ABSTRACT

WEERAKOON, DILAN R. Characterization of the Respiratory Donor Complex I in *Campylobacter jejuni*. (Under the direction of Dr. Jonathan W. Olson).

Campylobacter jejuni is an ε-proteobacterium that is one of the leading causes of human bacterial gastroenteritis, campylobacteriosis, in the world. *C. jejuni* does not utilize carbohydrates, does not have an intact glycolytic pathway, nor can it grow fermentatively. It uses oxidative phosphorylation and the electron transport chain to fulfill its energy requirements. Inspection of its electron transport chain reveals a highly branched electron transport chain that is comprised of a number of electron donor and acceptor enzymes. One of these electron donor enzymes in *C. jejuni* is the proton pump Complex I also known as NADH:Ubiquinone Oxidoreductase. This enzyme is encoded by the 14-gene *nuo* operon *nuoA-N*. However, two of the *nuo* genes that typically encode for the NADH dehydrogenase module, *nuoE* and *nuoF*, are missing and are replaced by two novel genes *Cj1575c* and *Cj1574c*, respectively. These genes share no homology at all to the *nuoE* and *nuoF* genes of other bacteria, and this provides evidence that NADH is not the electron donor to the complex I in *C. jejuni*. This study was conducted to characterize the complex I of *C. jejuni* and identify the electron donor to this enzyme. Twelve of the 14 *nuo* genes can be disrupted either via an insertion or deletion mutation. However, *Cj1575c* and *Cj1574c* are essential genes and could not be disrupted unless extra copies of these genes were provided at a separate location in the chromosome. Disruption of all 12 *nuo* genes results in strains with similar phenotypes: they will not grow in Mueller Hinton broth unless supplemented with an alternative respiratory substrate such as hydrogen or formate. We show here that flavodoxin,

another essential protein in *C. jejuni*, is the electron donor to complex I, and that flavodoxin accepts electrons from the enzyme 2-oxoglutarate:acceptor oxidoreductase (OOR).

From this data it appears that the machinery required for hydrogen and formate respiration operates separately from complex I. In order to determine the relative importance of these three respiratory pathways in host colonization, mutants in hydrogenase, formate dehydrogenase and a double mutant in these enzymes were constructed. These mutants as well as an OOR mutant (OorB::CM) and several complex I (*nuo*) mutants were used in chicken colonization assays. Individual mutations in hydrogenase or formate dehydrogenase do not display any defectiveness in colonizing chickens. However, a double mutant in these enzymes, OorB::CM and *nuo* mutants are significantly deficient in chicken colonization indicating that these enzymes may play an important role in *in vivo* colonization.

Characterization of the Respiratory Donor Complex I in *Campylobacter jejuni*

by
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DEDICATION

I would like to dedicate this work to my wife Niluka, our son Akein, my parents and my brother Dinesh.

BIOGRAPHY

Dilan Weerakoon was born and raised in Colombo, Sri Lanka. He attended S. Thomas' College from kindergarten (1983) until he completed his Advanced Level (A/L) examination in 1996. While taking a science class in 1992, Dilan developed a strong passion for both science and experimentation and gradually desired to study science and become a scientist. In the spring of 1998, he enrolled in the Institute of Technological Studies (ITS), a private university in Sri Lanka with an affiliation to the University of Houston-Clear Lake, which allowed students to transfer to universities in the U.S.A. In the spring of 2000, Dilan embarked to the U.S.A when he transferred from the ITS to Louisiana State University (LSU) to complete his B.S in Microbiology. In the spring of 2001, Dilan's passion for science and research led him to conduct volunteer research, and in the summer of 2001, he took part in active research when he was selected to participate in the Howard Hughes Medical Institute Summer Research Program. This research experience further enhanced his vision to obtain a postgraduate degree. Dilan graduated from LSU in December 2002, and joined the Department of Microbiology at North Carolina State University in 2003 where he pursued and completed his Ph.D. under the direction of Dr. Jonathan Olson in September 2008.

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CHAPTER 1

Literature Review

1.1 *Campylobacter jejuni* as a world wide pathogen

Campylobacters are epsilon proteobacteria that belong to the order Campylobacteriales (114). They are gram-negative spiral shaped organisms that currently compromise 18 species, 6 sub-species and 2 biovars (38), and have been known to be causative agents of disease since the late 1880's (70). The majority of the reported infections are caused by *Campylobacter jejuni* and *Campylobacter coli* (70) with *C. jejuni* known to be the cause of approximately 90% of these cases and *C. coli* being responsible for 5-10% of the additional identified cases (29, 94).

C. jejuni is a foodborne pathogen that is responsible for being the primary agent of human bacterial gastroenteritis, campylobacteriosis, in the world (3, 26, 52). It is estimated that 2-2.4 million cases of campylobacteriosis occurs in the United States alone each year (52, 84), accounting for 17.3% of all foodborne hospitalizations and 5.5% of food related deaths (52). In England and Wales, campylobacteriosis is the most commonly reported bacterial gastrointestinal disease (94) with an estimated 360,000 cases occurring in the year 2000 accounting for 27% of all food borne diseases (1). Studies conducted in the Netherlands revealed that *Campylobacter* species were the most common pathogens isolated from gastroenteritis patients who were between 5-14 (16% of patients) years of age and 15-59 (8-15% of patients) years of age (21). In New Zealand, campylobacteriosis has been identified as the most frequently notified communicable disease, and since 1992 *Campylobacter* infections has accounted for almost two thirds of all notified disease reports (24). In Japan, reports from 2003 indicate that 490 of the 1109 bacterial food borne outbreaks (44.2%) were caused by *C. jejuni* (83).

The incidence of campylobacteriosis in developing countries is much higher in comparison to developed countries with 40% children younger than five years old being infected in Thailand (95) and Mexico (11) in 1988. In other developing countries such as Nigeria (13), Tanzania (50), Bangladesh (31), China (23), Egypt (80) and Thailand (96), *Campylobacter jejuni* is the most commonly isolated bacterial pathogen from less than 2 year old children suffering from diarrhea. In contrast to developed countries where it is common among both adults and children, campylobacteriosis is less important among adult populations in developing countries (14). Despite these reports, it is likely that the number of campylobacteriosis cases reported are underestimated, since most of the cases go underreported. Data from population studies in England and Wales (109) and in The Netherlands (22) suggest that for every reported case of campylobacteriosis, between 7.6 and 19 cases were not reported (58). Also, the fact that most developing countries lack a national surveillance program for campylobacteriosis is indicative that the overall disease burden is much higher worldwide than what is reported (14).

1.2 Disease manifestation

Campylobacteriosis is a foodborne disease that causes an acute infection of the ileum, colon and rectum in humans (7). The disease is traditionally characterized by diarrhea (the main symptom) which can often vary between limited to voluminous stools that may either be watery or bloody (57). Usually a severe inflammatory diarrhea is the most common clinical symptom observed in industrialized countries while a milder non-inflammatory

diarrhea is present in developing countries (45). Other characteristic symptoms include acute abdominal pain, fever, malaise and myalgia (9).

The main virulence mechanisms utilized by *C. jejuni* include motility, adhesion, invasion, toxin production and subversion of host cell processes (19), of which the central factor is attributed to the production of the cytolethal distending toxin (CDT). In eukaryotic cells, CDT is known to cause cell cycle arrest, distention of the cytoplasm, chromatic fragmentation and ultimately the demise of the cell (46). *C. jejuni* infection is self limiting (14), and the disease is typically resolved within a week, although symptoms can last for up to two weeks (114). Antibiotics are administered usually only in cases where the host is immunocompromised or have an extra intestinal infection (14). Treatment therefore consists of administration of water and electrolytes to compensate for the loss of fluids in order to prevent dehydration (19).

Complications can still however occur, and the most important of which is the autoimmune disease Guillain-Barré syndrome (GBS) (2). GBS is characterized by limb weakness and loss of tendon reflexes (116), and is now recognized as the most frequent cause of acute flaccid paralysis in the world (115). Molecular mimicry between *C. jejuni* lipo-oligosaccharides (LOS) and human gangliosides is what actually causes GBS. Since *C. jejuni* synthesizes ganglioside like LOS structures, in an actual *C. jejuni* infection antibodies made against these structures of *C. jejuni* can ultimately act against the host thus giving rise to GBS (115).

Each year, GBS is known to affect 1-2 persons per 100, 000 population in the U.S (2) with *C. jejuni* being the frequent antecedent pathogen (115). Furthermore, 20-40% out of an

estimated 2628 to 9575 patients each year that are diagnosed with GBS in the US are initiated by an infection by *Campylobacter*. Two percent of GBS patients are known to die from the illness while another 65% report neurological problems (10). The loss of human productiveness at the work place and related medical costs due to *C. jejuni* infection can amount to billions of dollars. In 1995, the estimated total annual cost for campylobacter related GBS and campylobacteriosis in the U.S ranged between 1.3-6.2 billion dollars (10). Thus *Campylobacter* infection and the resulting campylobacteriosis and post complication expenses establishes it as a billion dollar disease making *Campylobacter* an important pathogen for research study.

1.3 *C. jejuni* sources, infection and transmission

Campylobacters have been isolated from the feces of an extensive range of domestic, wild and laboratory animals, thus indicating their broad presence (63). Investigation for *Campylobacter* species in Canada geese, migratory ducks and sandhill cranes resulted in the isolation of *C. jejuni* in all isolates. Although Canada geese displayed a low incidence (5%), a high carriage rate of *C. jejuni* was visible in both migratory ducks (81% incidence) and sandhill cranes (73% incidence) suggesting that due to their migratory habits wild birds may in fact play a major role in the environmental dispersion of this organism (68). Waterways commonly frequented by these birds may become contaminated and given the fact that some *Campylobacters* can survive in environmental water samples for months (82), open waters may prove to be a reservoir for other wild and domestic animals to acquire the organism (19).

Typically, the *Campylobacters* found in the gastrointestinal tract of poultry, swine, sheep, and cattle and do not cause the disease symptoms found in humans (38, 41). The desired ecological niche of *C. jejuni* however appears to be the intestines of avians that include wild birds, chickens, turkeys, quails, ducks, domestic pigeons, crows and ostriches (6, 20, 42, 51, 56, 67, 98, 104). The fact that these organisms are able to grow at 42 °C, the temperature that reflects the avian gut, highlights their adaptation to this particular niche (19). Being a commensal organism in avians, *C. jejuni* does not cause any disease in these birds with the exception of ostriches (79, 92, 102). Humans on the other hand, when ingested with *Campylobacter*, suffer from campylobacteriosis (16).

This disease is most frequently acquired by the consumption of improperly cooked chicken (26, 54, 114), and since a low dose of 500 organisms of *Campylobacter* is just enough to cause an illness, campylobacteriosis is a disease that can easily be contracted (26, 43, 81). Also, the fact that the majority of the retail poultry sold at supermarkets around the world are contaminated with *C. jejuni* is yet another reason why this disease is commonly contracted via the consumption of inadequately cooked chicken. For example, in Nairobi, Kenya it was found out that 77% of *C. jejuni* were isolated from retail poultry meat (66), while studies conducted in the greater Washington D.C area in the U.S revealed that 70.7% of chicken samples sold at 59 stores of four supermarket chains were contaminated with *Campylobacter* (117). In the UK, it is estimated that 80% of the raw chickens sold are contaminated with *Campylobacters* while a high level of these organisms can be found on carcasses that amounts to several thousand per cm^2 of chicken skin (18). Other factors that

contribute to campylobacteriosis includes the consumption of raw milk or untreated water, improper handling of raw chicken carcasses and via animal contact (35, 54, 88).

The contamination of poultry with *Campylobacter* usually occurs in the farm as well as in poultry processing facilities (43). The external environment too can serve as an important source of *Campylobacters* since these organisms are common contaminants in a farm environment (38). Usually, when a single chicken in a flock is contaminated with *Campylobacter*, the dissemination of the organism among hatch mates is rapid. Given the fact that chickens are coprophagic, the fecal shedding of *Campylobacters* among chickens is an important way of the dispersion of this bacterium (64). *C. jejuni* is know to colonize the lower gastro intestinal tract of chickens and are primarily found to dominate in the cecum (6), and once colonization has been achieved, *Campylobacters* can reach elevated numbers in the cecum, with numbers as high as 10^9 CFU in birds infected experimentally (105). Poultry processing too plays an important role in the contamination of poultry with *Campylobacters*. During poultry processing (defeathering and evisceration) the intestinal contents are subjected to being cut and ruptured and hence contaminate the carcass due to intestinal leakage (40). As a result, *Campylobacters* tend to be present in poultry parts through out the processing plant level and retail level (41). The fact that *Campylobacters* are still present in commercially available chicken meat (66, 117) is indicative that they can survive the harsh treatments faced during poultry processing thus making *C. jejuni* an important bacterium to study in order to ensure of its eventual removal from chickens.

1.4 Energy metabolism in *C. jejuni*

C. jejuni is unable to ferment carbohydrates, and neither does it have a complete glycolytic pathway (71, 101). It garners its energy solely by utilizing the electron transport chain and oxidative phosphorylation. A fundamental electron transport chain typically consists of an electron donor molecule, an electron donor enzyme that garners the electrons from the donor and feeds them on to a quinone pool, and into the electron transport chain and finally handing them to a terminal reductase, that will use the electrons to reduce an electron acceptor (44).

A number of electron donor enzymes have been identified that contribute electrons to the menaquinone pool in *C. jejuni* (Figure 1), which seems to be the only isoprenoid quinone present in this organism (12, 15). A number of donor enzymes present in *C. jejuni* include the integral membrane bound oxidoreductases that comprises a complex I type oxidoreductase, a hydrogenase, a formate dehydrogenase, and a succinate dehydrogenase while the oxidoreductases associated peripherally include a malate:quinone oxidoreductase, a proline dehydrogenase and a lactate dehydrogenase (44, 71). *C.jejuni* has also been shown to utilize sulfite as an electron donor via a monoheme cytochrome *c* and molybdopterin oxidoreductase (encoded by the genes *Cj0004c* and *Cj0005c* respectively) located in the periplasm, that displays homology to the sulfite:cytochrome *c* oxidoreductase of *Starkeya novella* (61).

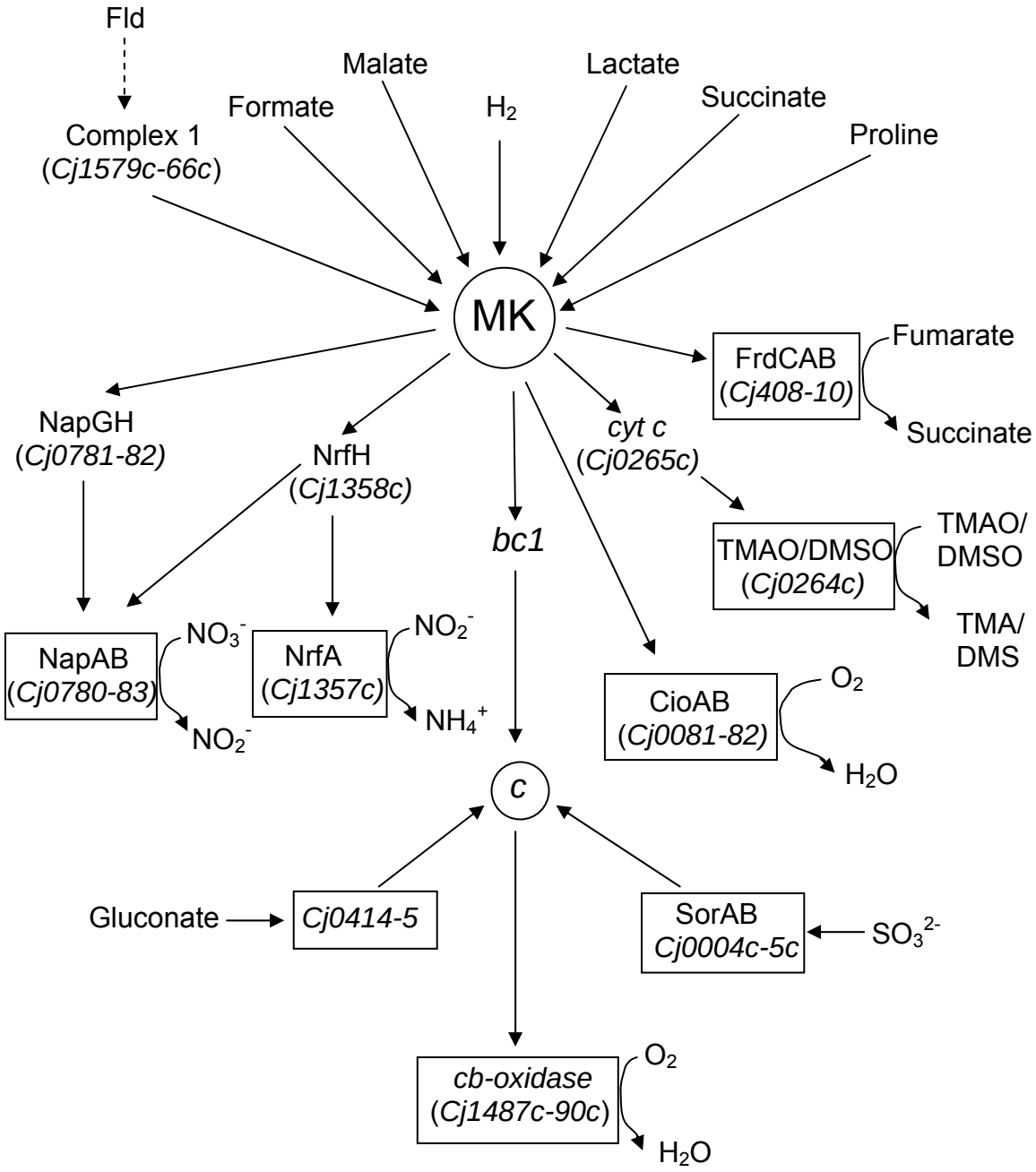

Figure 1: Schematic outline of the major electron transport pathways in the respiratory chain of *C. jejuni*.
MK – Menaquinone pool. Figure was adapted from (44).

Additionally gluconate has also been shown to be an electron donor in *C. jejuni* (69), and the genes that encode for gluconate dehydrogenase have been identified as *Cj0414* and *Cj0415* in *C. jejuni* strain 81-176 (33, 69) and these genes have been shown to be present in all *C. jejuni* strains (69).

The terminal reductases of *C. jejuni* include two oxidases, a cyanide-insensitive, low affinity quinol oxidase (CioAB) (39), and a *cb*-type cytochrome *c* oxidase that uses oxygen as a terminal electron acceptor, and several other alternative reductases that uses alternative electron acceptors to oxygen which include, a nitrate reductase that reduces nitrate to nitrite, a nitrite reductase that reduces nitrite to NH_4^+, a trimethylamine-N-oxide (TMAO)/dimethyl sulfoxide (DMSO) reductase that reduces TMAO/DMSO to trimethyl amine (TMA)/ dimethyl sulfide (DMS) respectively (44, 71, 108), and a fumarate reductase that is predicted to reduce fumarate to succinate (44). However it has been proposed that fumarate reductase primarily acts as a succinate dehydrogenase that converts succinate to fumarate and no respiratory activity could be detected (Weingarten personal communication).

When oxygen is the terminal electron acceptor, electrons extracted by the menaquinone pool will be passed either directly to the CioAB quinol oxidase or via the cytochrome bc_1 complex, which will then feed the electrons to a periplasmic cytochrome *c*, and finally to the high affinity *cb*-type cytochrome *c* oxidase (44). Studies conducted by Jackson et. al. reveal that in the presence of oxygen the preferred pathway appears to be via the high affinity *cb*-type cytochrome *c* in comparison to the low affinity CioAB quinol oxidase (39). This is in accordance to studies conducted by Weingarten et. al. who show that

despite *C. jejuni's* ability to use a wide range of electron acceptors, oxygen proves to be the most important respiratory acceptor in the chicken cecum (108).

However, in the presence of other alternative electron acceptors such as fumarate, nitrate, nitrite, and TMAO/DMSO, *C. jejuni* can utilize the alternative reductases fumarate reductase, nitrate reductase, nitrite reductase and TMAO/DMSO reductase respectively (77, 89, 108). With the exception of sulfite and gluconate, electrons liberated from other electron donors follow the pathway through the menaquinone in the inner membrane, to the cytochrome bc_1 complex, to cytochrome *c* and finally to the two terminal oxidases. The electrons released from sulfite have been shown to bypass both the menaquinone and cytochrome bc_1 complex and enter the respiratory chain at the level of cytochrome *c* (61), while studies suggest that the electrons generated from gluconate oxidation are likely to enter the electron transport chain at the cytochrome *c* level (69).

It thus appears that *C. jejuni* possesses a highly branched and complex electron transport chain that will certainly be beneficial for growth and survival to an organism that thrives in a fermentation rich, oxygen-limiting environment such as the chicken cecum.

1.5 Amino acid utilization in *C. jejuni*

The fact that *C. jejuni* has an incomplete glycolytic pathway and furthermore unable to ferment hexose sugars (71), it is highly likely that amino acids play key roles as carbon and energy sources since it possesses mechanisms to transport in and catabolize amino acids while containing only a few carbohydrate transport systems (44, 71). The fact that *C. jejuni* is routinely grown microaerobically in Mueller Hinton (MH) broth (an amino acid based

medium) is further evidence that amino acids play a crucial role as *C. jejuni's* carbon sources (30). However, experimental studies indicate that in rich complex media such as MH broth, the amino acids highly utilized by *C. jejuni* were serine, aspartate, glutamate and proline although both asparagine and glutamine were also shown to be utilized to a certain extent despite the fact their initial concentrations were low (30). This is in agreement with studies conducted by Leach et al. who showed that the most heavily consumed amino acids during the growth of *C. jejuni* in both complex (rich) and defined (minimal) media were serine, aspartate, glutamate and proline (47). This is certainly intriguing for it coincides with the fact that these amino acids are the most commonly found in chicken feces (72) and provides further evidence why they are important for *C. jejuni*.

