes* species. *J Bacteriol* **179**:1655-63.
42. **Levitt, M. D.** 1969. Production and excretion of hydrogen gas in man. *N Engl J Med* **281**:122-7.
43. **Luechtefeld, N. A., M. J. Blaser, L. B. Reller, and W. L. Wang.** 1980. Isolation of *Campylobacter fetus* subsp. *jejuni* from migratory waterfowl. *J Clin Microbiol* **12**:406-8.
44. **Ma, K., R. N. Schicho, R. M. Kelly, and M. W. Adams.** 1993. Hydrogenase of the hyperthermophile *Pyrococcus furiosus* is an elemental sulfur reductase or sulfhydrogenase: evidence for a sulfur-reducing hydrogenase ancestor. *Proc Natl Acad Sci U S A* **90**:5341-4.

45. **Ma, K., R. Weiss, and M. W. Adams.** 2000. Characterization of hydrogenase II from the hyperthermophilic archaeon *Pyrococcus furiosus* and assessment of its role in sulfur reduction. *J Bacteriol* **182**:1864-71.
46. **Magalon, A., and A. Bock.** 2000. Analysis of the HypC-hycE complex, a key intermediate in the assembly of the metal center of the *Escherichia coli* hydrogenase 3. *J Biol Chem* **275**:21114-20.
47. **Magalon, A., and A. Bock.** 2000. Dissection of the maturation reactions of the [NiFe] hydrogenase 3 from *Escherichia coli* taking place after nickel incorporation. *FEBS Lett* **473**:254-8.
48. **Meek, L., and D. J. Arp.** 2000. The hydrogenase cytochrome b heme ligands of *Azotobacter vinelandii* are required for full H(2) oxidation capability. *J Bacteriol* **182**:3429-36.
49. **Meyer, J., and J. Gagnon.** 1991. Primary structure of hydrogenase I from *Clostridium pasteurianum*. *Biochemistry* **30**:9697-704.
50. **Meyer, J., B. C. Kelley, and P. M. Vignais.** 1978. Nitrogen fixation and hydrogen metabolism in photosynthetic bacteria. *Biochimie* **60**:245-60.
51. **MS. Mutaftschiev, M. R. O. B. a. R. J. M.** 1983. Hydrogen Oxidation Activity in Membranes from *Rhizobium Japonicum*. *Biochimica et Biophysica Acta* **722**:372-380.
52. **Nachamkin, I., J. Liu, M. Li, H. Ung, A. P. Moran, M. M. Prendergast, and K. Sheikh.** 2002. *Campylobacter jejuni* from patients with Guillain-Barré syndrome preferentially expresses a GD(1a)-like epitope. *Infect Immun* **70**:5299-303.
53. **Newell, D. G., and C. Fearnley.** 2003. Sources of *Campylobacter* colonization in broiler chickens. *Appl Environ Microbiol* **69**:4343-51.
54. **Nicolet, Y., B. J. Lemon, J. C. Fontecilla-Camps, and J. W. Peters.** 2000. A novel FeS cluster in Fe-only hydrogenases. *Trends Biochem Sci* **25**:138-43.
55. **Olson, J. W., C. Fu, and R. J. Maier.** 1997. The HypB protein from *Bradyrhizobium japonicum* can store nickel and is required for the nickel-dependent transcriptional regulation of hydrogenase. *Mol Microbiol* **24**:119-28.
56. **Olson, J. W., and R. J. Maier.** 2000. Dual roles of *Bradyrhizobium japonicum* nickelin protein in nickel storage and GTP-dependent Ni mobilization. *J Bacteriol* **182**:1702-5.
57. **Olson, J. W., and R. J. Maier.** 2002. Molecular hydrogen as an energy source for *Helicobacter pylori*. *Science* **298**:1788-90.
58. **Osborne, J. P., and R. B. Gennis.** 1999. Sequence analysis of cytochrome bd oxidase suggests a revised topology for subunit I. *Biochim Biophys Acta* **1410**:32-50.
