ABSTRACT

WEINGARTEN, REBECCA ANN. The Characterization of *Campylobacter jejuni* Respiratory Oxidases and Reductases and their Role in Host Colonization. (Under the direction of Jonathan Olson).

Campylobacter jejuni is a foodborne human pathogen responsible for the gastroenteritis disease, campylobacteriosis. *C. jejuni* is a commensal microorganism in the digestive tract of birds and the majority of campylobacteriosis cases are linked to poultry products. Although the natural habitat of *C. jejuni* is the anaerobic ceca of birds, *C. jejuni* has long been considered a microaerophile and the requirement of oxygen is mandatory for growth. Surprisingly, the genome sequence of *C. jejuni* contains genes that should allow the bacterium to utilize anaerobic respiration. In order to characterize the respiratory chain, mutants were constructed in each of the following enzymes: nitrate reductase (*napA*::Cm), nitrite reductase (*nrfA*::Cm), SN oxide reductase (Cj0264c::Cm), cbb_3-terminal oxidase (*ccoN*::Cm), *bd*-terminal oxidase (*cydA*::Cm), and fumarate reductase (*frdA*::Cm).

Both oxidase mutants grow on plates, but *ccoN*::Cm is extremely oxygen sensitive. *C. jejuni* is able to grow anaerobically utilizing nitrate, nitrite, DMSO and TMAO as alternative electron acceptors; mutants in each enzyme are deficient in growth on their respective substrates. Fumarate is not an alternative electron acceptor for *C. jejuni*. Compared to the wild type strain, *frdA*::Cm had an affected growth phenotype under microaerobic conditions; it was only able to utilize half of the TCA intermediates for biomass. Because both fumarate reductase and succinate dehydrogenase can interconvert fumarate and succinate, a mutant was constructed in the succinate dehydrogenase (*sdhA*::Cm). Both enzymes equally contribute to fumarate reductase activity; however, *frdA*::Cm had no succinate

dehydrogenase activity and was unable to perform succinate-dependent respiration. It was concluded that the enzyme annotated as a fumarate reductase was the sole succinate dehydrogenase, which acts as a respiratory electron donor.

Wild type and mutant strains were assessed for their ability to colonize poultry. *cydA*::Cm and Cj0264c::Cm colonized similarly to wild type, while *frdA*::Cm, *napA*::Cm, and *nrfA*::Cm were significantly deficient in colonization ability. *ccoN*::Cm was completely unable to colonize the avian host. We concluded that oxygen is the most important respiratory acceptor to *C. jejuni* both *in vitro* and *in vivo*.

The Characterization of *Campylobacter jejuni* Respiratory Oxidases and Reductases and their Role in Host Colonization.

by
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A dissertation submitted to the Graduate Faculty of
North Carolina State University
In partial fulfillment of the
Requirements for the Degree of
Doctor of Philosophy

Microbiology

Raleigh, North Carolina

2008

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BIOGRAPHY

Rebecca Ann Weingarten was born May 16, 1981 in Stevens Point, WI. She attended area schools and graduated from Pacelli High School in 1999. From there, Rebecca began her undergraduate career at the University of Wisconsin, Madison where she eventually decided on a biology major. In her sophomore year at UW-Madison, she began to work in a Plant Pathology laboratory under the direction of Dr. Andrew Bent. It was in this laboratory that Rebecca became exposed to the exciting world of scientific research. A postdoctoral research fellow in the lab, Dr. Christine Pfund, encouraged Rebecca to pursue a graduate degree. In May 2003, she received her Bachelor of Science from UW-Madison, and in July moved to Raleigh, NC to pursue a PhD in Microbiology in Dr. Jonathan Olson's laboratory at North Carolina State University. Eventually, Rebecca came to appreciate *Campylobacter jejuni* and those never ending growth curves. More importantly, however, she made lifelong friends at NC State. Following completion of her PhD, Rebecca will move to Washington, DC and work under the direction of Dr. Alison O'Brien at the Uniformed Services University of Health Sciences studying uropathogenic *E. coli* and toxin production.