A closer look at the genome sequence of *C. jejuni* strain NCTC 11168 reveals several amino acid transport mechanisms. The PEB1a protein of *C. jejuni*, encoded by *Cj0921c* (71), has been shown to have homology to the periplasmic binding proteins that are associated with ABC transporters (73), By using purified recombinant PEB1a protein, and screening this protein with all 20 amino acids by fluorescence spectroscopy, studies conducted by Leon-Kempis et al., revealed that both L-aspartate and L-glutamate bound PEB1a, while PEB1a displayed a low affinity to L-asparagine and L-glutamine and no affinity at all to other amino acids (49). Furthermore, a mutant constructed in *Cj0921c* of *C. jejuni* strain NCTC 11168 (71), was totally defective in the transportation of glutamate while aspartate transport was reduced ~20 fold in comparison to the wild type strain. In addition, this mutant strain was unable to grow in minimal media when grown with either glutamate or aspartate as a carbon source under microaerobic conditions (49). Therefore, it is apparent that in *C.*

jejuni the preferred pathway for both aspartate and glutamate transportation under microaerobic conditions is via PEB1a. Furthermore, a mutant in the *peb1A* gene constructed in the sequenced *C. jejuni* strain 81-176 (33), showed 50- to 100-fold less adherence and to and 15-fold less invasion of epithelial cells in culture, while mouse colonization studies indicated that both the intestinal colonization rate and duration was significantly lower and shorter respectively in comparison with the wild-type strain (74). Apparently, the PEB1a protein is also an important factor in both virulence and colonization in *C. jejuni*. Consequently, it also strengthens the fact that both aspartate and glutamate may prove to play a crucial role in this organism. Even though western blot analysis reveals the presence of the PEB1a protein in all of the *C. jejuni* and *C. coli* strains, it is absent in *C. fetus*, *C. lari* and *Helicobacter pylori* (75). Although under microaerobic conditions the major channel of transport of aspartate is via the PEB1a transporter (49), under oxygen limiting conditions aspartate has also been shown to be transported via the two anaerobic C4-dicarboxylate transporters DcuA and DcuB encoded by *Cj0088* and *Cj0671* respectively in addition to PEB1a (30). Hence it seems that aspartate is indeed an important amino acid to *C. jejuni* since it has the ability to transport aspartate unhindered by oxygen limitations.

C. jejuni is also known to transport cysteine. Fluorescence titration experiments reveal that the protein *Cj0982* (*CjaA*) is a component of an ABC type transporter system that has been shown to bind cysteine with high affinity. Additionally, the crystal structure of *Cj0982* revealed a two-domain protein that contained a cysteine ligand in the enclosed cavity between the two domains further implying that this protein is part of a cysteine transport

system. *Cj0982* is also known to be major immunogenic protein (59), and hence cysteine transport may play an important role in the *C. jejuni's* physiology.

C. jejuni also discloses genes that are involved in the transport and catabolism of serine since it encodes homologs of the *E. coli* serine transporter (SdaC) and serine dehydratase (SdaA) encoded by the two-gene operon *Cj1624c* and *Cj1625c* respectively (44, 71). However, evidence implies that serine transport may be mediated by two ways that includes a low affinity serine transport via SdaC, and a high affinity transporter for residual transport of serine/threonine in an *sdaC* mutant background (100).

Proline too plays an important role as a carbon source for *C. jejuni* since it is an amino acid that is significantly consumed (30, 47). *C. jejuni's* ability to take in proline is via a proline symporter encoded by *putP* (*Cj1502c*) (71). Once transported inside the cell, proline can be broken down into glutamate via *putA* (*Cj1503c*), a putative proline dehydrogenase with Δ^1-pyrroline-5-carboxylate as an intermediate. (30, 71). The electrons liberated from this reaction may be transferred to the electron transport chain in the level of menaquinone, thus contributing for energy accumulation in *C. jejuni* (44).

Unsurprisingly, perusing the genome sequence of *C. jejuni* strain NCTC 11168 reveals the presence of a limited range of genes whose gene products are able to completely catabolize only a small set of amino acids that include aspartate, serine, glutamate, proline, asparagine and glutamine (44, 71). Typically, depending on the chemistry of each amino acid side chain, there appears to be four mechanisms to catabolize amino acids. These include, deamination, transamination via an oxo-acid acceptor (for example 2-oxoglutarate or oxaloacetate), β-elimination of ammonia via dehydratases or amino acid:ammonia lyases,

and decarboxylation (44). Apparently, with the exception of decarboxylation, all the three other amino acid catabolistic mechanisms are represented (44, 71).

Asparagine is thought to be deaminated to aspartate via a cytoplasmic L-asparaginase encoded by *Cj0029* (*ansA*), while aspartate is thought to be converted to fumarate via *β*-elimination of ammonia via the enzyme aspartate:ammonia-lyase (aspartase) encoded by *Cj0087* (*aspA*) (44, 71). The latter reaction is important in particular because fumarate can either enter the citric acid cycle or be utilized as a terminal electron acceptor *in vivo* (44).

Serine dehydratase activity (SdaA) has been documented previously in both intact cells and lysates by the formation of pyruvate and ammonia from L-serine, and the high deamination rates of serine observed certainly indicate that serine is indeed an important energy source (53). Also, the fact that the pyruvate generated from this reaction can then be utilized via the enzyme pyruvate:flavodoxin oxidoreductase in the tricarboxylic acid (TCA) cycle elucidates why this amino acid is certainly so well employed (30). Since *C. jejuni* 11168 also possesses the necessary genes (*serABC*) for the biosynthesis of this amino acid from 3-phosphoglycerate (71), indicates its importance as a carbon source. Furthermore, studies conducted by Leach et al. showed that even though the highly assimilated amino acids were serine, aspartate, glutamate and proline in both defined and complex media, serine appeared to be the central carbon and energy source utilized in the defined medium which is probably due to its high initial concentration in the fresh medium. Additionally, in a continuous complex culture medium serine and aspartate appeared to be the most utilized amino acids at the high dilution (growth) rate while glutamate and proline were only metabolized at a lower dilution rate (47). Therefore, it appears that the most sought after

amino acids appear to be serine and aspartate. Although an *sdaA* mutant was shown to not have any growth defect when grown in complex medium in comparison to the wild-type strain, thus suggesting that in nutrient replete conditions deaminating serine may not be of utmost importance to *C. jejuni*, serine however does appear to be an important growth factor *in vivo* since *sdaA* mutants were defective in colonizing chickens (100).

Although an *sdaA* mutant was shown to not have any growth defect when grown in complex media (100), a mutant in *aspA* displayed intense growth impairment when grown in complex media (MH broth) (30). In fact this mutant (*aspA*) was able to use serine although it was defective in utilizing aspartate, glutamate, glutamine and proline from MH broth while also failing to grow on these substrates in defined media. Furthermore, an *aspA*/*sdaA* double mutant that lacked both aspartase and serine dehydratase activity could not grow in MH broth unless supplemented with pyruvate and fumarate (30). Therefore, it seems that both aspartate and serine play a crucial role in *C. jejuni*. Additionally, under oxygen limiting conditions aspartase was shown to be upregulated both by enzymatic activity and proteomic analysis while aspartate also augmented the growth of *C. jejuni* in an *aspA*-dependent lifestyle. It seems that aspartase plays a dual role in addition to the role of aspartate catabolism, since stoichiometric aspartate consumption and release of succinate in *C. jejuni* demonstrate evidence for fumarate respiration. Moreover the fact that an *aspA* mutant constructed in the *C. jejuni* strain 81-176 was defective in colonizing chickens further elucidates the important role that aspartate plays in the survival of this organism in the intestines of chickens (30). These data underlies the key roles that aspartase plays in *C. jejuni* and also stresses the importance that amino acids engage in the survival of this organism.

Glutamate utilization is via a transamination reaction with oxaloacetate to form aspartate and 2-oxoglutarate using the enzyme aspartate aminotransferase encoded by *Cj0762* (*aspB*) (30, 44, 71). 2-Oxoglutarate is an important intermediate in amino acid metabolism of *C. jejuni* since it is a substrate in the TCA cycle, and most importantly plays a pivotal role in the reduction of flavodoxin in the cell (106). An *aspB* mutant was shown to have an intense growth deficiency when grown in MH broth, further highlighting the importance of aspartate and also glutamate in the growth and survival of *C. jejuni* (30).

Glutamate and glutamine metabolism is via a glutamine synthetase encoded by *Cj0699c* (*glnA*) which converts glutamate to glutamine and via a glutamate synthase enocoded by *Cj0007* (*gltB*) and *Cj0009* (*gltD*) also known as the glutamine:2-oxoglutarate amino transferase system which converts glutamine to glutamate. These reactions are important for being the major pathway in the inclusion of nitrogen into amino acids in the cells (30, 71). 2-Oxoglutarate plays the role of primary amino group acceptor in the interconversion between glutamine and glutamate, and is an important intermediate in both amino acid metabolism and central carbon metabolism (44).

In summary, amino acids are in fact important sources of both carbon and energy for *C. jejuni*. They also play a crucial role in the formation of various intermediates that can feed into either the TCA cycle or respiratory chain thus contributing heavily for the survival of this organism. The fact that these substrates are readily available in its preferred ecological niche, the avian cecum (44), certainly is a driving force that helps it thrive in such an environment.

1.6 Central carbon metabolism in *C. jejuni* - Glycolysis and the TCA cycle

In *C. jejuni* most of the intermediates formed during amino acid catabolization can enter either the Embden-Meyerhof pathway (glycolysis) or the TCA cycle. Although genes that encode for a glycolytic pathway as well as a pentose phosphate pathway are present in all of the *C. jejuni* strains sequenced thus far, they do not possess the necessary genes that encode for the important enzymes of the Entner-Doudoroff pathway (44, 71). A closer look at the glycolytic pathway in *C. jejuni* indicates that it is incomplete because it lacks the gene that encodes for 6-phosphofructokinase, which converts fructose-6-phosphate to fructose 1,6-bisphophate (Figure 2). However, it does contain the gene encoding a fructose-1,6-bisphophatase (*Cj0840c*/*fbp*) that carries out the reverse reaction converting fructose-1,6-bisphophate to fructose-6-phosphate (71), indicating that in *C. jejuni* the glycolytic chain operates exclusively in gluconeogenesis (101).

The presence of a pyruvate kinase encoded by *Cj0392c* (*pyk*) is somewhat surprising since it catalyzes the irreversible reaction of phosphoenol pyruvate (PEP) and ADP to pyruvate and ATP in the final reaction in glycolysis. A *pyk* mutant was shown to display only a slight growth defect when grown in complex media and interestingly was able to grow with pyruvate, lactate and malate in defined media. Furthermore, pyruvate kinase was shown to display a high specific activity of >800 nmol/min/mg of protein in cell extracts, was shown to have been activated by fructose 1,6-bisphophate and also displayed other regulatory activities (101), and hence Kelly suggests a catabolic role for the lower part of the glycolytic pathway in *C. jejuni* even though the absence of 6-phosphofructokinase results in dysfunction of the breakdown of glucose (44).

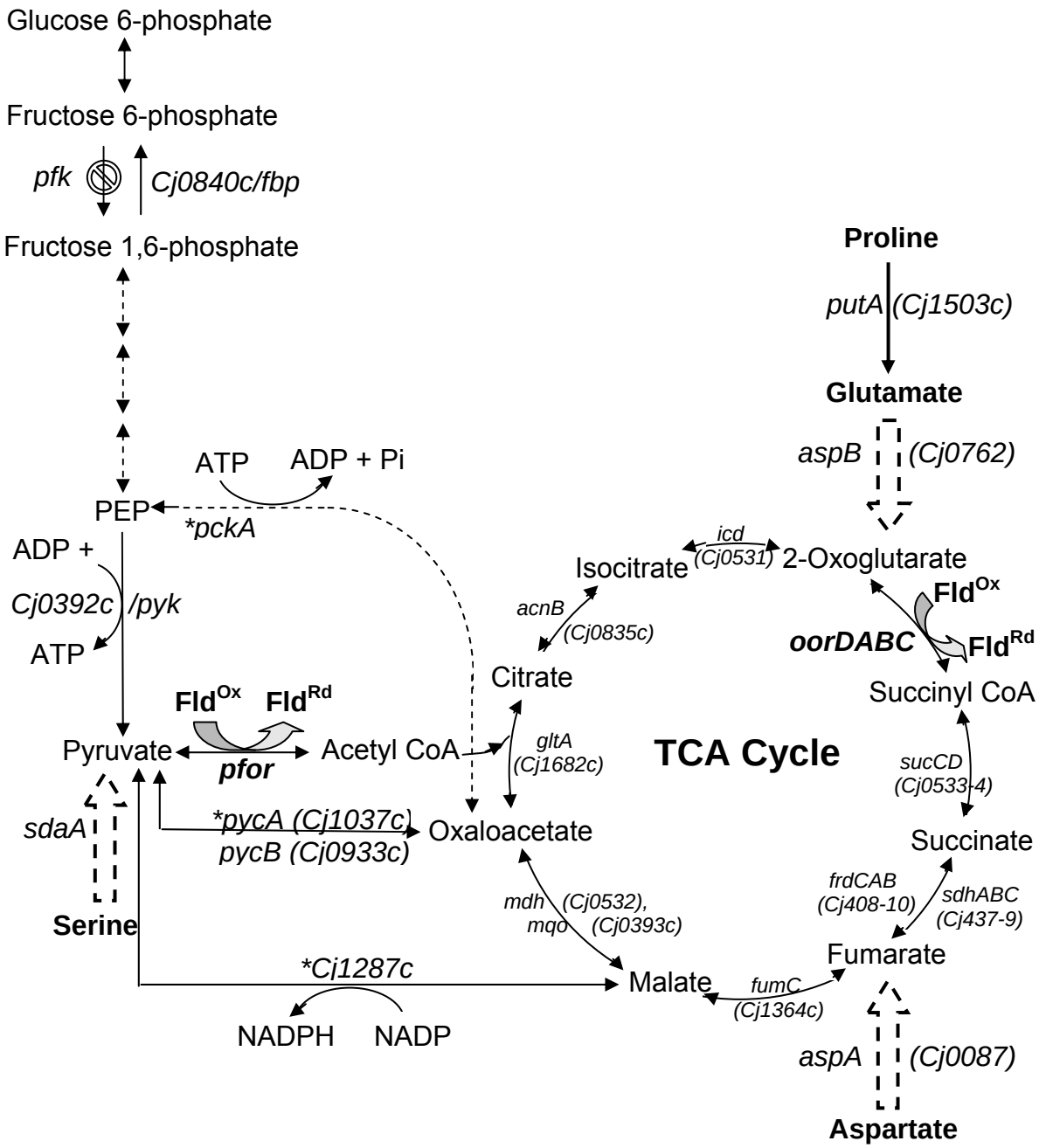

Figure 2: Schematic outline of gluconeogenesis, anapleurotic reactions, TCA cycle and amino acid catabolism in *C. jejuni* NCTC 11168 from available genome sequence data (71) and other studies (44, 101). * - Anapleurotic reactions.

C. jejuni also possesses anapleurotic enzymes that includes a phosphoenolpyruvate carboxykinase (PCK) encoded by *Cj0932c* (*pckA*), a pyruvate carboxylase (PYC) encoded by *Cj1037c* (*pycA*) and *Cj0933c* (*pycB*) and malate oxidoreductase encoded by *Cj1287c* (Figure 2) (71, 101). PCK allows for the ATP dependent decarboxylation of oxaloacetate (OAA) to PEP. This reaction is important for the functioning of gluconeogenesis since it will provide the necessary PEP required for gluconeogenesis to take place. However, the reaction may also be important for the TCA cycle since OAA is required as a substrate for the operation of this cycle. The failure to obtain a mutant in the *pck* gene indicates that it may be an essential gene for the survival of *C. jejuni* (101), thus playing a vital role for both gluconeogenesis and also for the TCA cycle. OAA synthesis is also taken place from pyruvate via the enzyme PYC. A *pycA* mutant was shown to have no growth defect when grown in rich complex media, but was unable to grow in a defined media with either pyruvate or lactate as carbon sources indicating that PYC does play an important anapleurotic role in *C. jejuni* (101). Therefore it appears that pyruvate plays an integral role as an important substrate in this junction.

The decarboxylation of malate to pyruvate with the reduction of NADP to NADPH is carried out by malate oxidoreductase. However, the fact that a mutant in this enzyme showed no growth phenotype in either rich or complex media and the fact that malate oxidoreductase displayed only a low affinity to malate suggests that this enzyme may only play a minor role in *C. jejuni* anapleurosis (101).

Although it possesses an incomplete glycolytic pathway, *C. jejuni* is known to have a complete TCA cycle (Figure 2). In fact all the *C. jejuni* strains sequenced thus far display a

complete oxidative TCA cycle with most of the major enzymes present (44). However, the genes that encode for pyruvate dehydrogenase and α–ketoglutarate (2-oxoglutarate) dehydrogenase are absent in *C. jejuni* and are replaced by pyruvate ferredoxin (flavodoxin) oxidoreductase (PFOR) and 2-oxoglutarate oxidoreductase (OOR) respectively (71). PFORs are iron sulfur proteins involved in the coenzyme A (CoA) dependent oxidative decarboxylation of pyruvate to form acetyl-CoA (36, 76), while OOR enzymes are involved in the decarboxylation of 2-oxoglutarate in the presence of CoA to form succinyl-CoA and CO_2 (37). Typically, the pyruvate dehydrogenase and α–ketoglutarate dehydrogenase enzymes are usually found in aerobes and they work in conjuction with NAD^+ that acts as the electron acceptor for the reducing equivalents generated by these enzymes (44). However, for both PFOR and OOR in *C. jejuni*, NAD does not seem to be the electron acceptor, and the reducing electrons are in fact transferred to low potential electron acceptors such as ferredoxin and flavodoxin since the genome sequence predicts for an oxidoreductase type enzyme (71). In accordance studies indicate that in *C. jejuni* the candidate electron acceptor from PFOR (91) and OOR (106) is indeed a flavodoxin. However, in *Helicobacter pylori*, a fellow ε-proteobacterium of *C. jejuni*, it was shown that while flavodoxin was indeed the electron acceptor from PFOR (36), it was not the case for OOR and the electron acceptor is yet to be identified (37).

These enzymes are usually sensitive to oxygen and are inactivated by the presence of molecular oxygen. In fact in *Helicobacter pylori*, both PFOR (36) and OOR (37) were shown to be sensitive to oxygen and in *C. jejuni* it may be the case as well (44). PFOR is encoded by *Cj1476 c* in *C. jejuni* strain NCTC 11168, and codes for a single protein subunit while OOR

is composed of 4 subunits encoded by the genes *oorDABC* (*Cj0535*-*Cj0538*) (71). PFOR is in fact an important enzyme because it catalyzes the reaction of pyruvate to acetyl Co-A, thus opening the gateway of carbon influx into the TCA cycle. Mutating *Cj1476c* has been possible, but the mutant displays a very strong phenotype and grows poorly (Olson lab unpublished data). This is not surprising since the major pathway to central carbon metabolism has been disrupted in such a mutant. An OOR mutant, termed OorB::CM has also been isolated (106). Although the oxidative TCA cycle has been disrupted in this mutant, the mutation does not appear to be lethal since *C. jejuni* possesses reductive TCA cycle enzymes such as fumarate reductase that will allow for the synthesis of important biosynthetic precursors.

The fact that both PFOR and OOR are involved in the reduction of flavodoxin is important in the physiology of *C. jejuni* since flavodoxin has been shown to be an essential gene in this bacterium. Furthermore, it is interesting to see how these reduced equivalents are reoxidized to serve as substrates to these enzymes and how the liberated electrons are used by the bacterium to garner energy (44). The answer actually lies within the complex I of *C. jejuni* and studies conducted in our laboratory indicate that flavodoxin is the electron donor to this enzyme (106).

1.7 Complex I

Complex I (also known as the NADH ubiquinone oxidoreductase) is the first enzyme in mitochondrial and bacterial respiratory chains (86). In addition to bacteria and the mitochondria of eukaryotes, complex I is also known to be widely found in archaea as well and hence found in all the three domains of life (28). This complex is composed of multi subunits and catalyzes the transfer of electrons from NADH to the quinone pool coupled to the translocation of protons across a membrane thus contributing to the proton motive force required for energy (adenosine triphosphate/ATP) synthesis (27).

The genes that encode for complex I in most bacteria are clustered in a conserved order (90), that consist of 14 different genes (*A*-*N* or *1*-*14*) designated either as *nuo* (*N*ADH:*u*biquinone *o*xidoreductase) (107) or *nqo* (*N*ADH:*q*uinone *o*xidoreductase) (113) (Figure 3).

Complex I is best characterized in *Escherichia coli* (8, 48) and *Paracoccus denitrificans* (110), and a model that represents the subunit structure and function is available. In *E. coli*, the protein subunits of complex I are designated as NuoA-N (48), and the genes that encode NuoC and NuoD are fused to form a single subunit protein NuoCD (8), while in *P. denitrificans* they are designated as Nqo1-14 and the genes *nuoC* (*nqo5*) and *nuoD* (*nqo4*) are not fused (93, 112).