59. **Parkhill, J., B. W. Wren, K. Mungall, J. M. Ketley, C. Churcher, D. Basham, T. Chillingworth, R. M. Davies, T. Feltwell, S. Holroyd, K. Jagels, A. V. Karlyshev, S. Moule, M. J. Pallen, C. W. Penn, M. A. Quail, M. A. Rajandream, K. M. Rutherford, A. H. van Vliet, S. Whitehead, and B. G. Barrell.** 2000. The genome sequence of the food-borne pathogen *Campylobacter jejuni* reveals hypervariable sequences. *Nature* **403**:665-8.
60. **Paschos, A., R. S. Glass, and A. Bock.** 2001. Carbamoylphosphate requirement for synthesis of the active center of [NiFe]-hydrogenases. *FEBS Lett* **488**:9-12.

61. **Pedroni, P., A. Della Volpe, G. Galli, G. M. Mura, C. Pratesi, and G. Grandi.** 1995. Characterization of the locus encoding the [Ni-Fe] sulfhydrogenase from the archaeon *Pyrococcus furiosus*: evidence for a relationship to bacterial sulfite reductases. *Microbiology* **141** (Pt 2):449-58.
62. **Peters, J. W.** 1999. Structure and mechanism of iron-only hydrogenases. *Curr Opin Struct Biol* **9**:670-6.
63. **Przybyla, A. E., J. Robbins, N. Menon, and H. D. Peck, Jr.** 1992. Structure-function relationships among the nickel-containing hydrogenases. *FEMS Microbiol Rev* **8**:109-35.
64. **Rey, L., J. Imperial, J. M. Palacios, and T. Ruiz-Argueso.** 1994. Purification of *Rhizobium leguminosarum* HypB, a nickel-binding protein required for hydrogenase synthesis. *J Bacteriol* **176**:6066-73.
65. **Ringoir, D. D., and V. Korolik.** 2003. Colonisation phenotype and colonisation potential differences in *Campylobacter jejuni* strains in chickens before and after passage in vivo. *Vet Microbiol* **92**:225-35.
66. **Sapra, R., M. F. Verhagen, and M. W. Adams.** 2000. Purification and characterization of a membrane-bound hydrogenase from the hyperthermophilic archaeon *Pyrococcus furiosus*. *J Bacteriol* **182**:3423-8.
67. **Sargent, F., E. G. Bogsch, N. R. Stanley, M. Wexler, C. Robinson, B. C. Berks, and T. Palmer.** 1998. Overlapping functions of components of a bacterial Sec-independent protein export pathway. *Embo J* **17**:3640-50.
68. **Sauter, M., R. Bohm, and A. Bock.** 1992. Mutational analysis of the operon (hyc) determining hydrogenase 3 formation in *Escherichia coli*. *Mol Microbiol* **6**:1523-32.
69. **Schneider, K., and H. G. Schlegel.** 1976. Purification and properties of soluble hydrogenase from *Alcaligenes eutrophus* H 16. *Biochim Biophys Acta* **452**:66-80.
70. **Schwartz, E., U. Gerischer, and B. Friedrich.** 1998. Transcriptional regulation of *Alcaligenes eutrophus* hydrogenase genes. *J Bacteriol* **180**:3197-204.
71. **Schwerer, B.** 2002. Antibodies against gangliosides: a link between preceding infection and immunopathogenesis of Guillain-Barré syndrome. *Microbes Infect* **4**:373-84.
72. **Skirrow, M. B.** 1994. Diseases due to *Campylobacter*, *Helicobacter* and related bacteria. *J Comp Pathol* **111**:113-49.
73. **Smith, M. A., M. Finel, V. Korolik, and G. L. Mendz.** 2000. Characteristics of the aerobic respiratory chains of the microaerophiles *Campylobacter jejuni* and *Helicobacter pylori*. *Arch Microbiol* **174**:1-10.
74. **Stephens, C. P., S. L. On, and J. A. Gibson.** 1998. An outbreak of infectious hepatitis in commercially reared ostriches associated with *Campylobacter coli* and *Campylobacter jejuni*. *Vet Microbiol* **61**:183-90.