ACKNOWLEDGEMENTS

I would like to thank Dr. Olson for his guidance, encouragement, advice and scientific knowledge. Your excitement about microbiology and your enthusiastic approach to draw out every metabolic pathway imaginable helped me to succeed in your lab. I would like to thank the Microbiology department, specifically my committee members, who helped me out whenever I got discouraged. To my labmates, Dilan and Mike, thank you for all of your support, friendship and very competent cell preps. To my NCSU friends: thank you for your ability to refrain from science talk while drinking beer. Most importantly, I would like to thank my family in Wisconsin. Thank you, Mom and Dad. Those yearly dentist and eye appointments kept me healthy, and those countless phone calls kept me sane. Thank you to my sisters, Gretchen and LB, as well as my friends scattered across the country: many of you did not understand half of what I was talking about, but you listened just the same.
Thank you.

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CHAPTER 1

Literature Review

Campylobacter jejuni

Campylobacter jejuni is the causative agent of campylobacteriosis, a bacterial gastroenteritis disease. It is a gram-negative, spiral shaped motile bacterium. *Campylobacter* species are members of the epsilon proteobacteria class and the order Campylobacterales, which is further divided into two families: Helicobacteraceae, composed of *Helicobacter* and *Wolinella* species and Campylobacteraceae, which includes *Campylobacter* species. *Wolinella succinogenes* is a facultative anaerobic, commensal ruminant whose genome was sequenced in 2003 (6). *Helicobacter pylori* is the causative agent of peptic and duodenal ulcers (56) and its genome was sequenced in 1997 (96). The annotated genome sequence of *C. jejuni* NCTC 11168 was published in 2000 (72). This strain was originally isolated in 1977 from feces of a patient experiencing diarrheal symptoms (29). The *C. jejuni* genome was predicted to encode for 1654 coding sequences (CDSs) and contained minimal repeated DNA sequences (72). In 2007, the *C. jejuni* NCTC 11168 genome was re-annotated and re-analyzed, which reduced the number of CDSs to 1643 (35). While *C. jejuni* and *H. pylori* are phylogenetically similar in reference to 16S rRNA sequence, *C. jejuni* has only 55% orthologous genes in *H. pylori* that results in highly divergent genes associated with survival, transmission and pathogenesis (72). Recently, more sequenced genomes of *C. jejuni* strains have been published and more are being assembled, which have provided scientists an excellent resource for continuing research on this fastidious pathogen (25, 37, 74).

C. jejuni is a foodborne human pathogen that causes bacterial gastrointestinal disease, which affects millions of people each year (4). It is a global issue in both developed and developing

nations. The World Health Organization (WHO) estimates that 1% of the inhabitants of Western Europe will be infected each year (38). The United States has an estimated two million cases every year (83). In developing nations, campylobacteriosis primarily affects children and according to the WHO, is the number one cause of bacterial gastroenteritis for children under the age of two (19). In addition, the incidence of campylobacteriosis is higher in HIV/AIDS patients, and the HIV/AIDS rates in developing nations is higher than developed nations (19). *C. jejuni* infection can be found in all ages, but children, the elderly, males, and those with a compromised immune system are most at risk. Seasonal variation has been noted for campylobacteriosis, with peaks during the summer months (27).

As early as the 18th century, spiral shaped bacterium were seen in the intestines of children who died from 'cholera infantum' (15). Unfortunately, culturing attempts were unsuccessful and documentation of the disease was ignored. By the 20th century, *Campylobacter* infections were considered a veterinarian disease by what is now classified as *Campylobacter fetus*, which causes abortions in ewes and bovines (57, 91). It was not until the 1950s that human *Campylobacter* infections were well documented, and while they could be visualized microscopically, standard culturing methods were still unable to isolate the microorganism. Then in 1968, a pure culture was isolated through multiple filtration steps of feces that were eventually passed onto selective media (23). From there, *C. jejuni* and *C. coli* were isolated from children experiencing diarrheal symptoms, and these symptoms disappeared with the administration of erythromycin (16). Later the development of more stringent selective media as well as microaerophilic incubation at 43°C made the filtration technique obsolete

(88). By the mid-1980s, *C. jejuni* was recognized as the causative agent of the most common form of gastroenteritis worldwide (15).