Complex I has a characteristic L-shaped structure that consists of a hydrophilic peripheral arm that protrudes into the cytoplasm while the hydrophobic membrane arm is embedded in the lipid bilayer of the membrane (Figure 4) (27, 85, 111). The peripheral arm consists of the subunits NuoB to NuoG and NuoI, while the remaining seven subunits NuoA,

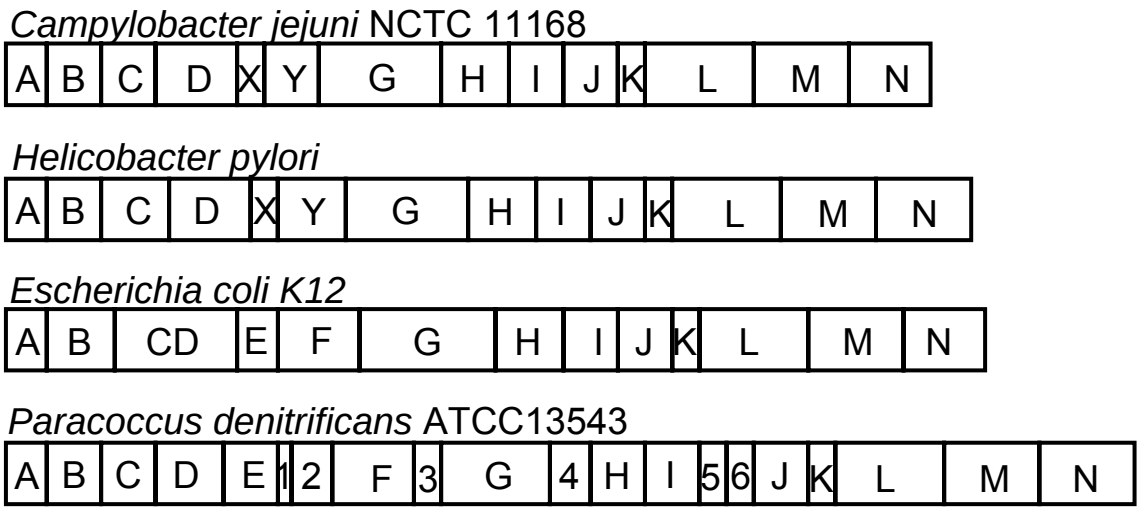

Figure 3: Organization of *nuo* genes (*A*-*N*) in *C. jejuni* NCTC 11168, *H. pylori* 22695, *E. coli* K12 and *P. denitrificans* ATCC13543. *X* and *Y* represent *C. jejuni* and *H. pylori* genes that correspond to *nuoE* and *nuoF*. Genes *1-6* of *P. denitrificans* ATCC13543 correspond to Pden_ 2245, Pden_ 2244, Pden_ 2242, Pden_ 2240, Pden_ 2237, Pden_ 2236.
Figure was adapted from (90)

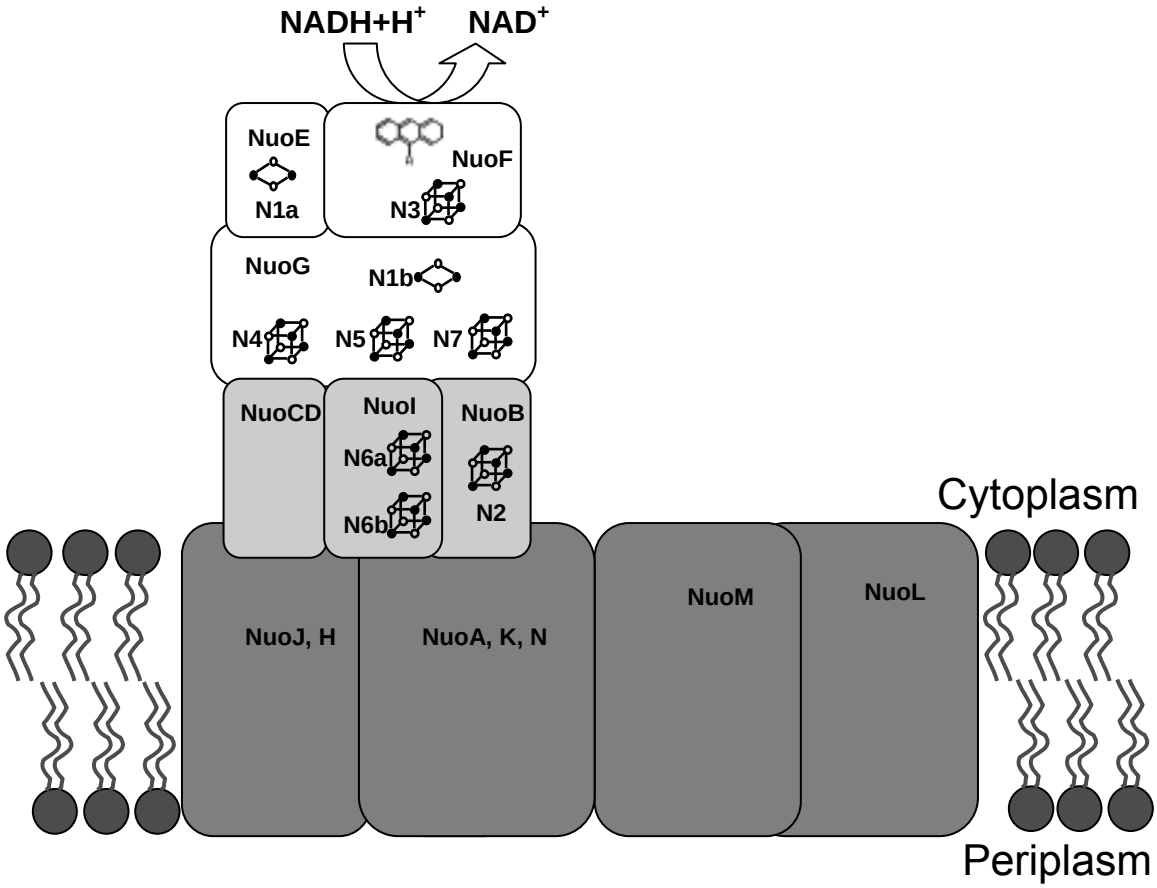

Figure 4: Schematic outline of complex I of *E. coli*. Iron-Sulfur [Fe-S] clusters are denoted N1-N7. The binuclear [Fe-S] cluster N1a is present in NuoE, while FMN and tetranuclear cluster N3 is present in NuoF. The binuclear [Fe-S] cluster N1b and tetranuclear [Fe-S] clusters N4, N5 and N7 are all present in NuoG. NuoB contains the tetranuclear [Fe-S] cluster N2 while NuoI contains the tetranuclear [Fe-S] clusters N6a and N6b (65, 78, 99). The NADH dehydrogenase fragment is shown in white, while the connecting fragment and membrane fragment are shown in light grey and dark grey respectively (48). Figure was adapted from (65).

NuoH, and NuoJ to NuoN consists of the membrane arm. All the known redox cofactors are found in the peripheral subunits, which include one flavin mononucleotide (FMN) and nine iron-sulfur [Fe-S] clusters (Figure 4) (65, 78, 86, 99). Hence all electron transfer should be via this domain (5). *In E.coli*, by using salts and raising the pH, this complex was cleaved into three fragments, that include a NADH dehydrogenase fragment, a connecting fragment, and a membrane fragment (48).

The soluble NADH dehydrogenase fragment includes NuoE, F and G and contains a NADH binding site, a FMN and six-iron-sulfur [Fe-S] clusters and is in fact the electron input part of the complex (8, 48, 99). NuoF contains the NADH binding site, the primary electron acceptor FMN and the tetra nuclear iron-sulfur cluster N3, while NuoE contains the binuclear iron-sulfur cluster N1a and NuoG contains the binuclear iron-sulfur cluster N1b and tetra nuclear iron sulfur clusters N4, N5 and N7 (48, 65, 78, 86, 99, 111). The connecting fragment consists of NuoB, C, D and I and contains three-iron-sulfur [Fe-S] clusters and connects the NADH dehydrogenase fragment with the membrane fragment (8, 48, 99). NuoB contains the tetra nuclear iron sulfur cluster N2, and NuoI contains the tetra nuclear iron sulfur clusters N6a and N6b while NuoC and NuoD does not contain any iron sulfur clusters (Figure 4) (48, 86, 99, 111).

The membrane fragment consists of NuoA, NuoH, and NuoJ to NuoN (8, 48) and are predicted to fold throughout the membrane into α–helices (5). Studies have yet to confirm the function of these seven subunits, however they are probably involved in the translocation of protons and the reduction of quinone (34, 87)

1.8 Complex I of *C. jejuni* and other *Epsilonproteobacteria*

Similar to the model systems of *E. coli* and *P. denitrificans*, the complex I genes of *C. jejuni* are organized in the 14 gene *nuo* operon (Figure 3). The fact that *C. jejuni* had a complex I was somewhat unexpected since previous studies had showed that NADH was a poor respiratory electron donor (32). Interestingly however, the genes that encode for the NADH dehydrogenase, *nuoE* and *nuoF*, are absent in *C. jejuni* and are replaced with two genes (*Cj1575c* and *Cj1574c*) of unknown function (71). With the exception of other ε-proteobacteria, these genes do not show homology to the *nuoE* and *nuoF* genes of other bacteria. However, like in *C. jejuni* all *Epsilonproteobacteria* do contain a *nuo* operon, but they all lack both the *nuoE* and *nuoF* genes with the exception of *Arcobacter butzleri* (55) which has a *nuoE* and *NuoF* gene (Figure 5). The loss of these genes however have been compensated either by the replacement of *Cj1575c* and *Cj1574c* homologs as in *Campylobacter* and *Helicobacter*, by deletion (including nuoG) with no replacement as in *C. mediatlanticus TB-2*, and one of the *Sulfurovum NBC37-1* operons, or by duplication of *nuoG* and the recruitment of a *Cj1575c* homolog that includes either *gltD* or *fdhB* as in *Wolinella succinogenes*, *Nitratiruptor SB155-2*, *Thiomicrospira denitrificans* ATCC 33889, and one of the *Sulfurovum NBC37-1* operons (Figure 5).

Thus it is apparent that NADH is not the donor to complex I of *C. jejuni*. However, the other 12 subunits of *C. jejuni* contain sequence similarities to complex I subunits of other bacteria that includes *E. coli* (48) and *P. denitrificans* (110) (Table 1).

The electron donor to the complex I of *C. jejuni* is yet to be identified. Previously, Finel had suggested that the proteins HP1264 and H1265 of *H. pylori*, which are homologs to

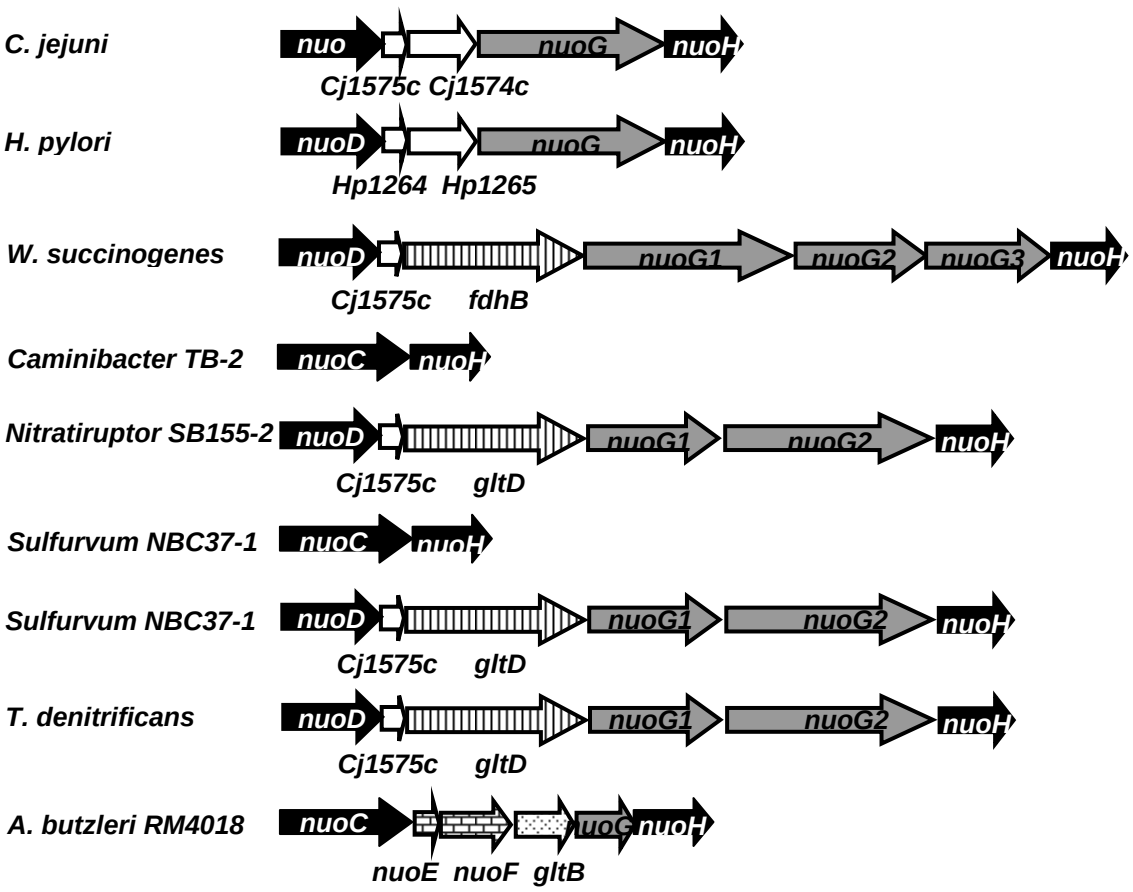

Figure 5: Genetic organization of the regions between *nuoD* and *nuoH* from various *Epsilonproteobacteria*. Each bacterium also contains *nuoA* to *nuoC* and *nuoI* to *nuoN*. References for each genome sequence are as follows: *C. jejuni* (71). *H. pylori* (97), *W. succinogenes* (4), *Caminibacter mediatlanticus TB-2* (103), *Nitratiruptor SB155-2* and the two operons from *Sulfurovum NBC37-1* (62), *Thiomicrospiria denitrificans ATCC 33889* (17), *Arcobacter butzleri RM4018* (55).

Table 1 Homologies of *C. jejuni* Nuo subunits to the *Paracoccus denitrificans* and *Escherichia coli* complex 1 subunits

C. jejuni	*Paracoccus denitrificans*			*Escherichia coli*		
	Subunit	% Identity	% Similarity	Subunit	% Identity	% Similarity
NuoA	NQO7	43	63	NuoA	28	52
NuoB	NQO6	52	73	NuoB	52	75
NuoC	NQO5	39	59	NuoCD	40	61
NuoD	NQO4	43	63	NuoCD	38	56
CJ1575		None	None		None	None
CJ1574		None	None		None	None
NuoG	NQO3	23	39	NuoG	22	38
NuoH	NQO8	32	52	NuoH	38	58
NuoI	NQO9	40	54	NuoI	35	47
NuoJ	NQO10	41	74	NuoJ	29	50
NuoK	NQO11	38	68	NuoK	39	66
NuoL	NQO12	45	62	NuoL	44	66
NuoM	NQO13	33	56	NuoM	33	56
NuoN	NQO14	30	50	NuoN	32	52

CJ1575 and CJ1574 in *C. jejuni*, provide a docking site to a protein that may feed its electrons directly to NuoG (Nqo3) (25), while Myers and Kelly suggested that this protein may in fact be a ferredoxin or flavodoxin (60). Importantly, studies conducted in our lab indicate that flavodoxin is probably the candidate donor enzyme to the complex I of *C. jejuni* (106). This may also help answer how reduced flavodoxin generated in the TCA cycle via the enzymes PFOR (91) and OOR (106) get reoxidized to form oxidized flavodoxin which will then serve as substrates for these enzymes.

The study of the physiology of *C. jejuni* will prove of importance for the identification of essential genes that could serve as probable drug targets for the future. Consequently, it may also help us study the behavior of this pathogen in its natural host such as the chicken and hence may eventually help in the elimination of this pathogen from the food supply.

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CHAPTER 2

The *Campylobacter jejuni* NADH:ubiquinone oxidoreductase (complex I) utilizes flavodoxin rather than NADH

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ABSTRACT

Campylobacter jejuni encodes 12 of the 14 subunits that make up the respiratory enzyme NADH ubiquinone oxidoreductase (also called complex I). The two *nuo* genes not present in *C. jejuni* encode the NADH dehydrogenase, and in their place in the operon are the novel genes designated as *Cj1575c* and *Cj1574c*. A series of mutants was generated in which each of the12 *nuo* genes (homologues to known complex I subunits) were disrupted or deleted. Each of the *nuo* mutants will not grow in amino acid-based media unless supplemented with an alternative respiratory substrate such as formate. Unlike the *nuo* genes, *Cj1574c* is an essential gene and could not be disrupted unless an intact copy of the gene was provided at an unrelated site on the chromosome. A *nuo* deletion mutant can efficiently respire formate but is deficient in α-ketoglutarate respiratory activity when compared to WT. In *C. jejuni,* α-ketoglutarate respiration is mediated by the enzyme 2-oxoglutarate:acceptor oxidoreductase, mutagenesis of this enzyme abolishes α-ketoglutarate-dependent O_2 uptake and fails to reduce the electron transport chain. The electron acceptor for 2-oxoglutarate:acceptor oxidoreductase was determined to be flavodoxin, which was also determined to be an essential protein in *C. jejuni*. A model is presented in which CJ1574 mediates electron flow into the respiratory transport chain from reduced flavodoxin and through complex I.

2.1 Introduction

Campylobacter jejuni is the leading cause of human bacterial gastroenteritis, campylobacteriosis, in the world (1, 12, 22). Humans are infected by ingestion of contaminated food, typically poultry, where *C. jejuni* is a frequent resident (6, 12). The physiology of *C. jejuni* has adapted to take advantage of life in the lower avian cecum, were *C. jejuni* predominates (3). Anaerobic fermentation appears to be the dominant lifestyle of the cecum (21), and *C. jejuni* utilizes fermentation byproducts such as small organic acids as both carbon and energy sources (19, 23, 24, 36, 40). *C. jejuni* itself is non-fermentative, and uses oxidative phosphorylation for all its energy demands. The genome sequence of *C. jejuni* encodes for several potential respiratory electron donor and acceptor enzymes to carry out electron transport (28). One of these electron donors in *C. jejuni* is a proton pump called complex I, which is widely found in bacteria, archaea, and the mitochondria of eukaryotes (13). This complex (also called NADH:ubiquinone oxidoreductase) is the first enzyme in many respiratory chains and catalyzes the transfer of electrons from NADH to the quinone pool, coupled with the translocation of protons across a membrane (13). The fact that complex I occurs in the *C. jejuni* genome sequence was somewhat surprising, as it has been shown that NADH is a poor respiratory electron donor (15).

The genes that encode for complex I of most bacteria are clustered in a conserved order (31) consisting of 14 different genes that are designated either as *nuo* (NADH:ubiquinone oxidoreductase) (39) or as *nqo* (NADH:quinone oxidoreductase) (13, 42). Complex I has been well characterized in both *Escherichia coli* (4, 20) and *Paracoccus denitrificans* (41), and a model of the subunit structure and function has emerged. Of the 14

subunits, 7 subunits (NuoA, H and NuoJ-N) are integral membrane proteins and the remaining 7 (NuoB-G and NuoI) are peripheral subunits. The known redox cofactors are found in the peripheral subunits (13), including those of the NADH dehydrogenase fragment which has been localized to NuoE, NuoF and NuoG (14). Like these model systems, the complex I genes of *C. jejuni* are organized in the 14 gene *nuo* operon, however, *nuoE* and *nuoF* are absent from this operon and are replaced with two genes (*Cj1575c* and *Cj1574c*) of unknown function (figure 1) (28). Since *nuoE* and *nuoF* encode the NADH dehydrogenase subunit, it is not surprising that NADH is not the donor to complex I of *C. jejuni*. The other 12 subunits of complex I in *C. jejuni* do contain sequence similarities to complex I subunits of other bacteria including *E. coli* and *P. denitrificans* (20, 41).

The electron donor to complex I of *C. jejuni* has yet to be identified. Finel has suggested that HP1264 and HP1265 (homologues to CJ1575 and CJ1574) in *H. pylori* may provide a docking site for a protein that may pass its electrons directly to NuoG (Nqo3) (10), and Myers and Kelly suggest a flavodoxin or ferredoxin as possible candidates (25). Flavodoxins are small acidic proteins that are involved in electron transfer and contain one molecule of flavin mononucleotide that acts as the redox active component (30), while ferredoxins are redox active iron-sulfur proteins classified by the number of Fe-S clusters (5). The genome sequence of *C. jejuni* encodes for several ferredoxins, and one flavodoxin (28). The genome also codes for two enzymes that typically use either ferredoxin or flavodoxin as the electron acceptor, pyruvate ferredoxin (flavodoxin) oxidoreductase (PFOR) and 2-oxoglutarate oxidoreductase (OOR). PFORs are iron sulfur proteins involved in the coenzyme A (CoA) dependent oxidative decarboxylation of pyruvate to form acetyl-CoA

(16, 29). OOR enzymes are involved in the decarboxylation of 2-oxoglutarate in the presence of CoA to form succinyl-CoA and CO_2 (17). In *C. jejuni*, PFOR is encoded by *Cj1476c*, and codes for a single protein subunit while OOR is composed of 4 subunits encoded by the genes *oorDABC* (*Cj0535-Cj0538*) (28). PFOR and OOR both play vital roles in central carbon metabolism in *C. jejuni*, serving the analogous functions of pyruvate dehydrogenase and α-ketoglutarate dehydrogenase, enzymes which are not found in the *C. jejuni* genome (28).

In this article, we demonstrate that complex I functions as a respiratory donor enzyme that accepts electrons from flavodoxin rather than NADH. We also show that OOR is responsible for α-ketoglutarate-dependent respiratory activity, and that flavodoxin is the electron acceptor for this enzyme. Furthermore, we show that both *Cj1574c* and *fldA* (which encode flavodoxin) are essential genes in *C. jejuni*. We suggest that flavodoxin is essential because of its role as a substrate for two important steps in central carbon metabolism, and that CJ1574 is essential in maintaining a pool of oxidized flavodoxin.

2.2 Materials and Methods

2.2.1 Bacterial strains and growth conditions.

C. jejuni strains are listed in table 1. Cells were grown on either tryptic soy agar plates supplemented with 10% sheep blood (called BA plates) or in Mueller Hinton (MH) broth. Formate (20 mM for liquid culture and 50 mM for plates), sodium nitrate (10 mM), chloramphenicol (25 μg/ml) and kanamycin (30 μg/ml) were added to the media as indicated. *C. jejuni* was routinely cultured microaerobically at 37°C in a tri gas incubator (model NU-4950, NuAire, Inc), in which the gas composition was constantly maintained at 12% O_2 and 5% CO_2 and balance N_2. *E. coli* strains are listed in table 1. Luria-Bertani broth and agar supplemented with ampicillin (at 150 μg/ml), chloramphenicol (at 25 μg/ml) and kanamycin (at 30 μg/ml) were used for growing various *E. coli* strains, as noted.

2.2.2 Cloning and construction of *nuo* mutants.

Oligonucleotide primers (table 2) were designed using the genome sequence of *C. jejuni* NCTC 11168 (28) using the DNA analysis program MacVector. Unless otherwise stated, PCR amplification was performed with *Taq* DNA polymerase (Promega) using *C. jejuni* NCTC 11168 genomic DNA as a template. Genomic DNA was isolated using the MasterPure™ Complete DNA & RNA Purification Kit (Epicentre® Biotechnologies). The PCR products were cloned into pBluescriptII$^+$ KS (Stratagene) and pCR®2.1 TOPO® (Invitrogen) vectors and confirmed by restriction analysis (plasmids are listed in table 3). The cloned *nuo* genes were then disrupted by insertion of a chloramphenicol acetyl transferase resistance cassette (*cat*), removed from pJMA-001 by *Sma* I digestion. pJMA-001 contains

cat from plasmid pRY111 (43), and cloned into the *Pvu* II site of pGEM-T Easy (Promega) to yield plasmid pJMA-001. Double mutants were obtained by using a kanamycin cassette, *aphA-3* (38). Insertion of the cassettes were confirmed by isolation of genomic DNA from the mutants and PCR amplification of the relevant *nuo* genes, followed by agarose gel electrophoresis to monitor the increase in size of the *nuo* genes due to the insertion of the cassettes (data not shown).

2.2.3 Transformation of *C. jejuni*.

C. jejuni electrocompetent cells were made by harvesting cells by swab from BA plates and washing the cells three times in an ice cold 9% sucrose and 15% glycerol solution. Cells were used immediately or frozen at -80°C for future use. For transformation, *C. jejuni* electrocompetent cells were incubated with 1-5 μg of plasmid DNA on ice for 10 minutes. The cells were then placed in a 2 mm electroporation cuvette, given a pulse of 2500 V in a ECM399 electroporator (BTX, San Diego, CA), and 50 μl of MH broth was added to the cuvette and subsequently incubated on ice for 10 minutes. The cells were then spotted onto a cold BA plate supplemented with formate and sodium nitrate, and incubated for 24 hours in an anaerobic jar containing an Anaerobic BBL™ GasPak Plus™ with palladium catalyst (anaerobic pack). After 24 hours on non-selective media, the cells were transferred onto BA plates supplemented with chloramphenicol, formate and sodium nitrate and incubated in an anaerobic jar with an anaerobic pack. Resistant colonies were selected after 3-5 days incubation, and the colonies were screened for recombination of the interrupted version of the gene by PCR of genomic DNA using primers against the targeted gene.