75. **Stern, N. J., P. Fedorka-Cray, J. S. Bailey, N. A. Cox, S. E. Craven, K. L. Hiett, M. T. Musgrove, S. Ladely, D. Cosby, and G. C. Mead.** 2001. Distribution of *Campylobacter* spp. in selected U.S. poultry production and processing operations. *J Food Prot* **64**:1705-10.
76. **Tersteegen, A., and R. Hedderich.** 1999. *Methanobacterium thermoautotrophicum* encodes two multisubunit membrane-bound [NiFe]

- hydrogenases. Transcription of the operons and sequence analysis of the deduced proteins. *Eur J Biochem* **264**:930-43.
77. **Turkson, P. K., K. J. Lindqvist, and G. Kapperud.** 1988. Isolation of *Campylobacter* spp. and *Yersinia enterocolitica* from domestic animals and human patients in Kenya. *Apmis* **96**:141-6.
 78. **Vignais, P. M., B. Billoud, and J. Meyer.** 2001. Classification and phylogeny of hydrogenases. *FEMS Microbiol Rev* **25**:455-501.
 79. **Vignais, P. M., A. Colbeau, J. C. Willison, and Y. Jouanneau.** 1985. Hydrogenase, nitrogenase, and hydrogen metabolism in the photosynthetic bacteria. *Adv Microb Physiol* **26**:155-234.
 80. **Vignais, P. M., B. Dimon, N. A. Zorin, M. Tomiyama, and A. Colbeau.** 2000. Characterization of the hydrogen-deuterium exchange activities of the energy-transducing HupSL hydrogenase and H(2)-signaling HupUV hydrogenase in *Rhodobacter capsulatus*. *J Bacteriol* **182**:5997-6004.
 81. **Volbeda, A., M. H. Charon, C. Piras, E. C. Hatchikian, M. Frey, and J. C. Fontecilla-Camps.** 1995. Crystal structure of the nickel-iron hydrogenase from *Desulfovibrio gigas*. *Nature* **373**:580-7.
 82. **Voordouw, G.** 2000. A universal system for the transport of redox proteins: early roots and latest developments. *Biophys Chem* **86**:131-40.
 83. **Voordouw, G., and S. Brenner.** 1985. Nucleotide sequence of the gene encoding the hydrogenase from *Desulfovibrio vulgaris* (Hildenborough). *Eur J Biochem* **148**:515-20.
 84. **Walker, C. C., and M. G. Yates.** 1978. The hydrogen cycle in nitrogen-fixing *Azotobacter chroococcum*. *Biochimie* **60**:225-31.
 85. **Wallace, J. S., K. N. Stanley, and K. Jones.** 1998. The colonization of turkeys by thermophilic *campylobacters*. *J Appl Microbiol* **85**:224-30.
 86. **Wang, R.** 1980. Amperometric Hydrogen Electrode, vol. 69. Academic Press.
 87. **Willison, H., G. Stoll, K. V. Toyka, T. Berger, and H. P. Hartung.** 2002. Autoimmunity and inflammation in the peripheral nervous system. *Trends Neurosci* **25**:127-9.
 88. **Wu, L. F., A. Chanal, and A. Rodrigue.** 2000. Membrane targeting and translocation of bacterial hydrogenases. *Arch Microbiol* **173**:319-24.
 89. **Wu, L. F., and M. A. Mandrand.** 1993. Microbial hydrogenases: primary structure, classification, signatures and phylogeny. *FEMS Microbiol Rev* **10**:243-69.
 90. **Yao, R., R. A. Alm, T. J. Trust, and P. Guerry.** 1993. Construction of new *Campylobacter* cloning vectors and a new mutational cat cassette. *Gene* **130**:127-30.
 91. **Yogasundram, K., S. M. Shane, and K. S. Harrington.** 1989. Prevalence of *Campylobacter jejuni* in selected domestic and wild birds in Louisiana. *Avian Dis* **33**:664-7.
 92. **Ziprin, R. L., R. E. Droleskey, M. E. Hume, and R. B. Harvey.** 2003. Failure of viable nonculturable *Campylobacter jejuni* to colonize the cecum of newly hatched leghorn chicks. *Avian Dis* **47**:753-8.