Disease manifestation

Campylobacteriosis is a self-limiting, acute gastrointestinal disease that occurs two to five days after the ingestion of *Campylobacter* species (15). Approximately, 80 to 85% of infections are caused by *C. jejuni*, and *C. coli* is associated with 10-15% (63). According to a volunteer study in the 1980s, as well as a scientist's own personal account, the disease can occur from ingestion of as few as 5 to 800 organisms (11, 81). Characteristic symptoms include diarrhea, abdominal cramping and fever. While the variety of symptoms associated with campylobacteriosis is not extensive, the severity of these symptoms is wide ranging. Mild cases will result in loose or watery diarrhea and mild fever; some patients report little diarrhea, but extensive cramping, which can often be misdiagnosed with acute abdomen and unnecessary surgical procedures are performed (3). More critical cases result in severe inflammatory diarrhea, with stool that contain blood, mucus, and inflammatory exudates with leukocytes (15). The symptoms can persist from one to seven days, but *Campylobacter* can be isolated several weeks after the symptoms have abated (47). Complications of *C. jejuni* infection include intestinal diseases such as cholecystitis, pancreatitis, peritonitis, and gastrointestinal hemorrhage. Extraintestinal manifestations include meningitis, endocarditis, septic arthritis, osteomyelitis and neonatal sepsis, but these diseases are very rare (3). The most serious post-infection complication associated with *Campylobacter* infection is Guillain-Barré syndrome (GBS) (3). For approximately every 1000 cases of

campylobacteriosis, one will develop into GBS (2). GBS is a temporary paralytic disorder that is marked by ascending paralysis from the demyelination of the peripheral nervous system. Antigenic molecular mimicry is believed to be the mechanism in how *C. jejuni* infections lead to GBS as one of the serotypes of *C. jejuni*, PEN19, has similar oligosaccharides to the GM1 ganglioside of the peripheral neuron membrane (105).

Methods of infection

C. jejuni is a commensal microorganism in the digestive tract of birds, both wild and domestic, and poultry represents the largest source of human *C. jejuni* infection. Campylobacteriosis has also been associated with the consumption of contaminated water supplies in both rural and urban settings in developed nations. To a lesser degree, consumption of unpasteurized milk can lead to *C. jejuni* infection (47). Globally, 3-50% of campylobacteriosis cases are linked to foreign travel (15). Person to person transmission is rare, but there have been reports of pet to human transmission (15). Because commercial poultry flocks are a large reservoir for *C. jejuni*, the consumption of undercooked poultry, improper handling of raw meat and cross-contamination are major routes of *C. jejuni* infection.

C. jejuni is transmitted horizontally in poultry. Chicks are born "*Campylobacter*-free" but are usually colonized by day ten. How these poultry become infected with *C. jejuni* remains unclear. External environmental contaminants seem to be a likely source. Studies have found identical subtypes of *Campylobacter* in puddles before placement of chicks in broiler

houses (13). Other contamination sources are wild and domestic animals, and contaminated water supplies. The process of "thinning" results in multiple contamination sources. For economic purposes, farmers overfill their broiler houses and then remove 10-50% of the birds after four to five weeks to comply with legal limitations. *Campylobacter* can be transported through a number of avenues during the thinning process, including crates, vehicles, as well as boots and gloves worn by workers (38). *Campylobacter* is excreted in the feces, and since poultry are coprophagic, the whole flock will be contaminated, once the bacteria are introduced to the flock. Within a week of detection, *Campylobacter* can be isolated from the feed, water, air, and drinkers (13). It is estimated that 90% of all flocks in the United States are infected with *Campylobacter* (94).