2.2.4 Construction of the *nuo* merodiploid strains

pET7574 contains *Cj1575c* and *Cj1574c* in-frame with a hexa-histidine epitope tag. The *cat* cassette was then inserted into a *Blp* I restriction site located downstream of *Cj1575c* and *Cj1574c* in pET7574 to yield pET7574::CM. The coding region of the two genes and cassette were excised from pET7574::CM by digestion with *Bgl* II and *Sty* I, and treated with T4 DNA polymerase (Promega) to ensure blunt ends. This fragment was then cloned into the *Nde* I restriction site of pHydA (table 3), which contains a 1193 bp fragment of *hydA*, to yield pHyd7574::CM. Electrocompetent *C. jejuni* cells were transformed with pHyd7574::CM to obtain 7574MD. Δ74MD was constructed as follows: A 2659 base pair fragment of *nuoC*, *nuoD*, *Cj1575c*, *Cj1574c* and *nuoG* was amplified by PCR using *C. jejuni* WT genomic DNA and primers NuoF and NuoR and cloned into pCR®2.1 TOPO® to yield pNuo. This plasmid was digested with *Ssp* I to create a deletion within *Cj1574c*, and *aphA-3* was inserted into the *Ssp* I sites of *Cj1574c* to yield pNuo::KAN. Restriction analysis revealed that *aphA-3* was in the same orientation as *Cj1574c*. This plasmid was then electroporated with competent 7574MD cells to obtain Δ74MD. PCR was carried out to confirm that *aphA-3* was inserted in the *Cj1574c* gene of the *nuo* operon and not in the *Cj1574c* gene cloned into *hydA* gene. ΔNuoMD was constructed as follows: *nuoN* was digested out of pNuoN (table 3) via *Eco*R I, blunt ended with T4 DNA polymerase and cloned into the *Bam*H I site of pNuoCD (table 3) in the same orientation as *nuoCD* to yield pNuoCDN. This plasmid was digested by creating two deletions between the *Acc*B7 I site of *nuoC* and *Cla* I site of *nuoD*, and the *Cla* I sites of *nuoD* and *nuoN*. Next, *aphA-3* was

inserted into the *Acc*B7 I/ *Cla* I site to yield pΔNuoCDN::KAN. Restriction analysis revealed that *aphA-3* was in the same orientation as *nuoC* and *nuoN*. This plasmid was mobilized via electroporation into competent 7574MD cells to obtain ΔNuoMD.

2.2.5 Construction of the flavodoxin merodiploid strains

A merodiploid flavodoxin strain (FldMD) was constructed as follows: primers designed to engineer a *Nde* I site to the first codon of the *fldA* gene (*Cj1382c*) (FldProF) and a *Xho* I site in place of the stop codon of *fldA* (FldProR) and platinum Pfu polymerase (Invitrogen) were used to amplify *fldA* using *C. jejuni* WT genomic DNA and cloned into pCR®2.1 TOPO® to yield pTOPOFld. *fldA* was excised from pTOPOFld by digestion with *Nde* I and *Xho* I and then cloned into the *Nde* I/*Xho* I digested pET-21a(+) in frame with the haxa-histidine tag to yield pETFld. *aphA-3* was then inserted into the *Blp* I site of pETFld to yield pET7574::KAN. *fldA* and *aphA-3* were then cut out of pET7574::KAN using *Bgl* II and Sty I, blunt ended with T4 DNA polymerase and cloned into the *Nde* I site of pHydA to yield pHydFld::KAN. This plasmid was used to transform competent *C. jejuni* to obtain FldMD. FldMD::CM was constructed as follows: The *fldA* gene (*Cj1382c*) and two flanking regions of 948 bp upstream of the start codon and 287 bp downstream of the stop codon respectively were amplified by PCR using *C. jejuni* WT genomic DNA and primers FldF and FldR, blunt ended with T4 DNA polymerase and phosphorylated with T4 polynucleotide kinase (Promega). This product was then cloned into the *Eco*R V site of pBluescriptII KS(+) where the *Eco*R I restriction site has been destroyed (pKSΔRI) to yield pΔRIFld. The *cat* cassette was then cloned into the *Eco*R I site of pΔRIFld. The resulting plasmid,

pΔRIFld::CM, was then electroporated into competent FldMD cells to obtain FldMD::CM. PCR was carried out to confirm that the original *fldA* gene in the genome contained the *cat* and not the *fldA* gene cloned into *hydA*.

2.2.6 Cloning of *oorB* and construction of OorB::CM

The *oorB* (*Cj0537)* gene was amplified by PCR using the primers OorF and OorR and cloned into pCR®2.1 TOPO® to yield pOorB. This plasmid was digested with *Ssp* I to create a deletion within *oorB*, and a *cat* was then inserted into the *Ssp* I sites of this plasmid to obtain pOorB::CM. This plasmid was electroporated into competent WT *C. jejuni* cells to obtain OorB::CM. Restriction analysis revealed that the *cat* was in the same orientation as the *oorB* gene.

2.2.7 Quantitative reverse transcriptase PCR (qRT)-PCR

qRT-PCR was performed using the Quantitect™ SYBR® Green RT-PCR kit (Qiagen, Valencia, CA). RNA was extracted from *C. jejuni* cells grown to mid log phase by using the MasterPure™ Complete DNA & RNA Purification Kit (Epicentre® Biotechnologies).

The RT-PCR reaction mixture included 40 ng RNA, 2 μM each of the forward and reverse primers, 1X QuantiTect SYBR Green RT-PCR Master Mix (Qiagen) and 0.2 μl / reaction QuantiTect RT Mix (Qiagen). The reverse transcriptase reaction was held at 50°C for 30 minutes, followed by a PCR initial activation step for 15 minutes at 95°C. The

mixtures were then amplified for 30 cycles consisting of 94°C for 15 seconds, 60°C for 30 seconds, and 72°C for 30 seconds in an automated thermal cycler (BIORAD iCycler^{TM}, Hercules, CA). The iCycler software was used to determine the threshold cycle for when each transcript can be detected. Threshold cycles were then compared to a standard curve, which was generated independently for each gene, to determine the number of starting RNA molecules. Total RNA in each sample was normalized by standardizing the copy number of that gene, to that of an internal control, *gyrA* (*Cj1027c*).

2.2.8 Flavodoxin expression, purification and characterization

E. coli strain BL21 Rosetta (Novagen) was transformed with pETFld. A 5 ml overnight culture of pETFld Rosetta grown at 37ºC in Luria-Bertani broth supplemented with ampicillin was used to inoculate a fresh 500 ml Luria-Bertani broth. This inoculated culture was shaken at 37ºC. When the OD of the culture at 600 nm was between 0.5 and 0.7, the culture was induced with a final concentration of 0.5 mM IPTG. Two hours post induction the cells were harvested by centrifugation, washed two times with lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole) and resuspended with 40 mls lysis buffer. The cells were then broken by passage through a French Pressure Cell (Thermo Spectronic) three times at 20,000 lbs/in^2. The crude extract was cleared by centrifugation at 12,000 x g for 5 minutes. The supernatant was collected and passed over a Ni-NTA agarose (5 ml bed volume) column (Qiagen) pre-equilibrated with lysis buffer. The column was then washed with 5-column volumes wash buffer (50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole). Protein was eluted with elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 250 mM

imidazole), and 2 ml fractions were collected. Elution fractions were assayed for protein by SDS-PAGE as described by Laemmli (18). Protein containing fractions were dialyzed overnight in a 1 L solution of 10 mM Tris, 10 mM NaCl, 1 mM DTT and 10% glycerol at 4ºC. Spectra were obtained using this purified flavodoxin in a Shimadzu UV-1650PC spectrophotometer. Spectra from commercially purchased flavin mononucleotide (MP Biomedicals, Inc) and flavin adenine dinucleotide (Alexis Biochemicals) were used to compare the flavodoxin spectrum.

2.2.9 Cytochrome Reduction Assays

Cells were harvested by swab into N_2 sparged phosphate buffered saline (PBS), washed once and sonicated in sealed tubes under N_2 to obtain cell free extracts (CFE). One ml each of CFE was put into two quartz cuvettes, which were subsequently sealed with rubber stoppers, flushed with N_2 gas for 3 minutes, and then placed in a Shimadzu UV-1650PC spectrophotometer. A baseline spectra was produced first without the addition of any substrates, and second with the simultaneous addition of a 5 mM final concentration of α-ketoglutarate and a 0.5 mM final concentration of CoA. Protein concentrations were determined by BCA™ protein assay kit (Pierce).

2.2.10 Reduction of Flavodoxin

Purified flavodoxin and *C. jejuni* CFE were mixed in a 4:1 ratio in a capped quartz cuvette to obtain a total volume of 1 ml, a blank cuvette contained only *C. jejuni* CFE and buffer. The cuvettes were flushed with N_2 for 3 minutes, and placed in a Shimadzu UV-

1650PC spectrophotometer. A baseline spectrum was produced without the addition of substrate to verify the characteristic flavodoxin spectra. Next, a mixture of α-ketoglutarate and CoA were added to both quartz cuvettes simultaneously to give a final concentration of 5 mM and 0.5 mM respectively. The cuvettes were inverted several times and a spectral analysis was conducted immediately, 1 minute and 5 minutes after the addition of these substrates. To determine the flavodoxin reduction kinetics, the absorbance at 460 nm was monitored following addition of α-Ketoglutarate and CoA.

2.2.11 Oxygen Uptake Experiments

O_2 was quantified using a YSI Model 5300 Biological Oxygen Monitor (Yellow Springs instrument Co, Yellow Springs, OH) and Clark-type electrode. The electrode was inserted into a 5 ml capacity glass chamber that was continuously stirred. 5 ml of whole cells or CFE were added to the chamber and allowed to equilibrate until no change in dissolved O_2 was observed for several minutes. Upon equilibration, substrate was added through a capillary tube via a Hamilton syringe into the chamber and the dissolved O_2 was recorded by chart recorder. After each experiment the chamber was calibrated using known concentrations of dissolved O_2. Substrate concentrations used were: α-ketoglutarate and CoA, 5 mM and 0.5 mM, respectively; formate, 5 mM; NADH, 5 mM; NADPH, 5 mM.

2.2.12 SDS-PAGE and Immunoblotting

To prepare membrane particles, ten plates of each strain were harvested by sterile swab into cold PBS, washed twice with PBS, and resuspended to 10 mls in cold PBS. The

cells were broken by passage three times through a French Pressure Cell (Thermo Spectronic) at 20,000 lbs/in^2 and the lysate was cleared of unbroken cells by centrifugation at (12,000 x g) for 5 minutes. The cleared supernatant was then subjected to fractionation by ultra centrifugation (150,000 x g for 90 minutes) in a Beckman L8-55 ultracentrifuge to isolate the membranes. The supernatant (soluble fraction) was saved for immunoblot. The membrane fraction was washed once by resuspension in 10 ml cold PBS by dounce homogenization, followed by centrifugation (150,000 x g for 90 minutes), and finally resuspended by dounce homogenizer. 10 μg of the soluble and membrane fractions were subjected to SDS-PAGE and transferred electrophoretically onto 0.2 μm nitrocellulose membranes (Bio-Rad Laboratories). The membranes were incubated in blocking solution (5% nonfat dry milk powder in tris buffered saline for 30 minutes at room temperature. The primary antibody (purified mouse monoclonal Tetra•His™ Antibody [Qiagen]) was diluted 1:2000 in blocking solution and incubated overnight at 4°C. The membranes were washed 3x for 5 minutes each time with tris buffered saline, and then incubated with a 1:3000 dilution of purified goat anti mouse alkaline phosphatase conjugate antibody (Bio-Rad Laboratories) for two hours at room temperature. The membranes were washed twice with tris buffered saline and a final wash was conducted with alkaline tris buffer (100 mM Tris, 100 mM NaCl, 50 mM $MgCl_2$). After washing, the membranes were developed with the alkaline phosphatase color development reagents 5-bromo-4-chloro-3-indoyl phosphate p-toluidine salt and p-nitro blue tetrazolium chloride purchased from Bio-Rad Laboratories.

2.3 Results

2.3.1 Twelve *nuo* genes of complex I are dispensable.

12 of the 14 genes in the complex I operon were disrupted either via an insertion or deletion mutation (figure 1A). ΔNuoAB contains a deletion comprising 34% of *nuoA* and 53% of *nuoB*; the deleted DNA was replaced with *cat* (43). *nuoC* was disrupted both by an insertion with *cat* within the coding region (NuoC::CM) and also as part of a deletion comprising *nuoC* (15% deletion) and *nuoD* to obtain ΔNuoCD. Two *nuoD* mutants were isolated with *cat* insertions near the middle (NuoD::CM) and near the end of the gene (Nuo::CM). *nuoG* was disrupted by insertion of *cat* (in both orientations) within the coding region, and the resulting mutants were designated NuoG::CM^+ and NuoG::CM^- (for all mutants, a superscript + indicates the cassette is transcribed in the same orientation as the gene, a superscript – indicates cassette transcription opposite the gene). *nuoG* was also disrupted as part of a deletion comprising *nuoG* (56% deletion), *nuoH*, and *nuoI* (23% deletion) to obtain Δ$NuoGI^+$ and Δ$NuoGI^-$. *nuoI* was disrupted by insertion of a *cat* (in both orientations) within the coding region (NuoI::CM^+ and NuoI::CM^-) and also as part of a deletion comprising 77% of *nuoI*, all of *nuoJ*, *nuoK,* and *nuoL*, and 89% of *nuoM* to obtain Δ$NuoIM^+$ and Δ$NuoIM^-$. *nuoN* was disrupted by insertion of a *cat* (in both orientations) to obtain NuoN::CM^+ and NuoN::CM^-. Two versions of a double mutant were obtained (figure 1B), using ΔNuoCD as the parent strain and insertion of *aphA-3* (in both orientations) into a deleted region consisting of *nuoI*, *nuoJ, nuoK, nuoL,* and *nuoM*, to yield Δ$NuoCDIM^+$ and Δ$NuoCDIM^-$. Each of these mutants were isolated on BA plates supplemented with formate (50 mM) and nitrate (10 mM) under an anaerobic atmosphere created by a GasPak Plus ™

anaerobic pouch. All *nuo* mutants display a similar growth phenotype: they will not grow in liquid culture (cultures fail to double twice in 30 hours) or on plates (no isolated colonies) unless the media is supplemented with an alternative respiratory substrate such as formate. When formate is provided, these mutants displayed growth rates similar to WT (table 4 and figure 2). Growth is entirely dependent on formate in these strains, which is shown graphically for strain ΔNuoMD in figure 2. Final culture optical density (OD 600) is proportional to the initial concentration of formate (10 mM supplemented cultures grow to final OD 600 of 0.18, 20 mM supplemented cultures to 0.36), and after the formate has been consumed growth ceases.

2.3.2 Expression of *Cj1574c* is required for viability.

Despite many attempts, we were unsuccessful at mutating *Cj1574c* either via either an insertion or deletion mutation. We also noticed that *nuo* genes upstream of *Cj1574c* could only be mutated with *cat* that was in the same orientation as the *nuo* genes, while genes downstream of *Cj1574c* could be mutated with *cat* in either orientation (figure 1). Quantitative RT-PCR (qRT-PCR) reveal that expression of the genes downstream of the *cat* are affected differentially dependent on the *cat* orientation. To show this effect quantitatively, we measured the transcription of *nuoH* in NuoG::CM^+ and NuoG::CM^-, strains that are identical except for the orientation of *cat*. When *cat* is in the same orientation as the operon (NuoG::CM^+) transcription is reduced 8 fold in comparison to WT, but down 50 fold when *cat* is transcribed opposite (NuoG::CM^-) from the *nuo* genes (table 5). That no

strains with (-) orientation *cat* cassettes upstream of *Cj1574c* could be isolated gives support to the conclusion that expression of *Cj1574c* is required for viability in *C. jejuni*.

2.3.3 The *Cj1574c* merodiploid strains.

A merodiploid *Cj1575c* and *Cj1574c C. jejuni* strain was constructed by cloning the coding regions of both into the coding region of *hydA* (*Cj1267c*). This strain, (designated 7574MD), contains two copies of *Cj1575c* and *Cj1574c*. Insertions into *hydA* have no phenotype relevant to these studies, and transcription of *hydA* is constitutive (data not shown), making this a useful locus unrelated protein expression. The resulting strain (designated 7574MD) contain two copies of *Cj1575c* and *Cj1574c*. When 7574MD was used as the parent strain, it was possible to delete a portion of *Cj1574c* found within the *nuo* operon, yielding Δ74MD. Furthermore, a large deletion of the *nuo* operon could be made, in which *nuoC-nuoN* (including *Cj1574c* and *Cj1575c*) are deleted and replaced with the *aphA-3* cassette (designated ΔNuoMD, [figure 1C]). qRT-PCR of WT and ΔNuoMD strains indicate that *Cj1574c* expression levels are roughly similar in both strains (table 5). Δ74MD is unable to grow in MH broth without formate supplementation (table 4). This result was unexpected, as this strain contains intact copies of all 12 *nuo* genes and is also complemented with *Cj1575c* and *Cj1574c*. qRT-PCR assays of *nuoH* in WT and Δ74MD, however reveals that the *aphA-3* cassette within *Cj1574c* reduced the expression of downstream genes ~80 fold (table 5). The requirement for formate in this strain can be attributed to the loss of transcription of *nuoG-nuoN*.

2.3.4 Localization of CJ1574.

Purified CJ1575 and CJ1574 expressed from *E. coli* were not immune reactive when inoculated into two separate rabbits, and did not provide antisera able to identify these proteins (data not shown). We therefore used an epitope tagged (hexa-histidine) version of CJ1574 to create the merodiploid strains. Since this tag has been cloned in-frame with *Cj1574c*, the resulting protein product would contain a hexa-histidine epitope tag on the C-terminus. Anti•His antibodies recognize an appropriate sized protein expressed in all three strains expressing the CJ1574 from the *hyd* operon (figure 3). To determine CJ1574 localization, membrane and soluble fractions of each strain were prepared and blotted (figure 3). The results indicate that CJ1574 was present in both the membrane and soluble fractions in all three strains, however the ratio of membrane-associated protein was highest in strain ΔNuoMD.

2.3.5 Respiratory activities of WT and ΔNuoMD.

Respiration rates on various substrates were determined by O_2 uptake using a Clark-type O_2 electrode on both whole cells and CFE in WT and ΔNuoMD. In agreement with previous studies (8, 15), formate was the preferred substrate for both WT and ΔNuoMD (table 6). The only substrate tested that was significantly affected by the *nuo* deletion was α-ketoglutarate, a known respiratory substrate in *C. jejuni* (24, 40). WT CFE respired α-ketoglutarate at a rate of 16.4 nmoles of O_2 consumed $minute^{-1}$ mg^{-1} of protein vs. 3.7 nmoles of O_2 consumed $minute^{-1}mg^{-1}$ of protein for ΔNuoMD (table 6). Significantly, *C. jejuni* CFE

had low rates of respiratory activity with either NADH or NADPH, and these rates were unaffected by the *nuo* deletion (table 6).

2.3.6 OOR is responsible for α-ketoglutarate respiration in *C. jejuni*.

The decreased ability of ΔNuoMD to respire α-ketoglutarate prompted us to look at this substrate as a possible donor to complex I. Previous studies have shown that α-ketoglutarate supports respiration in *C. jejuni* strain 11168 (24) and *C. jejuni* strain ACC 29428 (40), however the enzymes responsible have not been characterized. We targeted the OorB subunit of the enzyme OOR for mutagenesis by insertion of a *cat* into the *oorB* (*Cj0537*) coding region to yield OorB::CM. CFE of OorB::CM displayed negligible α-ketoglutarate and CoA dependent O_2 uptake (table 6), indicating OOR is the sole enzyme responsible for α-ketoglutarate respiration. Furthermore, we were able to show that α-ketoglutarate (plus CoA) initiated the reduction of the electron transport chain, as evidenced by the emergence of peaks at 421 nm, 524 nm, and 553 nm, characteristic of reduced cytochromes. Addition of α-ketoglutarate and CoA to CFE of OorB::CM results in no cytochrome reduction, even after a 30' incubation (data not shown). These data indicate that α-ketoglutarate is oxidized by OOR, and electrons liberated are transferred to O_2 via the respiratory electron transport chain.

2.3.7 Flavodoxin is the electron acceptor of OOR and an essential protein.

Attempts to mutate flavodoxin (*fldA*, *Cj1382c*) in *C. jejuni* were unsuccessful despite many attempts. We used the same strategy to make an *fldA* merodiploid strain as 7574MD, and *fldA* was cloned in-frame with a His•Tag coding sequence into the *hyd* operon, disrupting *hydA* to obtain FldMD. When FldMD was used as the parent strain, we were able to interrupt the original *fldA* gene to obtain FldMD::CM, indicating that flavodoxin is required for viability in *C. jejuni*. A his-tagged version of *C. jejuni* flavodoxin was expressed at high levels in *E. coli* and purified to near homogeneity in one step using nickel-chelate affinity chromatography (figure 4A). The recombinant flavodoxin contains a flavin cofactor as determined by visible spectra, however the nature of the cofactor remain ambiguous. Comparison of purified flavodoxin to a commercially purchased flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD) (figure 4B), reveal that although the gross spectra are similar, the absorption maxima for flavodoxin (460 nm) is shifted in relation to both FMN (445 nm) and FAD (450 nm). Addition of dithionite to the protein leads to reduction of the flavin, which can be monitored by a decrease in absorbance at 460 nm (data not shown). We used the redox state of the flavodoxin to determine if α-ketoglutarate (via OOR) was the electron donor. Purified flavodoxin was incubated with CFE of WT and OorB::CM along with α-ketoglutarate and CoA in a stoppered quartz cuvette. Flavodoxin reduction was monitored by a decrease in absorbance of the peak at 460 nm (figure 5A). WT CFE reduce recombinant flavodoxin at a rate of 213 ± 19 nmoles min^{-1} mg^{-1} of protein (figure 5A), OorB::CM CFE are unable to reduce flavodoxin (figure 5B). These data indicate that in *C. jejuni*, (unlike in *H. Pylori*) flavodoxin is an efficient electron acceptor of

OOR (17). Previous studies have shown that the flavodoxin of *H. pylori* is essential for the survival for this organism (11).