C. jejuni will colonize the ceca of inoculated poultry (10, 67), and *C. jejuni* has been naturally found in the ceca, thymus, spleen, gallbladder and liver (21). In intact birds, colonization is limited to internal organs and *C. jejuni* has not been recovered from the meat or skin of poultry pre-harvest, yet, *C. jejuni* can be isolated from every part of a chicken post-harvest. This discrepancy can be explained by the automated equipment that is used for processing steps such as scalding, plucking and evisceration of poultry, which results in the diffusion of pathogens normally contained in the digestive tract (63). The slaughtering and processing actions can increase bacterial counts on carcasses up to 1000-fold (93). *C. jejuni* is able to survive on fresh, chilled or frozen meat for long periods of time (15). In fact, *Campylobacter* can be recovered from meat that has been refrigerated for seven days (45). Surveys worldwide have been performed on retail meat for the detection and isolation of

pathogens. *C. jejuni* is a prevalent pathogen on poultry products found in retail stores; *C. jejuni* isolates can be recovered in upwards of 70% of poultry tested in grocery stores in the United States (107). In New Zealand, the presence of *Campylobacter* species in retail stores is even more significant. Not only has *C. jejuni* been isolated from the external packaging of chicken, nearly 90% of chicken tested were found to be contaminated with *C. jejuni* and *C. coli* (104).

Chicken microflora

C. jejuni colonizes the intestinal tract of chicks, in particular the ceca (10). The ceca are paired blind ended sacs at the junction of the small and large intestine (18). Newly hatched chicks can be experimentally infected with *C. jejuni* with as little as 35 CFU (92), and a fully colonized bird contains up to 10^9 CFU/g cecal content. The function of the ceca is multi-fold, including: fermentation of cellulose and other food products, absorption of nutrients and water, and the utilization of nitrogenous products, all of which are made available by the action of bacteria and fungi (18). The predominant culturable bacteria are obligate anaerobes greater than 10^{11} per gram of cecal content, and facultative anaerobes rarely occur above 10^9/g (7, 8). However, it is believed that culturable bacteria only represent a fraction of total bacteria. Recent advances in molecular methods have given a clearer picture to the microbial makeup of the cecum. The cecal ecology is an ever changing environment, which can be altered by multiple factors, and can take up to six weeks to become established (7); whereas the small intestine will have an established adult normal gut flora by two weeks (5). 16S rRNA sequences have revealed that the ceca are predominated by *Clostridiaceae*-related

bacteria (65%), followed by *Fusobacterium* (14%), *Lactobacillus* (8%), and *Bacteroides* (5%), which all represent facultative or obligate anaerobes (54). The anaerobic metabolism of the cecal community leaves the cecum rich in fermentation products as well as undigested amino acids (58). Anaerobes isolated from the ceca formed lactate, acetate, hydrogen and formate (82). Parsons studied the amino acid composition of the ceca by comparing amino acid excretion of intact and caecectomized laying hens. He found that the caecectomized hens excreted more amino acids, in particular, the excretion of aspartate, threonine, proline, valine, isoleucine, leucine and lysine than the control hens (73).

Amino acid catabolism

The NCTC 11168 sequenced genome revealed the inability of *C. jejuni* to utilize glycolysis because it is missing two key kinases: glucokinase, which converts glucose to glucose 6-phosphate and 6-phosphofructokinase, which is required for the conversion of fructose 6-phosphate to fructose 1,6-diphosphate (72). It does have the reversible enzymes to perform gluconeogenesis, however (72). *C. jejuni* encodes for a complete cyclic oxidative tricarboxylic acid (TCA) cycle, but 2-oxoglutarate is converted to succinyl CoA by 2-oxoglutarate ferredoxin oxidoreductase encoded by OorDABC (Cj0535-0538) instead of the more typical 2-oxoglutarate dehydrogenase (72).

Because *C. jejuni* is unable to use glycolysis, anapleurotic reactions are especially important alternative pathways for the replenishment of TCA intermediates. Pyruvate caboxylase encoded by *pycA* (*Cj*1037c) and *pycB* (*Cj*0933c) performs the anaplerotic reaction that is an

ATP-dependent carboxylation of pyruvate to oxaloacetate (100). Phosphoenolpyruvate carboxykinase (encoded by *Cj*0932) catalyzes the ATP-dependent reaction of oxaloacetate to phosphoenolpyruvate, which is required for gluconeogenesis. Pyruvate is incorporated into the TCA cycle by pyruvate:flavodoxin oxidoreductase (*Cj*1476c), which is required for the oxidative decarboxylation of pyruvate, to form acetyl-CoA. Pyruvate can be formed by *Cj*1287c, which encodes a probable malate oxidoreductase that converts malate to pyruvate that is coupled to NADP reduction.