2.4 Discussion

In order to fulfill its energy demands, *C. jejuni* relies solely on the respiratory chain and oxidative phosphorylation. Known respiratory substrates for *C. jejuni* include hydrogen, formate, succinate, malate, lactate, sulfite, and α-ketoglutarate (15, 24, 26, 40). Curiously, despite the fact that NADH is a poor respiratory substrate in *C. jejuni* (15), the genome sequence predicts the presence of 12 *nuo* (for NADH ubiquinone oxidoreductase) genes, also called complex I (28). This apparent paradox is explained by the fact the *nuo* genes that encode the NADH dehydrogenase module subunits (*nuoE* and *nuoF*) are absent from the operon. In the place of *nuoE* and *nuoF* (between *nuoD* and *nuoG*, see figure 1A) are two novel genes, *Cj1574c* and *Cj1575c*. We present a model (figure 6) whereby these novel proteins act as electron acceptors from a flavodoxin rather than NADH. In this model flavodoxin acts (like NADH) as an intermediate between central carbon metabolism and the electron transport chain.

Despite the lack of NADH dehydrogenase activity, *C. jejuni* complex I is a major point of entry of electrons into the respiratory chain. *nuo* mutants fail to grow in MH broth, but growth can be restored when provided with an alternative respiratory substrate such as formate (table 4). In contrast to the 12 *nuo* genes, *Cj1574c* (the 6th gene of the *nuo* operon, see figure 1A) is essential for viability of *C. jejuni*. We draw this conclusion based on three criteria. First, repeated attempts to interrupt or delete the gene via allelic replacement were unsuccessful, despite being able to interrupt or delete the other 12 genes of the operon. Second, genes "upstream" of *Cj1574c* can only be interrupted when the drug cassette is in an

orientation that allows transcription of downstream genes. Third, when a second copy of *Cj1574c* was provided on a second location in the genome (a *Cj1574c* merodiploid), the entire *nuo* operon is dispensable.

Strain ΔNuoMD contains a large (10.5 Kb) deletion from the middle of *nuoC* to the beginning of *nuoN*. (figure 1C). Importantly, *Cj1574c* is expressed in this strain from the inserted copy both transcriptionally (table 5) and translationally (as measured by immunoblot, figure 3). We used this strain to identify the physiological donor to complex I. Respiratory activities (as measured by O_2 uptake) were similar to the parent strain for all respiratory substrates tested except for α-ketoglutarate (table 6). α-ketoglutarate is a TCA cycle intermediate and the entry point for many amino acids into central carbon metabolism, making it especially important in an asacchrolytic organism such as *C. jejuni* that garners most of its carbon and energy from amino acids (19, 23, 36). OOR (encoded by *oorDABC* (*Cj0535-Cj0538*) is a functional equivalent to α-ketoglutarate dehydrogenase in that it catalyzes the decarboxylation of α-ketoglutarate to form succinyl-CoA (17). The OOR mutant strain supports neither α-ketoglutarate dependent cytochrome reduction nor α-ketoglutarate dependant O_2 uptake (table 6). Although the oxidative TCA cycle is interrupted in OorB::CM, the mutation is not lethal due to the presence of reductive TCA enzymes (e.g., fumarate reductase) which allows for the synthesis of important biosynthetic precursors. One major difference between OOR and α-ketoglutarate dehydrogenase is that the reducing equivalents from OOR are transferred to a low potential protein electron acceptor rather than NADH. In search of the physiological electron acceptor to OOR, we

mutated two ferredoxin genes (*Cj0333c* and *Cj0369c*) and found no effect on α-ketoglutarate respiratory activity (data not shown). Attempts to mutate the *C. jejuni* flavodoxin gene (*fldA*) proved unsuccessful unless a second copy of *fldA* was provided on the chromosome. We conclude that *fldA,* (like *Cj1574c*) is an essential gene in *C. jejuni*. Flavodoxin has also been shown to be essential in *H. pylori* (11). *C. jejuni* flavodoxin expressed heterologously in *E. coli* and purified by nickel chelate affinity chromatography (figure 4A) contains a flavin cofactor that can be monitored spectrophotometrically (figure 4B). Extracts of WT *C. jejuni* reduce flavodoxin when provided with α-ketoglutarate and CoA (figure 5A), extracts of OorB::CM do not (figure 5B). These data indicate that the electron acceptor for OOR in *C. jejuni* is flavodoxin, in contrast to *H. Pylori* where the electron acceptor was determined to not be flavodoxin (17).

Taken together, these data indicate that complex I in *C. jejuni* is an entry point for electrons into the respiratory electron transport chain from a reduced flavodoxin. We also conclude that CJ1574 (and possibly CJ1575, but we will only speculate on CJ1574 here) is required for viability of *C. jejuni* and involved in both electron transport and flavodoxin redox cycling. In our model (figure 6), we believe that in WT cells CJ1574 is part of the complex I, and facilitate the transfer of electrons into the complex. Furthermore, in the absence of a functional complex I (such is the case in all the *nuo* mutants), CJ1574 retains its role in facilitating the oxidation of flavodoxin. This model explains why CJ1574 is essential, as without this activity the flavodoxin pool would remain reduced. Oxidized flavodoxin is required for the activity of both OOR and PFOR (16, 32), both of which are important

enzymes of central carbon metabolism. The importance of a pool of oxidized flavodoxin is indicated by the essential nature of both flavodoxin and CJ1574.

O_2 is the final electron acceptor of flavodoxin regardless of whether complex I is intact. Although greatly reduced, the *nuo* deletion strain retains partial α-ketoglutarate-dependent O_2 uptake activity (table 6), and CJ1574 is still membrane associated (figure 3). We conclude that this low level of CJ1574 mediated electron flow in the *nuo* mutants is sufficient to provide a pool of oxidized flavodoxin, but does not provide enough energy to support growth of the cultures in the absence of formate (table 4). A recent study has shown that in *H. Pylori* flavodoxin interacts with a flavin quinone reductase the authors termed FqrB (32). FqrB was shown to mediate the transfer of electrons from flavodoxin to NADP to form NADPH. FqrB is also present in *C. jejuni* and can accept electrons from reduced flavodoxin (32). Although we feel it most likely that CJ1574 and FqrB accept electrons from flavodoxin independently, we cannot rule out the possibility that FqrB is an intermediate in electron flow from flavodoxin to the respiratory chain. We do not believe, however, that NADPH is involved in the electron transfer to the respiratory chain. The level of NADPH-dependent respiratory activity observed (table 6) is too low to account for the rate of α-ketoglutarate-dependent O_2 uptake.

C. jejuni belongs to the ε-class of the proteobacteria, the diversity of which has only recently been recognized. In addition to the well studied pathogens of the genera *Campylobacter* and *Helicobacter,* the ε-proteobacteria also include many marine and terrestrial aquatic species (7), and may be the dominant bacterial species in deep sea hydrothermal vent systems (34). Abandoning the NADH dehydrogenase module form

appears to be an early event in the evolution of the ε- proteobacteria, as *nuoE* and *nuoF* are absent from all ε-proteobacterial genome sequences, despite the presence of *nuo* operons (figure 7). Three different strategies have been employed by these bacteria to cope with the loss of *nuoE* and *nuoF*: replacement with *Cj1575c* and *Cj1574c* homologues (as in *Campylobacter and Helicobacter* species); deletion (along with *nuoG*) without replacement (as in *C. mediatlanticus TB-2*, and one of the *Sulfurovum NBC37-1* operons); and duplication of *nuoG* and recruitment of a *Cj1575c* homologue and either *gltD* or *fdhB* (as in *Wolinella succinogenes*, *Nitratiruptor SB155-2*, *Thiomicrospira denitrificans* ATCC 33889, and one of the *Sulfurovum NBC37-1* operons) (figure 7). The different strategies employed by the ε-proteobacteria likely reflect the different needs of each bacterium adapted to their specific environments. The ε-proteobacters of the hydrothermal vents systems grow autotrophically using the reductive TCA cycle to fix CO_2 and relying heavily on inorganics for energy (7, 33). The presence of the reversible enzymes PFOR and OOR (both of which are required for the reductive TCA cycle) in the ε-proteobacteria is likely the legacy of this autotrophic lifestyle (33). *C. jejuni*, on the other hand, thrives in the resource rich environment of the animal intestinal tract and employs an oxidative TCA cycle to garner energy from organic compounds found in its ecological niche. In this evolutionary context, the adaptation of complex I to accepts electrons from flavodoxin is a logical response to inheriting a TCA cycle that employs PFOR and OOR. A better understanding of this unusual respiratory pathway (of which at least two of the components are essential for *C. jejuni* viability) could prove vital in devising strategies to eliminate this important human pathogen from the food supply.

2.5 Acknowledgements

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We thank Debbie Threadgill and Jay Andrus for the kind gift of pJMA-001.

Table 1. Strains used in this study

Strains	Description and orientation of antibiotic cassette if any	Source
C. jejuni		
NCTC 11168	Parent strain for *C. jejuni* strains (WT)	NCTC[a]
ΔNuoAB	*cat* replaces *nuoA* and *nuoB* deletion	This Study
NuoC::CM	*cat* inserted within *nuoC*	This Study
NuoD::CM	*cat* inserted within *nuoD*	This Study
ΔNuoCD	*cat* replaces *nuoC* and *nuoD* deletion	This Study
Nuo::CM	*cat* inserted within *nuoD*	This Study
NuoG::CM^{+}	+ orientation *cat* inserted within *nuoG*	This Study
NuoG::CM^{-}	– orientation *cat* inserted within *nuoG*	This Study
ΔNuoGI^{+}	+ orientation *cat* replaces *nuoG-nuoI* deletion	This Study
ΔNuoGI^{-}	– orientation *cat* replaces *nuoG-nuoI* deletion	This Study
NuoI::CM^{+}	+ orientation *cat* inserted within *nuoI*	This Study
NuoI::CM^{-}	– orientation *cat* inserted within *nuoI*	This Study
ΔNuoIM^{+}	+ orientation *cat* replaces *nuoI-nuoM* deletion region	This Study
ΔNuoIM^{-}	– orientation *cat* replaces *nuoI-nuoM* deletion region	This Study
NuoM::CM^{+}	*cat* inserted within *nuoM*	This Study
NuoM::CM^{-}	*cat* inserted within *nuoM*	This Study
ΔNuoCDIM^{+}	*cat* replaces *nuoC-nuoD* deletion; + orientation *aphA-3* replaces *nuoI-nuoM* deletion	This Study
ΔNuoCDIM^{-}	*cat* replaces *nuoC-nuoD* deletion; – orientation *aphA-3* replaces *nuoI-nuoM* deletion	This Study
NuoN::CM^{+}	+ orientation *cat* inserted within *nuoM*	This Study
NuoN::CM^{-}	– orientation *cat* inserted within *nuoM*	This Study
7574MD	*Cj1575c*, *Cj1574c* merodiploid strain	This Study
Δ74MD	*aphA-3* inserted within *Cj1574c* in the *nuo* operon in 7574MD	This Study
ΔNuoMD	*aphA-3* inserted between *nuoC*, *nuoN* 7574MD	This Study
FldMD	*fldA* merodiploid strain	This Study
FldMD::CM	*cat* inserted within *fldA* (*Cj1382c*) in FldMD	This Study
OorB::CM	*cat* inserted within *OorB* (*Cj0537*)	This Study
FdxA::CM	*cat* inserted within *fdxA* (*Cj0333c*)	This Study
0369::CM	*cat* inserted within *Cj0369c*	This Study
E. coli		
DH5α	Cloning strain	Lab Stock
BL21 Rosetta	Expression strain	Lab Stock
GM2163	*dam*$^{-}$ strain	NEB

[a]NCTC – National Collection of Type Cultures
$^{+}$ Denotes *cat* / *aphA-3* in the same orientation as the gene
$^{-}$ Denotes *cat* / *aphA-3* in the opposite orientation as the gene

Table 2. Primers used in this study

Primers	Description	Source
NuoABF	5'-AAAAATCCTATGAGTTAAGCGGAG-3'	IDT[b]
NuoABR	5'-AGTTTTCAAAAGCGGATGTCC-3'	IDT
NuoCF	5'-CGCAAAGAAAAAGCAAGTCGC-3'	IDT
NuoCR	5'-AAATCAAGCATAACCCCGCC-3'	IDT
NuoDF	5'-TGATTGGTTTGGACATCCGC-3'	IDT
NuoDR	5'-AAAGCAAGCACTTCGCCTGC-3'	IDT
NuoF	5'-ATGAAGTGCCAAAAGGTGGTC-3'	IDT
NuoR	5'-AATGTTTATGTGGTTTATGCGTGTC-3'	IDT
NuoGF	5'-GCATAAACCACATAAACATTGGGG-3'	IDT
NuoGR	5'-CAAATCACAAATCATAGCCACACC-3'	IDT
NuoIF	5'-TTATGGAACGGAGTATTCGGG-3'	IDT
NuoIR	5'-GCCAAAAAAGCCTAAAACCACTAC-3'	IDT
NuoMF	5'-CCACTTCATACTTGGGCACCTAAG-3'	IDT
NuoMR	5'-GAAATAGCATTATCACTTCCACGC-3'	IDT
NuoNF	5'-CTTCATACTTGGGCACCTAAGG-3'	IDT
NuoNR	5'-CACAAAGAACAAAAATCATCACTCC-3'	IDT
HydF	5'-GTTTAGAAAAAAAATACATCCACGC-3'	IDT
HydR	5'-CATAGGCTCTTCAAAAGGTCCC-3'	IDT
1575F	5'-CGCATATGAGAAGGGTAGATTTAAGAAAAAGC-3'	IDT
1574R	5'-GGCTCGAGTTTTGCTTCCTTTATGCGAATTG-3'	IDT
FldProF	5'-GGCATATGTCAGTAGCAGTAATCTATGGT-3'	IDT
FldProR	5'-CCCTCGAGAGCAAAATAAGGTTTGATTTGTTC-3'	IDT
FldF	5'-GGTGCTAAAAAAATAGAACACTGGG-3'	IDT
FldR	5'-TGCTCAAAAAATCACAACTTCAGC-3'	IDT
OorF	5'-TCCGTCCGATTACTCTTTATCCAG-3'	IDT
OorR	5'-CCTCCTTCTTTTACACCCCCAC-3'	IDT
FdxF	5'-TACTCTCAGCAAAATCAGCCC-3'	IDT
FdXR	5'-TTGGATAAAAGAATACTACCGCTC-3'	IDT
0369F	5'-CTGCTTTGATTTTTTCTTGTCTGG-3'	IDT
0369R	5'-CACAGTCCTCACCACCTTTCT-3'	IDT
GyrAF-RT	5'-TGGTTGTAACTATCACACATCGTGG-3'	IDT
GyrAR-RT	5'-AATCATCATCATAAGTCGTAACGGC-3'	IDT
NuoHF-RT	5'-CACCTTTGATTTCTGCTATTTGTGC-3'	IDT
NuoHR-RT	5'-AAGTCCTGAAGTGCCGATGAC-3'	IDT
1574F-RT	5'-TGACGGGTTAGTGCTTTTTAGAG-3'	IDT
1574R-RT	5'-TCATTTTGCTTCCTTTATGCG-3'	IDT

[b]IDT - Integrated DNA Technologies, Coralville, IA

Table 3. Plasmids used in this study

Plasmids	Description	Source
pCR®2.1-TOPO®	Cloning vector	Invitrogen
pBluescriptII KS(+)	Cloning vector	Stratagene
pJMA-001	pGEM-T containing *cat* insert	Jay Andrus
pHP1	*aphA-3* containing construct	David McGee
pET-21a(+)	Cloning vector	Novagen
pKSΔRI	pBluescriptII KS(+) with destroyed *Eco*R I recognition site	This study
pNuoAB	*nuoA*, *B* cloned from WT into *Eco*R V digested pBluescriptII KS (+)	This study
pKSΔNuoAB::CM	*cat* inserted into *Sty* I and *Bcl* I digested pNuoAB	This study
pNuoC	*nuoC* cloned from WT into pCR®2.1-TOPO®	This study
pNuoC::CM	*cat* inserted into *Acc*B7 I digested pNuoC	This study
pNuoD	*nuoD* cloned from WT into pCR®2.1-TOPO®	This study
pNuoD::CM	*cat* inserted into *Bsa*M I digested pNuoD	This study
pNuoCD	*nuoC*, *D* cloned from WT into pCR®2.1-TOPO®	This study
pΔNuoCD::CM	*cat* inserted into *Acc*B7 I and *Cla* I digested pNuoCD	This study
pNuo	*nuoC*, *D*, *Cj1575c*, *Cj1574c*, *nuoG* cloned from WT into pCR®2.1-TOPO®	This study
pNuo::CM	*cat* inserted into *Cla* I digested pNuo	This study
pNuoG	*nuoG* cloned from WT into pCR®2.1-TOPO®	This study
pNuoG::CM$^+$	+ orientation *cat* inserted into *Eco*47 III digested pNuoG	This study
pNuoG::CM$^-$	– orientation *cat* inserted into *Eco*47 III digested pNuoG	This study
pNuoGI	*nuoG*, *H* and *I* cloned from WT into pCR®2.1-TOPO®	This study
pΔNuoGI::CM$^+$	+ orientation *cat* inserted into *Eco*47 III digested pNuoGI	This study
pΔNuoGI::CM$^-$	– orientation *cat* inserted into *Eco*47 III digested pNuoGI	This study
pNuoI	*nuoI* cloned from WT into pCR®2.1-TOPO®	This study
pNuoI::CM$^+$	+ orientation *cat* inserted into *Eco*47 III digested pNuoI	This study
pNuoI::CM$^-$	– orientation *cat* inserted into *Eco*47 III digested pNuoI	This study
pNuoIM	*nuoI*, *J*, *K*, *L*, and *M* cloned from WT into pCR®2.1-TOPO®	This study
pΔNuoIM::CM$^+$	+ orientation *cat* inserted into *Eco*47 III digested pNuoIM	This study
pΔNuoIM::CM$^-$	– orientation *cat* inserted into *Eco*47 III digested pNuoIM	This study
pNuoM	*nuoM* cloned from WT into pCR®2.1-TOPO®	This study
pNuoM::CM$^+$	+ orientation *cat* inserted into *Eco*47 III digested pNuoM	This study
pNuoM::CM$^-$	– orientation *cat* inserted into *Eco*47 III digested pNuoM	This study
pΔNuoIM::KAN$^+$	+ orientation *aphA-3* inserted into *Eco*47 III digested pNuoIM	This study
pΔNuoIM::KAN$^-$	– orientation *aphA-3* inserted into *Eco*47 III digested pNuoIM	This study
pNuoN	*nuoN* cloned from WT into pCR®2.1-TOPO®	This study
pNuoN::CM$^+$	+ orientation *cat* inserted into *Cla* I digested pNuoN	This study
pNuoN::CM$^-$	– orientation *cat* inserted into *Cla* I digested pNuoN	This study
pTOPO7574	*Cj1575c*, *Cj1574c* cloned from WT into pCR®2.1-TOPO®	This study
pET7574	*Cj1575c*, *Cj1574c* from pTOPO7574 inserted into Nde I/*Xho* I digested pET-21a(+)	This study
pET7574::CM	*cat* inserted into *Blp* I digested pET7574	This study
pHydA	*hydA* cloned from WT into pCR®2.1-TOPO®	This study
pHyd7574::CM	*Cj1575c*, *Cj1574c* and *cat* inserted into *Nde* I digested pHydA	This study
pNuo::KAN	*aphA-3* inserted into *Ssp* I digested pNuo	This study
pNuoCDN	*nuoN* from pNuoN inserted into *Bam*H I digested pNuoCD	This study
pΔNuoCDN::KAN	*aphA-3* inserted into *Acc*B7 I and *Cla* I digested pNuoCDN	This study
pTOPOFld	*fldA* cloned from WT into pCR®2.1-TOPO®	This study
pETFld	*fldA* from pTOPOFld inserted into Nde I/*Xho* I digested pET-21a(+)	This study
pETFld::KAN	*aphA-3* inserted into *Blp* I digested pETFld	This study
pHydFld::KAN	*fldA* and *aphA-3* inserted into *Nde* I digested pHydA	This study
pΔRIFld	*fldA* cloned from WT into *Eco*R V digested pKSΔRI	This study
pΔRIFld::CM	*cat* inserted into *Eco*R I digested pΔRIFld	This study
pOorB	*oorB* cloned from WT into pCR®2.1-TOPO®	This study
pOorB::CM	*cat* inserted into *Ssp* I digested pOorB	This study
pFdxA	*fdxA* (*Cj0333c*) cloned from WT into pCR®2.1-TOPO®	This study

Table 3 Continued

pFdxA::CM	*cat* inserted into *Bsa*M I digested pFdxA	This study
p0369	*Cj0369c* cloned from WT into pCR®2.1-TOPO®	This study
p0369::CM	*cat* inserted into *Bsa*M I digested p0369	This study

[+] Denotes *cat* / *aphA-3* in the same orientation as the gene
[-] Denotes *cat* / *aphA-3* in the opposite orientation as the gene

Table 4 – Generation times[a] of various strains in MH broth and MH broth + 20 mM formate

Strain	Condition	
	MH + Formate	MH
WT	1.59	2.48
Hyd:CM	1.14	1.81
NuoAB:CM	1.51	NG[a]
NuoC:CM	1.43	NG
NuoD:CM	1.57	NG
Nuo:CM	1.36	NG
NuoG:CM$^+$	1.33	NG
NuoG:CM$^-$	1.59	NG
NuoI:CM$^+$	1.62	NG
NuoI:CM$^-$	1.59	NG
NuoM:CM$^+$	1.73	NG
NuoM:CM$^-$	1.29	NG
NuoN:CM$^+$	1.60	NG
NuoN:CM$^-$	1.52	NG
ΔNuoCD	1.71	NG
ΔNuoGI$^+$	1.48	NG
ΔNuoGI$^-$	1.49	NG
ΔNuoIM$^+$	1.70	NG
ΔNuoIM$^-$	1.45	NG
ΔNuoCDIM$^+$	1.78	NG
ΔNuoCDIM$^-$	1.52	NG
Δ74MD	1.32	NG
ΔNuoMD	1.25	NG

[a]NG – No Growth (cultures failed to double twice in 30 hours).

Table 5. Transcription of *nuoH* and *Cj1574c* in various strains as measured by qRT-PCR

Strain	Transcript Abundance[a]	
	nuoH	*Cj1574c*
WT	2,410 ± 1,260	1,146 ± 220
NuoG::CM$^+$	288 ± 33	ND[b]
NuoG::CM$^-$	49 ± 11	ND
Δ74MD	29 ± 11	ND
ΔNuoMD	ND	3,250 ± 302

[a] expressed as mRNA copies / ng total RNA ± standard deviation
[b] Not Determined

Table 6 – Respiration rates[a] in strains (CFE) with various substrates

Strain	Formate	α-Ketoglutarate + CoA	NADH	NADPH
WT	33.87 ± 19.9	16.43 ± 3.6	2.57 ± 0.25	1.50 ± 0.12
ΔNuoMD	30.83 ± 19.0	3.70 ± 1.3	2.11 ± 0.49	1.56 ± 0.24
OorB::CM	23.67 ± 3.9	0.49 ± 0.32	ND[b]	ND

[a]Rates are expressed as nmoles of O_2 consumed/min/mg of protein
[b]ND – Not Determined

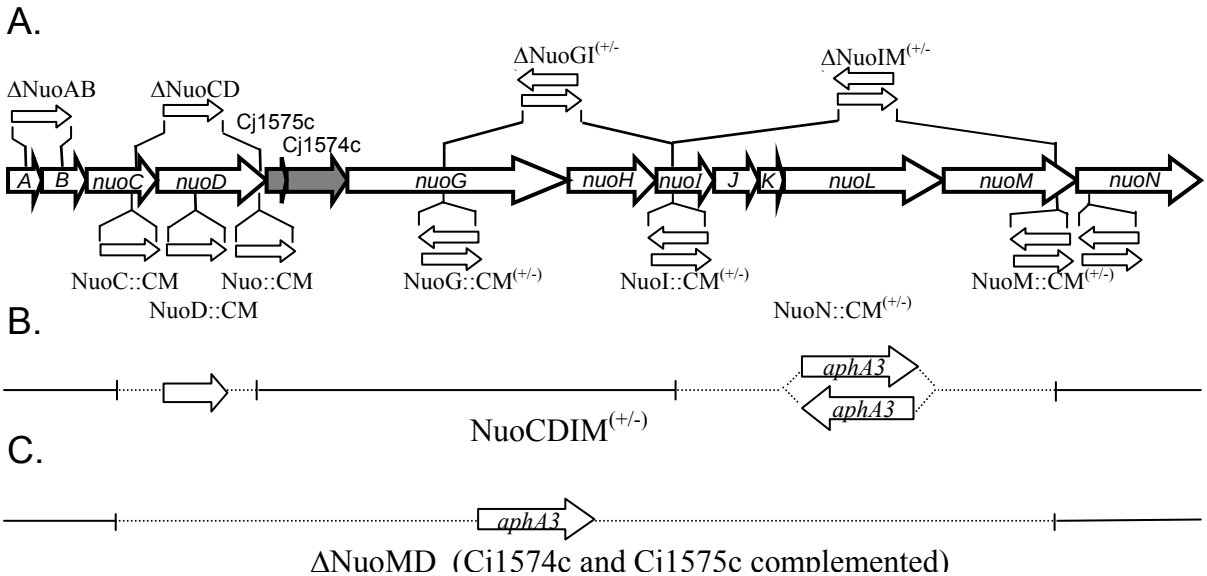

Figure 1: Genetic organization of the *nuo* operon and graphical representation of mutants. A. Deletions are indicated above the genes, sites of insertions are indicated below the genes. The arrow indicates the chloramphenicol resistance cassette *cat*, direction of the arrow indicates the orientation of *cat* within the gene. **B.** Representation of the deleted regions (dotted lines) in the double mutant ΔNuoCDIM^{+}. **C.** Representation of the deleted region (dotted line) in ΔNuoMD.

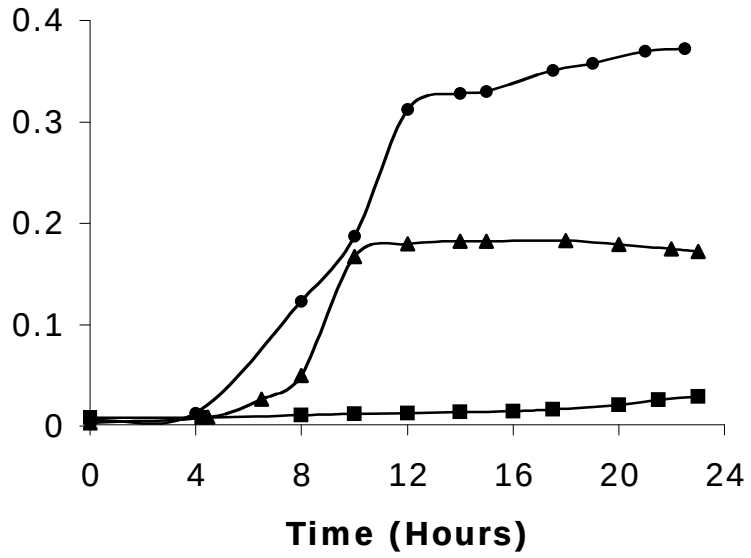

Figure 2: Growth curve of ΔNuoMD in MH broth in a microaerobic atmosphere (5% CO_2, 12% O_2, balance N_2). Conditions: unsupplemented MH broth (squares), MH broth + 10 mM formate (triangles), MH broth + 20 mM formate (circles). The Y axis is in a linear scale to show the difference in the terminal optical density.

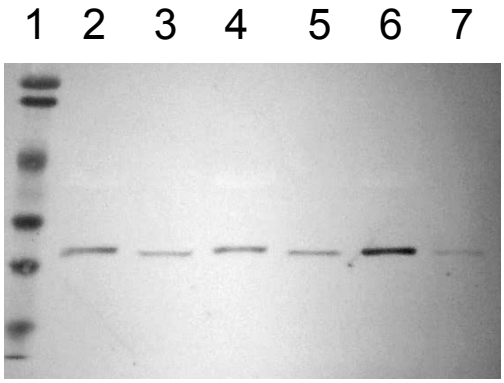

Figure 3: Immunoblot of *C. jejuni* extracts using anti-HIS primary antibody. Lane 1 - prestained low range standards composed of phosphorykase b (113 kDa), bovine serum albumin (92 kDa), ovalbumin (52.3 kDa), carbonic anhydrase (35.3 kDa), soybean trypsin inhibitor (28.7 kDa), and lysozyme (21.3 kDa); Lane 2 ,7574MD membrane fraction; Lane 3 , 7475MD soluble fraction; Lane 4, Δ74MD membrane fraction; Lane 5, Δ74MD soluble fraction; Lane 6, ΔNuoMD membrane fraction; Lane 7, ΔNuoMD soluble fraction.

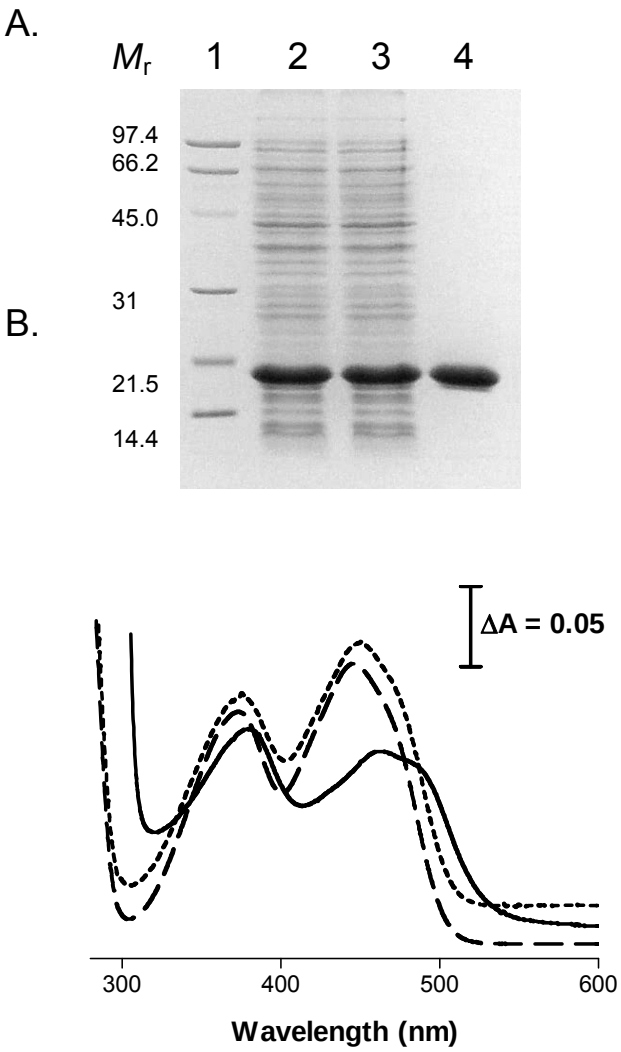

Figure 4: Purification of flavodoxin. **A.** SDS PAGE Lane 1- Low range standards (M_r of each standard is to the left of the gel). Lane 2, Crude extract; Lane 3, Supernatant of crude extract; Lane 4, Purified flavodoxin. **B.** Absorption spectra of purified flavodoxin (solid line), commercially purchased FMN (dashed line), and commercially purchased FAD (dotted line).

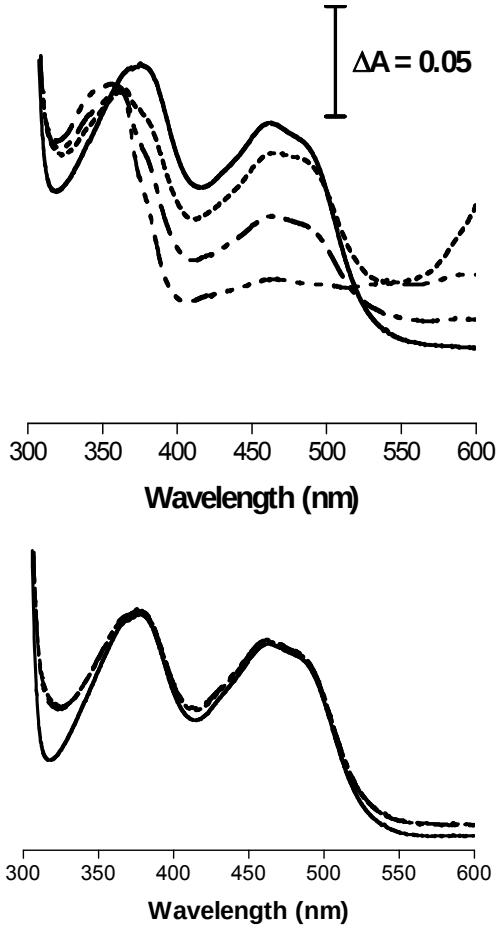

Figure 5: Reduction of flavodoxin with CFE and α-ketoglutarate and CoA. A. WT CFE. Spectrum with no addition (solid line), immediately after the addition of substrates (dashed line), 1 minute after the addition of substrates (dashed line with dots), and spectra run 5 minutes after the addition of substrates (dashed line with double dots). **B.** OorB::CM CFE. Spectra with no addition (solid line), immediately after the addition of substrates (dashed line), 1 minute after the addition of substrates (dashed line with dots) and spectra run 5 minutes after the addition of substrates (dashed line with double dots).

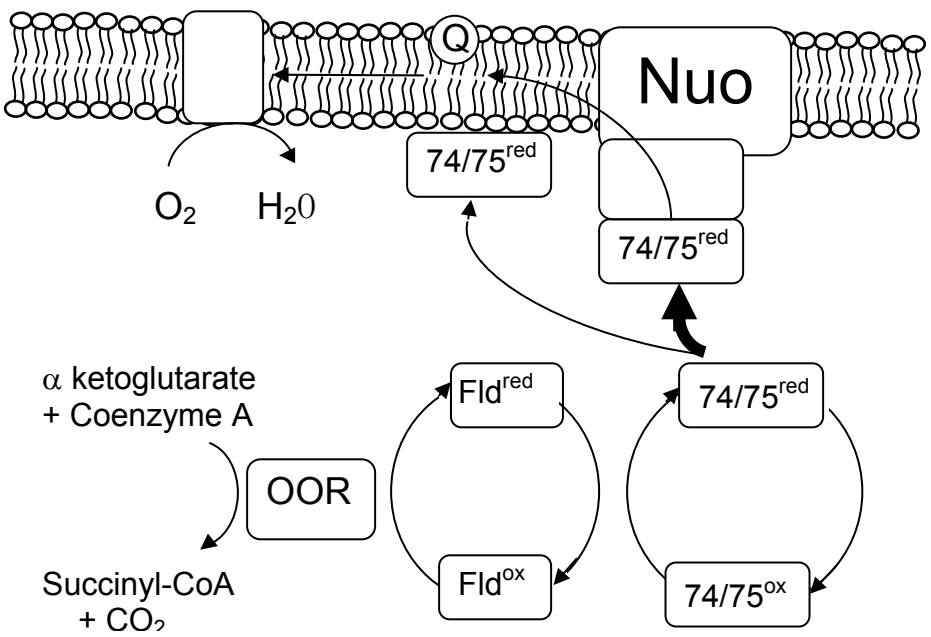

Figure 6: Model of the *C. jejuni* respiratory pathway through complex 1. Arrows indicate the flow of electrons.

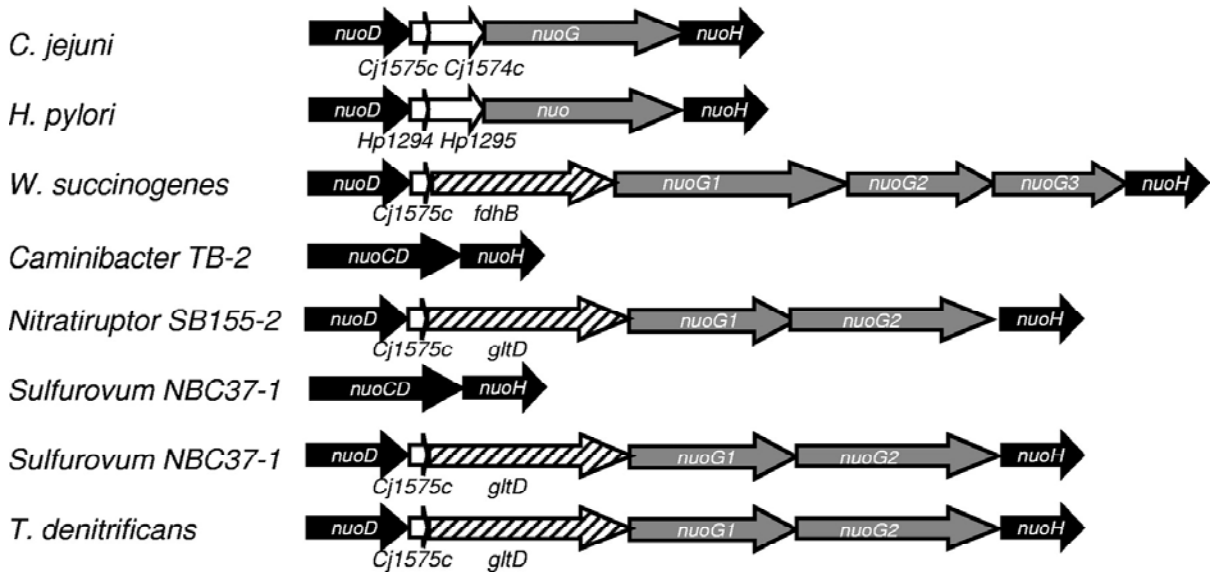

Figure 7: Genetic organization of the *nuo* operons from various ε-proteobacteria. Only the regions between *nuoD* and *nuoH* are shown, each bacterium also encodes *nuoA-C* and *nuoI-N*. References for each genome sequence: *C. jejuni*, (28); *H. Pylori* (35); *Wolinella succinogenes* (2); *Caminibacter mediatlanticus* TB-2 (37); *Nitratiruptor* SB155-2 and the two operons from *Sulfurovum* NBC37-1 (27); *Thiomicrospiria denitrificans* ATCC 33889 (9).

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CHAPTER 3

The Role of Respiratory Donor Enzymes in *Campylobacter jejuni* Host Colonization and Physiology

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ABSTRACT

The human pathogen *Campylobacter jejuni* utilizes oxidative phosphorylation to meet all of its energy demands. The genome sequence of this bacterium encodes a number of respiratory enzymes in a branched electron transport chain that predicts the utilization of a number of electron transport chain donor and accepter molecules. Three of these electron donor enzymes: hydrogenase, formate dehydrogenase, and 2-oxoglutarate:acceptor oxidoreductase (OOR), oxidize hydrogen, formate and α-ketoglutarate as electron donors, respectively. Mutations were created in these donor enzymes to isolate mutants in hydrogenase (HydB::CM), formate dehydrogenase (Fdh::CM), and OOR (OorB::CM) mutants, as well as a strain with insertions in both hydrogenase and formate dehydrogenase (Hyd:Fdh). These mutants are deficient in their respective enzyme activities and do not reduce the components of the electron transport chain when provided with their respective substrates. The presence of either hydrogen or formate in the media stimulated the growth of wild type (WT) *C. jejuni* (but not the associated mutant strains) and absolutely required for growth of the OOR mutant strain OorB::CM. Finally, the importance of hydrogenase, formate dehydrogenase and OOR as well as the complex I of *C. jejuni* are elucidated by chicken colonization assays, where the double mutant Hyd::Fdh, OorB::CM and *nuo* mutants are severely impaired in host colonization.

3.1 Introduction

Campylobacter jejuni is the leading cause of human bacterial gastroenteritis, campylobacteriosis both in the western and developing world [2, 5, 7, 19]. Although *C. jejuni* is considered a commensal in chickens and other avians, humans can suffer from campylobacteriosis when infected by the consumption of contaminated chicken products [37]. Contamination commonly occurs in processing plants due to the rupturing of the lower gastro intestinal tract, where *C. jejuni* is primarily found in birds [4, 12]. The preferred area for *C. jejuni* is the cecum, a pouch-like organ, that serves as a haven to multiple strains of bacteria, and allows for an anaerobic fermentation dominant lifestyle [16]. *C. jejuni* is well equipped to utilize fermentation byproducts, which include small organic acids, as carbon and energy sources [15, 20, 22, 30, 35]. Fermentation products such as lactate, formate [18, 28] and hydrogen [27, 28] produced by cecal microbes have been shown to serve as respiratory substrates in *C. jejuni* [10, 22, 35]. Since *C. jejuni* neither ferments nor contains an intact glycolytic pathway [26], oxidative phosphorylation serves as the major route for energy accumulation in this organism.

Experimentally, *C. jejuni* has been shown to exhibit hydrogenase [6, 10], formate dehydrogenase [10], and 2-oxoglutarate:acceptor oxidoreductase (OOR) activities [34], with the highest respiratory activities in terms of H^+/O ratios observed with hydrogen and formate [10]. NADH however, was shown to be a poor respiratory substrate [10]. These data were further validated when the genome sequence of *C. jejuni* NCTC 11168 was released [26], for it revealed the presence of genes that encoded for a hydrogenase, a formate dehydrogenase

and OOR, enzymes which will allow for the utilization of hydrogen, formate and α-ketoglutarate, respectively [26].

Hydrogenases are present in all domains of life and catalyze the reversible reaction $H_2 \Leftrightarrow 2H^+ + 2e^-$ [32]. Hydrogenases that catalyze the forward reaction are known as uptake hydrogenases while those that catalyze the reverse reaction are known as evolutionary hydrogenases [32]. When functioning in the forward (hydrogen oxidation) reaction, the electrons liberated will enter the electron transport chain contributing to the proton motive force. *C. jejuni* is known to contain a [Ni/Fe] hydrogenase and the genes that encode it are annotated as *hydABCD* (*Cj1267c-Cj1264c*) [26]. *hydA* (*Cj1267c*) encodes a 41.4 kDa small subunit protein which is a ligand to the Fe-S cluster, while *hydB* (*Cj1266c*) encodes the 63.5 kDa Ni/Fe active site [26]. *hydC* (*Cj1265c*) encodes the 26.7 kDa Ni/Fe-hydrogenase B-type cytochrome subunit, which is anchored in the membrane, while *hydD* (*Cj1264c*) encodes a 20.1 kDa protease involved in enzyme maturation [26]. The presence of the HydC cytochrome subunit in the genome predicts that hydrogenase in *C. jejuni* is a hydrogen uptake-type and would be energy conserving.

Formate dehydrogenase catalyzes the oxidation of formate to yield CO_2, protons and electrons. The electrons generated in this reaction will enter the electron transport chain thus contributing to the proton ion gradient. The *C. jejuni* formate dehydrogenase is encoded by the *fdhABCD* (*Cj1511c-Cj1508c*) operon [26]. *fdhA* (*Cj1511c*) encodes the 104 kDa selenocysteine containing large subunit while *fdhB* (*Cj1510c*) encodes the 23.7 kDa iron-sulfur subunit. *fdhC* (*Cj1509c*) encodes the 35.4 kDa putative formate dehydrogenase

cytochrome B subunit, and *fdhD* (*Cj1508c*) encodes the 28.6 kDa FdhD protein that is required for formate dehydrogenase activity [26].

OOR enzymes are involved in the decarboxylation of α-ketoglutarate (2-oxoglutarate) to form succinyl-CoA and CO_2 in the presence of CoA [11]. In *C. jejuni* OOR is encoded by the genes *oorDABC* (*Cj0535-Cj0538*) [26]. A mutant strain in the *oorB* gene termed OorB::CM has been isolated [34]. Previous studies have shown that α-ketoglutarate can be used as an electron donor in *C. jejuni* [22, 34, 35] while OorB::CM has been shown to be defective in OOR activity, and thus, deficient in α-ketoglutarate respiration [34].

C. jejuni is also known to contain a donor enzyme which is a proton pump called complex I (also termed NADH:ubiquinone oxidoreductase), that is broadly distributed among bacteria, archaea and the mitochondria of eukaryotes [8]. This complex is typically the first enzyme found in many respiratory chains and is known to catalyze the transfer of electrons from NADH to the quinone pool [8]. However, unlike most other organisms, in *C. jejuni* the donor to complex I has been identified as flavodoxin, rather than NADH [34]. Flavodoxin is the electron acceptor from OOR, and α-ketoglutarate respiration links the electron transport chain (ETC) through complex I. Mutations within complex I (*nuo* mutants) are only tolerated when the growing cultures are supplemented with an alternative electron donor such as hydrogen or formate [34], indicating the importance an intact respiratory chain in *C. jejuni* survival.

Thus far, the individual roles of the respiratory donor enzymes hydrogenase, formate dehydrogenase and OOR have not been characterized, and a suitable animal model study has also not been performed. The goal of this study was to characterize these enzymes and

determine their importance in the overall physiology of *C. jejuni*, as well as the role of each enzyme in host colonization. Host colonization assays were also performed with *nuo* mutant strains from a previous study [34] to test their ability in colonizing chickens. An animal model study such as chickens will be essential to identify genes that are important in host colonization, as it will pave the way for the eventual elimination of this microorganism from chickens.

3.2 Results

3.2.1 Growth of C. jejuni strains

When grown in Mueller Hinton (MH) broth under microaerobic conditions (5% CO_2, 12% O_2, balance N_2), Fdh::CM and CUMHyd::CM had generation times similar to that of their respective wild type (WT) strains NCTC 11168 [26] and CUM001 [21] (Table 3). Addition of the respiratory donor formate to NCTC 11168 and hydrogen to CUM001 cultures promoted growth as evidenced by the decrease in generation times (Table 3). The addition of formate to Fdh::CM and the addition of hydrogen to CUMHyd::CM did not promote growth indicating their inability to utilize these substrates (Table 3). OorB::CM displayed similar growth phenotypes to all the *nuo* mutants isolated previously [34]. It did not grow in MH broth (cultures fail to double twice in 30 h) or on plates (no isolated colonies) unless the growth medium was supplemented with an alternative respiratory donor such as formate (Figure 4) or hydrogen (data not shown). Growth was totally dependent on formate, and once the consumption of formate had taken place this mutant ceased to grow (Figure 4).

3.2.2 Cytochrome spectra

Membrane vesicle particles were prepared from NCTC 11168, Hyd::CM, Fdh::CM and Hyd::Fdh. Reduced minus oxidized difference spectra were obtained for each membrane preparation utilizing hydrogen, formate or dithionite as reductants and air as the oxidant. The spectra observed in WT NCTC 11168 *C. jejuni* membranes when exposed to hydrogen and formate indicate the reduction of cytochromes as evidenced by the formation of peaks (Figure 2A), and confirms the electrons released from the oxidation of these substrates are

destined to the electron transport chain. The cytochrome scans reveal an absorption peak of 551 nm (indicating the presence of reduced *c*-type cytochromes), and a prominent shoulder at 560 nm that indicates the presence *b*-type cytochromes (Figure 2A). Hyd::CM (Figure 2C) and Fdh::CM (Figure 2D) do not reduce membrane bound cytochromes in response to the addition of their respective substrates. Hyd::Fdh is defective in both of these enzymes and does not reduce membrane-bound cytochromes when exposed to either formate or hydrogen (Figure 2B). The addition of dithionite to the membrane preparations confirm that each of these strains retain a full complement of *b*- and *c*-type cytochromes (Figure 2B, C and D). α-Ketoglutarate has also been shown to be a respiratory donor in *C. jejuni* [22, 34, 35]. The OOR enzyme complex is soluble, so reduced minus oxidized spectra were performed on whole cell extracts rather than isolated membrane particles. The addition of 2-oxoglutarate and CoA to cell extract of NCTC 11168 initiated the reduction of the electron transport chain, as evidenced by the emergence of peaks at 421 nm, 524 nm, and 553 nm, characteristic of reduced cytochromes (Figure 3). Addition of α-ketoglutarate and CoA to cell extract of OorB::CM results in no cytochrome reduction (Figure 3). These data indicate that α-ketoglutarate is oxidized by OOR, and the electrons liberated are transferred to oxygen via the respiratory electron transport chain.

3.2.3 Respiratory activities of NCTC 11168 and mutant strains Hyd::CM, Fdh::CM, Hyd::Fdh and OorB::CM

Respiration rates were determined by oxygen uptake via a Clark-type oxygen electrode of NCTC 11168 and the mutant strains using hydrogen, formate and α-ketoglutarate as

substrates (Table 2). As previous studies have confirmed [10], the WT NCTC 11168 strain respired hydrogen and formate while the respective mutants were unable to utilize these substrates (Hyd::CM with H_2, Fdh::CM with formate and Hyd::Fdh with formate) (Table 2). No difference was observed in the NCTC 11168 and Hyd::Fdh strains when using α-ketoglutarate as a substrate (Table 2).

3.2.4 Chicken colonization of respiratory chain mutants

The NCTC 11168 WT strain, NCTC 11168 mutants identified in this study (Hyd::CM, Fdh::CM, Hyd::Fdh and OorB::CM) and NCTC 11168 mutants from a previous study (*nuo* mutants) [34] were evaluated for their ability to colonize chickens. Both the single mutants Hyd::CM and Fdh::CM colonized at slightly lower levels in comparison to the WT strain (Figure 5A). The colonization levels of the Hyd::CM strain were not statistically significant in comparison to WT, while the colonization levels of Fdh::CM were significantly lower. In comparison to both the WT as well the individual mutant strains, Hyd::Fdh displayed a statistically lower colonization level (Figure 5A). OorB:CM and all three *nuo* mutants were significantly decreased in colonization in comparison to the WT. However, ΔNuoGI$^+$ showed increased levels of colonization in comparison to Nuo::CM and ΔNuoCDIM$^+$ (Figure 5B, C ,D). No *C. jejuni* was isolated in the control birds that were given PBS as the inoculum (data not shown)

3.3 Discussion

C. jejuni is non fermentative and does not oxidize hexose sugars, nor does it express a complete glycolytic pathway [26, 31]. All of its energy demands must be met via oxidative phosphorylation and the ETC. *C. jejuni* contains a highly branched ETC able to utilize a variety of electron donors that include succinate, malate, lactate, sulfite, gluconate and α-ketoglutarate [6, 10, 22, 23, 25, 35] and is also versatile in extracting carbon and energy from amino acids [9, 14, 15, 20, 30]. The complex I in *C. jejuni* has also been shown to be an entry point of electrons into the respiratory chain, and flavodoxin, the electron acceptor from OOR has been shown to be the donor to complex I [34]. Furthermore, mutants in complex I have been shown to be unable to grow in MH broth unless supplemented with an alternative respiratory substrate such as hydrogen and formate [34]. Other studies have shown that both hydrogen [6, 10] and formate [10] serve as good energy substrates in *C. jejuni*. These findings were further verified when the genome sequence of *C. jejuni* 11168 was released since it encoded for a hydrogenase and formate dehydrogenase, two enzymes that can utilize hydrogen and formate respectively as substrates [26]. To date, however, the roles of hydrogenase and formate dehydrogenase enzymes have not been studied *in vitro* or *in vivo*. This study was an attempt to characterize these enzymes and show their importance as well as depict the significance of complex I and OOR in the physiology and host colonization of *C. jejuni*

Both formate and hydrogen greatly enhance the growth rates of *C. jejuni* when grown in MH broth. This is observed by the lower generation times witnessed in the presence of these substrates (Table 3). However, the presence of an intact complex I may prove to be an

advantage to the WT and mutants strains, Hyd::CM and Fdh::CM, since it may help to compensate for the loss of one donor enzyme such as either a hydrogenase and formate dehydrogenase. This can be further validated when considering that both Hyd::CM and Fdh::CM displayed similar growth rates in comparison to WT when grown in unsupplemented MH Broth (Table 3). In addition, none of the complex I (*nuo*) mutants can grow in an amino acid medium such as MH broth unless supplemented with an alternative respiratory donor (hydrogen or formate) [34], provides further evidence for this phenotype.

In order to investigate the phenotype of a mutation in both hydrogenase and formate dehydrogenase, a hydrogenase/formate dehydrogenase double mutant termed Hyd::Fdh was isolated. This mutant was defective in both enzymes and displayed no hydrogenase or formate dehydrogenase activity (Table 2, Figure 2B). This strain shows no growth defects in comparison to the NCTC 11168 WT strain in unsupplemented MH broth, and addition of either hydrogen or formate does not stimulate growth (data not shown). This provides additional proof that the lack of both these donor enzymes can be compensated by complex I when grown in MH broth. Growth of OorB::CM in MH broth however was dependent on the addition of an alternative electron donor such as formate (Figure 4). This is interesting because it coincides with the phenotype of *nuo* mutants which are growth dependent on the addition of formate as well [34], and provides further evidence that the electrons from OOR feed through the complex I of *C. jejuni*.

Further confirmation that both hydrogen and formate serve as respiratory substrates can be evidenced by the reduction of the components of the ETC. Typically in *C. jejuni*, the electrons generated from an electron donor are fluxed via a quinone to the cytochrome bc_1

complex (encoded by *petABC*), to a one of several periplasmic *c*-type cytochromes, and finally to a cbb_3 type terminal oxidase (*ccoNOPQ*) [14, 26]. The reduction of these components can be deduced by the exposure to substrates such as hydrogen and formate, whose oxidation results in the liberation of electrons that are fluxed through the ETC. Therefore, since both the hydrogenase and formate dehydrogenase activities are confined to the membranes [10], the reduction of the ETC in *C. jejuni* can be monitored by the reduction of cytochromes using their respective respiratory substrates (Figure 2), revealing the presence of *b*- and *c*-type cytochromes with characteristic peaks observed at 560 and 551 nm respectively. Hyd::CM, Fdh::CM and Hyd::Fdh, are unable to reduce these cytochromes (Figure 2B, C, D). These mutants do however express a full complement of cytochromes as shown with dithionite-reduced membranes, signifying a functional ETC in these strains (Figure 2B, C, D). α-Ketoglutarate respiration by *C. jejuni* has also been documented [22, 34, 35], and a mutant in OOR termed OorB::CM has been isolated [34]. OOR activity is not confined to membranes, however, by using cell extracts we show that α-ketoglutarate reduces the ETC in NCTC 11168 while OorB::CM is unable to reduce cytochromes in the presence of α-ketoglutarate (Figure 3).

Another measure of the importance of formate and hydrogen come from chicken colonization experiments. Although both the Hyd::CM mutant and Fdh::CM mutant showed only a modest decrease in their colonizing abilities in comparison to the NCTC 11168 WT strain (Figure 5A), the Hyd::Fdh double mutant was significantly more impaired in its colonizing ability. The chicken cecum provides an abundant source of hydrogen and formate [27, 28], which may prove to be an advantage to the individual mutants since Hyd::CM can

still utilize formate, and Fdh::CM can still deplete the hydrogen. Unable to oxidize either of these substrates in its ecological niche would prove to be a costly disadvantage to the Hyd::Fdh double mutant as the lack of these genes may weaken this strain to a greater extent that may result in other microorganisms out competing this *C. jejuni* mutant strain for colonization. However, the presence of a complex I may add a stimulus for the survivability of this double mutant strain in the chicken cecum. The colonization ability of the *C. jejuni* hydrogenase is a stark contrast to its fellow ε-proteobacterium *H. pylori*. Although *H. pylori* lacks a formate dehydrogenase, it does possess a set of hydrogenase genes similar to that of *C. jejuni* [1, 26, 29]. Studies by Olson and Maier have shown that the *H. pylori* uptake hydrogenase plays an important role in *in vivo* host colonization [24], where mutants of hydrogenase are significantly decreased in the ability to colonize mice.

The importance of both OOR and the complex I of *C. jejuni* are further elucidated by investigating the ability of OorB:CM and several complex I mutants (*nuo* mutants Nuo::CM, ΔNuoGI$^+$ and ΔNuoCDIM$^+$) isolated previously [34] in colonizing chickens. All three *nuo* mutants exhibited statistically lower colonization levels than the parent strain NCTC 11168 (Figure 5), indicating that a functioning complex I is required for efficient host colonization. However, there were differences between the *nuo* strains used in this study that may shed light on the importance of CJ1574, an essential protein in *C. jejuni* [34], in host colonization. Of the three tested *nuo* mutant strains, ΔNuoGI$^+$ was the least impaired, and the only mutant strain tested that would have wild type *Cj1574c* expression. This is because the *cat* cassette that replaces the *nuoG-nuoI* deletion in this strain is located downstream of *Cj1574c*, while both Nuo::CM and ΔNuoCDIM$^+$ contain the *cat* cassette (in the same transcriptional

orientation) upstream of *Cj1574c*, reducing transcription downstream about 8 fold [34]. The lower colonization levels displayed by Nuo::CM$^+$ and ΔNuoCDIM$^+$ imply that *Cj1574c* expression affects host colonization in *C. jejuni* and provides further insight of the importance of this gene. However, the fact that the ΔNuoGI$^+$ strain was a much more efficient colonizer than the two other *nuo* mutants is an indication that the *nuo* genes may not be vital for survival in the avian host, since this mutant has access to other electron donors abundant in the cecum, and further due to the fact that *Cj1574c* is expressed at normal levels. OorB:CM too displayed a deficiency in full colonization of chickens which may be attributed to the loss of flavodoxin reduction as well as the inability to use α-ketoglutarate as respiratory substrate. However, the chicken cecum has been identified as an anaerobic environment with the presence of anaerobic bacteria [3, 16, 28]. Flavodoxin reduction in OorB:CM can be compensated by the activity of PFOR playing an active role in near anoxic conditions. Additionally, since the chicken cecum harbors other electron donors such as formate, hydrogen and lactate [18, 27, 28] which serve as excellent respiratory donors in *C. jejuni* [10] will certainly prove to be of advantage for *C. jejuni* in chicken colonization. Importantly, the diminished levels of colonization witnessed in this mutant, stresses the importance that OOR plays a central role *in vivo*.

Mutating hydrogenase and formate dehydrogenase, OOR and complex I result in poor chicken colonization exemplifies the important role these enzymes may play in *C. jejuni's* physiology *in vivo*. The chicken cecum is a haven for many bacterial species including *C. jejuni*, and devising strategies that may help target these enzymes may prove worthy to the ultimate removal of this organism from the chicken. The evidence that both formate and

hydrogen are produced in the human colon [13, 17] may thus play a crucial role for the survival and infectivity of *C. jejuni* in humans, and is yet another reason why these genes may be important as objects for drug design in the future.

3.4 Materials and Methods

3.4.1 Bacterial strains and growth conditions

C. jejuni strains are listed in Table 1. Cells were grown on either tryptic soy agar (Difco) supplemented with 10% sheep blood (called BA plates) or in MH broth. Formate (5 mM, 10 mM, 20 mM and 25 mM for liquid culture and 20 mM for plates), chloramphenicol (25 μg/ml) and kanamycin (30 μg/ml) were added to the media as indicated. *C. jejuni* was routinely cultured microaerobically at 37°C in a tri gas incubator (model NU-4950, NuAire, Inc), in which the gas composition was constantly maintained at 12% O_2 and 5% CO_2 and balance N_2. For growth experiments of strains grown under a hydrogen atmosphere, cultures were grown in MH broth at 42°C under a constant stream of gas, which was continually sparged through a glass dispersion tube. The conditions were 12% O_2, balance N_2 and 12% O_2, 10% H_2 and balance N_2. Gas flow was controlled by flowmeters. 12% O_2 conditions were obtained by continually sparging cultures with a cylinder containing 12% O_2, 5% CO_2 and balance N_2 (blood gas). Under these conditions the flow meter read 100. MH broth was sparged with 90% blood gas and 10% H_2, as read by the flow meters to achieve 12% O_2 and 10% H_2 conditions. MH broth was allowed to equilibrate to both temperature and gas conditions for 10 minutes prior to inoculation. Genetic manipulations were performed with *Escherichia coli* strain DH5α. Luria-Bertani (LB) broth and agar supplemented with ampicillin (at 150 μg/ml), chloramphenicol (at 25 μg/ml) and kanamycin (at 30 μg/ml) were used for growing *E. coli* DH5α.

3.4.2 Cloning and construction of a hyd mutant, fdh mutant and hyd/fdh double mutant

Oligonucleotide primers (Table 1) for cloning genes of interest were designed from the sequenced strain *C. jejuni* NCTC 11168 [26] using the DNA analysis program MacVector. Polymerase chain reaction (PCR) amplification was performed with *Taq* DNA polymerase (Promega) and using *C. jejuni* 11168 genomic DNA as a template. Genomic DNA was isolated using the MasterPure™ Complete DNA & RNA Purification Kit (Epicentre® Biotechnologies). Hyd::CM was constructed as follows: The *hydB* gene was amplified by PCR, blunt ended with T4 DNA polymerase and phosphorylated with T4 polynucleotide kinase (Promega). This product was then cloned into the *Hin*d III site of pKSΔRI to yield pHydB. pKSΔRI was obtained by destroying the *Eco*R I site of pBluescript II KS (+)(Strategene). pHydB was digested with *Eco*R I to create a 263 bp deletion within *hydB*, blunt ended with T4 DNA polymerase, and a chloramphenicol acetyl transferase resistance cassette (*cat*) was inserted into the *Eco*R I sites of pHydB to obtain pHydB::CM. The *cat* cassette was removed from pJMA-001 by *Sma* I digestion. pJMA-001 was created by cloning the *cat* cassette from plasmid pRY111 [36] into the *Pvu* II site of pGEM-T Easy (Promega). pHydB::CM was then introduced into competent WT *C. jejuni* 11168 by electroporation to obtain HydB::CM. pHydB::CM was also naturally transformed into CUM001 to obtain CUM001::Hyd::CM. Fdh::CM was constructed as follows: A 999 base pair fragment of *fdhA* was amplified by PCR using NCTC 11168 WT genomic DNA and primers FdhAF and FdhAR. The PCR product was then cloned into the blunt ended *Hin*d III site of pBluescript II KS to yield pFdhA. pFdhA was digested with *Hin*d III creating a 69 bp deletion within *fdhA*, filled with T4 DNA polymerase, and ligated with the *cat* cassette to obtain pFdhA::CM. This

plasmid was naturally transformed into *C. jejuni* NCTC 11168 cells to isolate FdhA::CM. Hyd::Fdh double mutant was obtained by using the kanamycin cassette *aphA-3* [33]. This strain was isolated by insertion of *aphA-3* into the *Hin*d III sites of pFdhA to yield pFdhA::KAN, and electroporating this plasmid with electrocompetent Hyd::CM cells to obtain Hyd::Fdh. Insertion of the antibiotic cassettes were confirmed by genomic DNA isolation of all mutants strains and amplifying the relevant genes by PCR followed by subjection of the PCR products to agarose gel electrophoresis to observed the increase in size of the genes due to the insertion of the antibiotic cassettes (data not shown).

3.4.3 Transformation of C. jejuni.

C. jejuni electrocompetent cells were made by harvesting cells by swab from BA plates and washing the cells three times in an ice cold 9% sucrose and 15% glycerol solution. Cells were used immediately or frozen at -80°C for future use. Two transformation protocols were followed. For transformation using an electroporator, *C. jejuni* electrocompetent cells were incubated with 1-5 µg of plasmid DNA on ice for 10 minutes. The cells were then placed in a 2 mm electroporation cuvette, given a pulse of 2500 V in a ECM399 electroporator (BTX, San Diego, CA), and 50 µl of Mueller Hinton (MH) broth was added to the cuvette and subsequently incubated on ice for 10 minutes. The cells were then spotted onto a cold BA plate supplemented and incubated microaerobically at 37°C for 24 hours (12% O_2, 5% CO_2 and balance N_2). After 24 hours on non-selective media, the cells were transferred onto BA plates supplemented with chloramphenicol and incubated microaerobically. Resistant colonies were selected after 3-5 days incubation, and were

screened for recombination of the interrupted version of the gene by PCR of genomic DNA using primers against the targeted gene. The natural transformation protocol was conducted as follows: *C. jejuni* cells were harvested off a single BA plate into sterile PBS and the cell number was determined by measuring the Optical Density (OD) at 600 nm (OD 1 = 7.5 x 10^8 cells). A cell suspension of 5 x 10^8 was next spotted onto a BA plate and incubated microaerobically for 3 hours. Three hours post-incubation, the cells were spotted with 1-5 μgs of plasmid DNA and incubated microaerobically for 5 hours. After this incubation, the cells were transferred onto BA plates supplemented with chloramphenicol and incubated microaerobically. Resistant colonies were selected and screened for the recombination of the disrupted version of the gene by PCR.

3.4.4 H_2 uptake assays

H_2 uptake was measured amperoterically using a modified Clark-type electrode on whole cells. Ten Brucella agar plates supplemented with 10% defibrinated sheep's blood of strains *C. jejuni* CUM001 and CUMHyd::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 μLs of hydrogen sparged deionized water to 5 ml of stirred deionized water. The chamber was rinsed twice with deionized water. Five mls of the cell suspension was added to the chamber and allowed to equilibrate. Upon equilibration, 100 μL of hydrogen sparged water was added. The chamber and electrode were connected to a chart recorder and the hydrogen

uptake rate was calculated based on the slope of the disappearance of hydrogen. Hydrogen concentration was determined using the standard value of hydrogen dissolved in water at room temperature. The chamber was again rinsed twice with deionized water and the procedure was repeated for the mutant strain CUMHyd::CM. Activities were expressed as nanomoles of H_2 consumed per minute per 10^8 cells. Cell counts were obtained by OD 600 using the conversion factor 1 OD = $7.5x10^8$ cells.

3.4.5 O_2 uptake experiments

O_2 was quantified using a YSI Model 5300 Biological Oxygen Monitor (Yellow Springs instrument Co, Yellow Springs, OH) and Clark-type electrode. The electrode was inserted into a 5 ml capacity glass chamber that was continuously stirred. 5 ml of whole cells or CFE were added to the chamber and allowed to equilibrate until no change in dissolved O_2 was observed for several minutes. Upon equilibration, substrate was added through a capillary tube via a Hamilton syringe into the chamber and the dissolved O_2 was recorded by chart recorder. After each experiment the chamber was calibrated using known concentrations of dissolved O_2. Substrate concentrations used were: α-ketoglutarate and CoA, 5 mM and 0.5 mM, respectively; formate, 5 mM.

3.4.6 Preparation of membranes

Ten BA plates of each strain were harvested by sterile swab into cold PBS (pH 7.4) and washed twice with PBS, and resuspended to 10 mls in a 50 mM cold phosphate buffer (pH 7.0), 2.5 mM $MgCl_2$, 1 mM PMSF solution. The cells were broken by passage three times

through a French Pressure Cell (Thermo Spectronic) at 20,000 lbs/in^2 and the lysate was cleared of unbroken cells by centrifugation at (12,000 x g) for 5 minutes. The cleared supernatant was then subjected to fractionation by ultra centrifugation (150,000 x g for 90 minutes) in a Beckman L8-55 ultracentrifuge to isolate the membranes.

3.4.7 Difference spectral analysis of membranes

Membranes were cleared of the supernatant and resuspended by dounce homogenization in a 50 mM phosphate buffer (pH 7.0), 2.5 mM $MgCl_2$ solution. One ml each of this membrane suspension was added to two quartz cuvettes. One cuvette was sealed with a rubber stopper, while the unsealed air oxidized membrane suspension cuvette was used as a reference. The sealed cuvette was used to add various substances, and difference spectra from 600-450 nm were performed in a Shimadzu UV-1650PC spectrophotometer. For hydrogen experiments: the sealed quartz cuvette was sparged with 100% hydrogen for 15 minutes. For formate experiments: formate was added to a final concentration of 5 mM to the sealed cuvette. Dithionite-reduced spectra were obtained by adding several grains of sodium dithionite to the sealed cuvette.

3.4.8 Cytochrome reduction assays

Cells were harvested by swab into N_2 sparged phosphate buffered saline (PBS), washed once and sonicated in sealed tubes under N_2 to obtain cell extracts (CE). One ml each of CE was put into two quartz cuvettes, which were subsequently sealed with rubber stoppers, flushed with N_2 gas for 3 minutes, and then placed in a Shimadzu UV-1650PC spectrophotometer. A

baseline spectra was produced first without the addition of any substrates, and second with the simultaneous addition of a 5 mM final concentration of α-ketoglutarate and a 0.5 mM final concentration of CoA. Protein concentrations were determined by BCA™ protein assay kit (Pierce).

3.4.9 Chicken Colonization

Day old broiler chicks free of *Campylobacter* were obtained from the Lake Wheeler Poultry Facility, operated by the North Carolina State University Poultry Science Department. Chicks were housed for 21 days in isolation rooms at the Dearstyne Avian Health Center (Department of Poultry Science at North Carolina State University) using isolation brooder batteries (Petersime Incubator Co., Gettysburg, OH) that contained 10 chicks each. The birds were fed ad libidum with water and Purina Mills Start & Grow SunFresh Recipe Feed (Purina Mills LLC, St. Louis, MO). On day seven chicks were dosed with 0.1 ml of PBS containing 1 x 10^6 CFU of *C. jejuni*, negative control chicks were only given 0.1 ml of sterile PBS. Fourteen days post challenge the birds were euthanized by CO_2 asphyxiation. Approximately one gram of cecal contents were removed and assayed for the presence of these strains by plating serial dilutions of the cecal contents in PBS on BA plates supplemented with 10 μg/ml Trimethoprim, 40 μg/ml Cefoperazone, 40 μg/ml Vancomycin, and 100 μg/ml Cyclohexamide for the WT, Hyd::CM, FdH::CM and Hyd::Fdh strains. BA plates supplemented with these antibiotics and 20 mM formate for were used for the OorB::CM and *nuo* mutant strains. Samples from the chickens inoculated with the Nuo::CM strain and 8 out of 20 WT samples were incubated at 2% O2, 5% CO2, Balance N2 at 37°C.

All other samples were incubated at 37°C microaerobically. After 2 days of incubation, colonies were counted and the CFU/g of cecal content was calculated. The mutant strains Hyd::CM, Fdh::CM and Hyd::Fdh were further sub-cultured on BA plates supplemented with chloramphenicol, while OorB::CM and the *nuo* mutant strains were sub-cultured on BA plates supplemented with chloramphenicol and formate. The data were analyzed by use of a one-tailed Mann-Whitney test, and the 95% confidence interval. This study was conducted under the guidelines established by the North Carolina State Animal Care and Use Committee.

3.5 Acknowledgements

This work was supported by USDA-NRI Grant # 2004-04553.

Table 1. Strains, plasmids and primers used in this study

Strain	Description	Source
C. jejuni		
CUM001	Clinical isolate from a Guillian Barré Syndrome patient	[21]
CUMHyd::CM	*cat* inserted within *hydB* of CUM001	This Study
NCTC 11168	Parent strain for *C. jejuni* strains (WT)	NCTC[a]
Hyd::CM	*cat* inserted within *hydB*	This Study
Fdh::CM	*cat* inserted within *fdhA*	This Study
Hyd::Fdh	*cat* inserted within *hydB*, *aphA-3* inserted within *fdhA*	This Study
Nuo::CM	*cat* inserted within *nuoD*	[34]
ΔNuoGI$^+$	+ orientation *cat* replaces *nuoG-nuoI* deletion	[34]
ΔNuoCDIM$^+$	*cat* replaces *nuoC-nuoD* deletion; + orientation *aphA-3* replaces *nuoI-nuoM* deletion	[34]
E. coli		
DH5α	Cloning strain	Lab Stock
Plasmids		
pCR®2.1-TOPO®	Cloning vector	Invitrogen
pBluescriptII KS(+)	Cloning vector	Stratagene
pJMA-001	pGEM-T containing *cat* insert	Jay Andrus
pHP1	*aphA-3* containing construct	David McGee
pKSΔRI	pBluescriptII KS(+) with destroyed *Eco*R I recognition site	This study
pHyd	*hydB* cloned from CUM001 into *Hin*d III digested pKSΔRI	This Study
pHyd::CM	*cat* inserted into *Eco*R I digested pHyd	This Study
pHydB	*hydB* cloned from WT into *Hin*d III digested pKSΔRI	This Study
pHydB::CM	*cat* inserted into *Eco*R I digested pHydB	This Study
pFdhA	*fdhA* cloned from WT into *Hin*d III digested pBluescriptII KS (+)	This Study
pFdhA::CM	*cat* inserted into *Hin*d III digested pFdh	This Study
pFdhA::KAN	*aphA-3* inserted into *Hin*d III digested pFdh	This Study
pOorB	*oorB* cloned from WT into pCR®2.1-TOPO®	[34]
pOorB::CM	*cat* inserted into *Ssp* I digested pOorB	[34]
pNuo	*nuoC*, *D*, Cj1575c, Cj1574c, *nuoG* cloned from WT into pCR®2.1-TOPO®	[34]
pNuo::CM	*cat* inserted into *Cla* I digested pNuo	[34]
pNuoGI	*nuoG*, *H* and *I* cloned from WT into pCR®2.1-TOPO®	[34]
pΔNuoGI::CM$^+$	+ orientation *cat* inserted into *Eco*47 III digested pNuoGI	[34]
pNuoIM	*nuoI*, *J*, *K*, *L*, and *M* cloned from WT into pCR®2.1-TOPO®	[34]
pΔNuoIM::KAN$^+$	+ orientation *aphA-3* inserted into *Eco*47 III digested pNuoIM	[34]
Primers		
HydBF	5'-TGAATGCGGCTTTATTTTTACACG-3'	IDT[b]
HydBR	5'-TCAAGTGGTTGTTTTACATCAGCG-3'	IDT
FdhAF	5'-GTGAAGAAGCAGCAAAATGGAC-3'	IDT
FdhAR	5'-AAGGAAGCCCGTAATACTCGC-3'	IDT
OorF	5'-TCCGTCCGATTACTCTTTATCCAG-3'	IDT
OorR	5'-CCTCCTTCTTTTACACCCCCAC-3'	IDT
NuoF	5'-ATGAAGTGCCAAAAGGTGGTC-3'	IDT
NuoR	5'-AATGTTTATGTGGTTTATGCGTGTC-3'	IDT
NuoGF	5'-GCATAAACCACATAAACATTGGGG-3'	IDT
NuoIF	5'-TTATGGAACGGAGTATTCGGG-3'	IDT
NuoIR	5'-GCCAAAAAAGCCTAAAACCACTAC-3'	IDT
NuoMR	5'-GAAATAGCATTATCACTTCCACGC-3'	IDT

[a]NCTC – National Collection of Type Cultures;
[b]IDT - Integrated DNA Technologies, Coralville, IA

Table 2. Respiration rates[a] in strains with various substrates

	Substrate	
Strains	Formate	α-Ketoglutarate + CoA
NCTC 11168	18.06 ± 3.3	7.19 ± 2.0
Fdh::CM	0.60 ± 0.6	ND[b]
Hyd::Fdh	0.18 ± 0.02	7.50 ± 3.8

[a] Rates are expressed as nmoles of O_2 consumed/min/mg/protein
[b]ND – Not Determined

Table 3. Generation times[a] of various strains in MH broth and MH broth supplemented with either formate or hydrogen

	Generation time (h) under condition:			
Strain	No addition	10 mM Formate	25 mM Formate	10% Hydrogen
NCTC 11168	2.40 ± 0.17	0.76 ± 0.07	0.94 ± 0.11	ND[b]
Fdh::CM	2.50 ± 0.17	2.92 ± 0.17	4.03 ± 0.28	ND
CUM001	1.78 ± 0.09	ND	ND	0.96 ± 0.12
CUMHyd::CM	1.77 ± 0.38	ND	ND	1.38 ± 0.10

[a] Generation time expressed in hours ± standard deviation
[b]ND, not determined

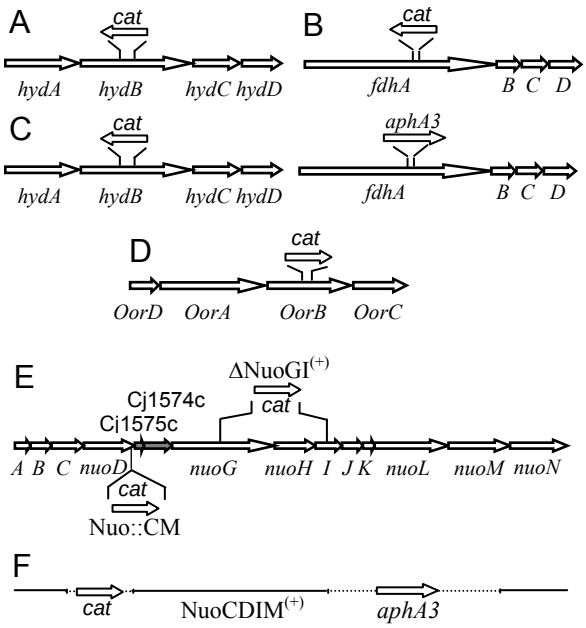

Figure 1. Graphical representation of mutants used in this study. Antibiotic resistance cassettes include the chloramphenicol resistance cassette *cat* and kanamycin resistance cassette *aphA3*: the direction of each antibiotic cassette indicates which orientation the cassette is in regard to the gene. **(A)** Hyd::CM and CUMHyd::CM **(B)** Fdh::CM **(C)** Hyd::Fdh **(D)** OorB::CM **(E)** Mutants from the *nuo* operon: the deletion mutant is shown above the *nuo* genes (ΔNuoGI^{+}), the insertion mutant is shown below the *nuo* genes (Nuo::CM) **(F)** Representation of the deleted Regions (dotted lines) in the double mutant ΔNuoCDIM^{+}.

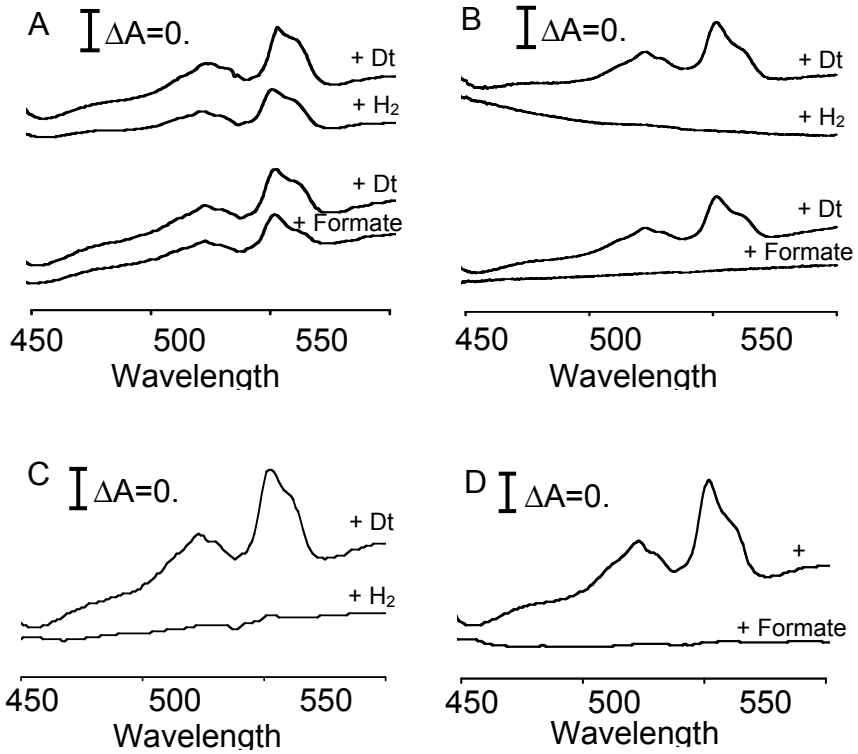

Figure 2. Reduced minus oxidized spectra on membranes isolated from **(A)** WT, **(B)** Hyd::Fdh, **(C)** Hyd::CM, **(D)** Fdh::CM

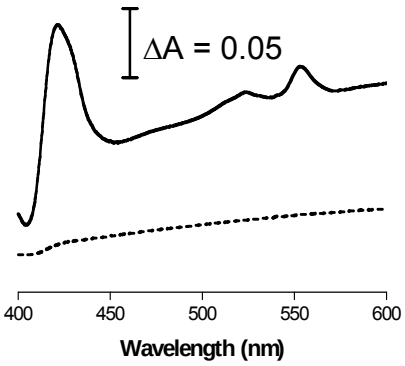

Figure 3: α-Ketoglutarate-dependent cytochrome reduction. **A.** WT CFE and CoA as substrates. **B.** OorB::CM CFE and CoA as substrates.

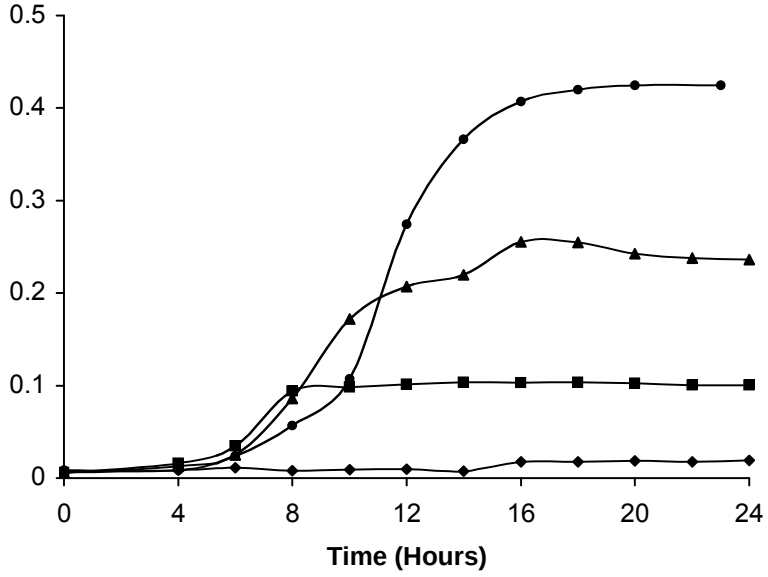

Figure 4. Growth curve of OorB::CM in MH broth in a microaerobic atmosphere (5% CO_2, 12%O_2, balance N_2). Conditions: unsupplemented MH broth (diamonds), MH broth plus 5 mM formate (squares), MH broth plus 10 mM formate (triangles), MH broth plus 20 mM formate (circles). The *y* axis is in a linear scale to show the difference in the terminal optical density.

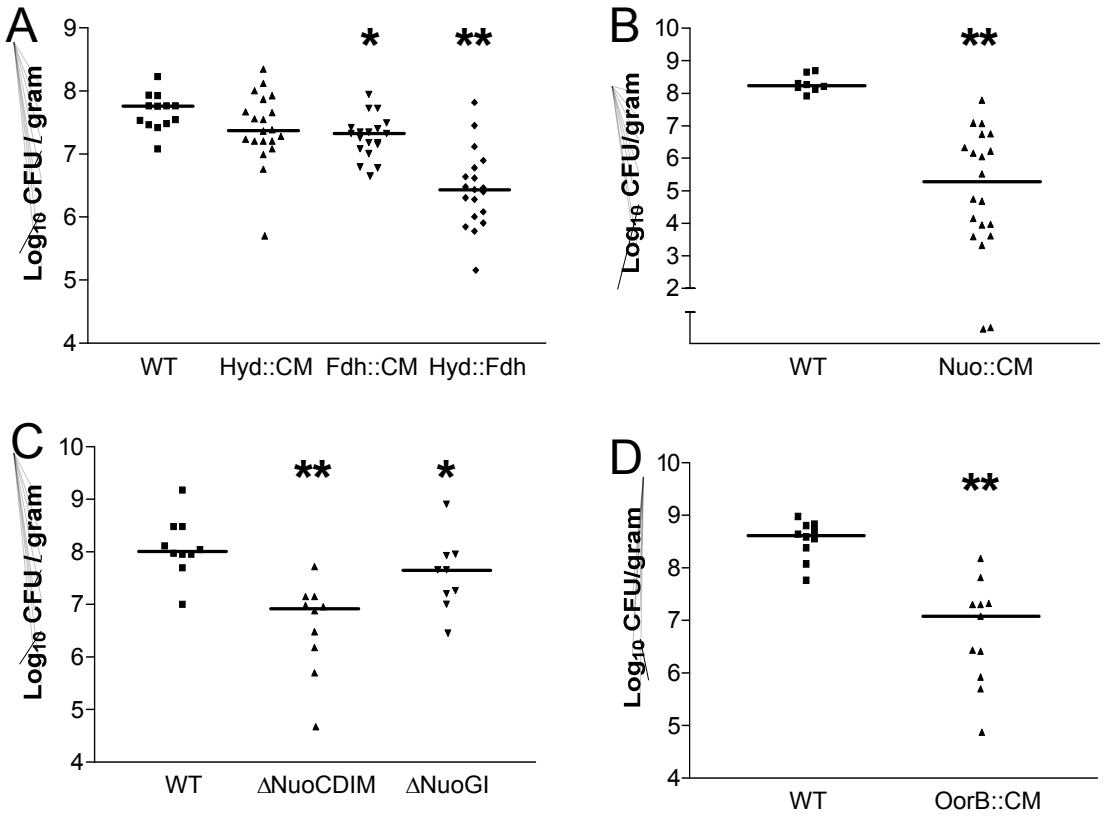

Figure 5: Chicken colonization abilities of various strains. (A) CFU/ gram of cecal contents of *C. jejuni* wild type (squares) and the Hyd::CM (triangles), and the Fdh::CM (inverted triangles), and Hyd::Fdh (diamonds) strains. (B) CFU/ gram of cecal contents of *C. jejuni* wild type (squares) and the Nuo::CM (triangles) strain. (C) CFU/ gram of cecal contents of *C. jejuni* wild type (squares) and the ΔNuoCDIM$^+$ (triangles) and ΔNuoGI$^+$ (inverted triangles) strains. (D) Nuo::CM (D) CFU/ gram of cecal contents of *C. jejuni* wild type (squares) and the OorB::CM (triangles) strain. The horizontal bars represent the median value for each group.

***,** $P < 0.05$ compared to the wild type (WT). ****** $P < 0.0001$ compared to the wild type.

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Appendix

***Cj1575c* is required for viability**

Previous studies conducted in our laboratory have shown that *Cj1574c* is an essential gene in *Campylobacter jejuni* (1). However, no attempts had been made previously to investigate whether *Cj1575c* was essential as well. Therefore, we attempted to mutate *Cj1575c* via a deletion mutation using the construct pΔ1575::CM. This plasmid contains an in-frame deletion of *Cj1575c* and a chloramphenicol resistance cassette (*cat*) that is in the same orientation as the *nuo* genes so that expression of *CJ1574c*, although reduced will still take place. However, despite many attempts mutating *Cj1575c* via a deletion mutation using WT *C. jejuni* NCTC 11168 proved to be unsuccessful. In order to determine whether *Cj1575c* was required for viability, we attempted to construct a mutant in *Cj1575c* in the *nuo* operon using the construct pΔ1575::KAN. This construct is identical to pΔ1575::CM, with the exception of it having an *aphA-3* cassette in place of *cat*.and using the 7574MD strain. This strain has an additional copy of *Cj1575c* and *Cj1574c* cloned into the coding region of *hydA* (1). When 7574MD was used as the parent strain, we were able to delete *Cj1575c* in the *nuo* operon to yield Δ75MD. Therefore, like *Cj1574c*, it seems that *Cj1575c* is required for viability as well.

Cloning and construction of Δ75MD

Primers P1DF and P275R (Table 1) were used to amplify a region of *nuoD* and the beginning of *Cj1575c*, while primers P375F and P474R (Table 1) were used to amplify a region of the end of *Cj1575c* and *Cj1574c* using WT genomic DNA to yield NuoD Pcr #1 and 1574 Pcr #2 respectively. Since the primers P275R and P375F were engineered to have *Sma* I restriction

sites, NuoD Pcr #1 and 1574 Pcr #2 would each contain a *Sma* I restriction site at the end and beginning of the PCR products respectively. PCR products, NuoD Pcr #1 and 1574 Pcr #2, were then used as a template along with primers P1DF and P274R to amplify a region of *nuoD* and *Cj1574c* to obtain the PCR product Δ*75*, which now contains a *Sma* I restriction site with an in-frame deletion of *Cj1575c*. This product was cloned into pCR2.1-TOPO to yield pΔ75. This plasmid was then digested with Sma I, and a *cat* cassette as well as an *AphA-3* cassette was cloned separately to yield the plasmids pΔ75::CM and pΔ75::KAN respectively.

Transformation of *C. jejuni*: for Δ75MD

C. jejuni electrocompetent cells were incubated with 1-5 μg of plasmid DNA on ice for 10 minutes. The cells were then placed in a 2 mm electroporation cuvette, given a pulse of 2500 V in a ECM399 electroporator (BTX, San Diego, CA), and 50 μl of MH broth was added to the cuvette and subsequently incubated on ice for 10 minutes. The cells were then spotted onto a cold BA plate supplemented with formate and sodium nitrate, and incubated for 24 hours in an anaerobic jar containing an Anaerobic BBL™ GasPak Plus™ with palladium catalyst (anaerobic pack). After 24 hours on non-selective media, the cells were transferred onto BA plates supplemented with chloramphenicol, formate and sodium nitrate and incubated in an anaerobic jar with an anaerobic pack. Resistant colonies were selected after 3-5 days incubation, and the colonies were screened for recombination of the interrupted version of the gene by PCR of genomic DNA using primers against the targeted gene.

Table 1. Strains, plasmids and primers

Strain	Description	Source
C. jejuni		
NCTC 11168	Parent strain for *C. jejuni* strains (WT)	NCTC[a]
7574MD	*Cj1575c* and *Cj1574c* merodiploid strain	(1)
Δ75MD	*aphA-3* inserted into the deleted region of Cj1575c in 7574MD	This Study
E. coli		
DH5α	Cloning strain	Lab Stock
Plasmids		
pCR®2.1-TOPO®	Cloning vector	Invitrogen
pJMA-001	pGEM-T containing *cat* insert	Jay Andrus
pHP1	*aphA-3* containing construct	David McGee
pΔ75	*nuoD*, ΔCj1575c, Cj1574c cloned from WT into pCR®2.1-TOPO®	This Study
pΔ75::CM	*cat* inserted into *Sma* I digested pΔ75	This Study
pΔ75::KAN	*aphA-3* inserted into *Sma* I digested pΔ75	This Study
Primers		
P1DF	5'-GTGTGATGTTAAGAGGAAGTGGCA-3'	IDT
P275R	5'-CCAATCCCCGGGTACCCTTCTCATCGATCTATCTCA-3'	IDT
P375F	5'-AGGGTACCCGGGGATTGGACTATTGTTATAAAAAAGGAG-3'	IDT
P474R	5'-CGAATTGGAGTAAAATCAAAACCATTA-3'	IDT

[a]NCTC – National Collection of Type Cultures;
[b]IDT - Integrated DNA Technologies, Coralville, IA

References

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