Instead of metabolizing sugar for its carbon source, *C. jejuni* can utilize amino acids that are incorporated into the TCA cycle. Interestingly, it only has the transport and enzyme capabilities for six amino acids (33, 72). *C. jejuni* preferentially utilizes certain amino acids. Amino acid composition of spent rich media was measured when *C. jejuni* was grown in continuous culture (51). At high dilution rates, serine and aspartate were depleted, and it was not until a lower dilution (growth) rate did *C. jejuni* utilize glutamate and proline (51). The biosynthesis of serine begins with 3-phosphoglycerate. Aspartate is synthesized by the transamination of oxaloacetate, a four carbon dicarboxylic acid. Glutamate and proline are part of the 2-oxoglutarate family of amino acids. More recently, it was confirmed by the analysis of spent media (Mueller Hinton Broth) that *C. jejuni* has preference for specific classes of amino acids, specifically the utilization of aspartate, glutamate, proline and serine (33). According to the genome of *C. jejuni,* it only encodes for the enzymes necessary to catabolize aspartate, asparagine, glutamate, glutamine, serine and proline (33) (Figure 1). A proline dehydrogenase (Cj1503c) is predicted to oxidize proline to glutamate, forming an

intermediate Δ^1-pyrroline-5-carboxylate and the electrons are predicted to be donated to menaquinone. Glutamine and glutamate can be interconverted through two enzyme systems, a glutamine synthetase (Cj0699c), and glutamate synthase (Cj007/009). AnsA, (Cj0029) encodes an asparaginase, which deaminates asparagine to aspartate. AspA (Cj0087) is an aspartase used to convert aspartate to fumarate. An aspartase mutant exhibits no specific aspartase activity, and is unable to grow in minimal media containing aspartate, glutamate, glutamine or proline (33). With serine as a carbon source, the *C. jejuni* mutant exhibited wild type growth. The mutant was also deficient in poultry colonization (33). This AspA mutant, however, did not have a defect in aspartate transport.

C. jejuni has the capability of transporting aspartate and glutamate by way of the amino acid ABC-transporter PEB1a (Cj0921c) (53). The protein PEB1 has high, but equal, affinity to both aspartate and glutamate as shown through radiolabelled ligand binding and fluorescence spectroscopy studies. The disruption of *Cj*0921c resulted in a mutant that was unable to support growth on glutamate as its carbon source, nor was it able to uptake glutamate. The *Cj*0921c mutant was also unable to grow on aspartate as its primary carbon source, but there was still some aspartate metabolism, as confirmed by NMR, which resulted in significantly lower, but measurable uptake of aspartate (53). Aspartate can also be transported via DcuA/B, C_4-dicarboxylate transporters (33).

Transport of cysteine utilizes another potential ABC-type transporter, which contains a cysteine-binding protein via CjaA (Cj0982). The crystal structure of Cj0982 revealed that it

is a two-domain protein with a pocket containing bound cysteine (65). Fluorescence titration confirmed the high affinity to cysteine, as well as serine; however, the addition of a small amount of cysteine could replace excess serine. No other amino acid was able to bind the protein (65).

Serine metabolism of *C. jejuni* has been studied extensively (99). *C. jejuni* has a serine dehydratase (SdaA) encoded by *Cj*1624c and a serine transporter (SdaC) encoded by *Cj*1625c, which have homologues to *E. coli* (72). Studies have looked at the transport of serine and it has been shown that serine is transported through SdaC as well as through an unidentified high-affinity transporter (99). An SdaA mutant did not grow in media that contained serine as the major carbon source. The enzymatic activity of the SdaA mutant was 10-fold lower than wild type and an intact SdaA is required for poultry colonization. The purified SdaA protein was oxygen sensitive. The SdaC mutant was unable to uptake serine as measured by [^{14}C]L-serine (99).

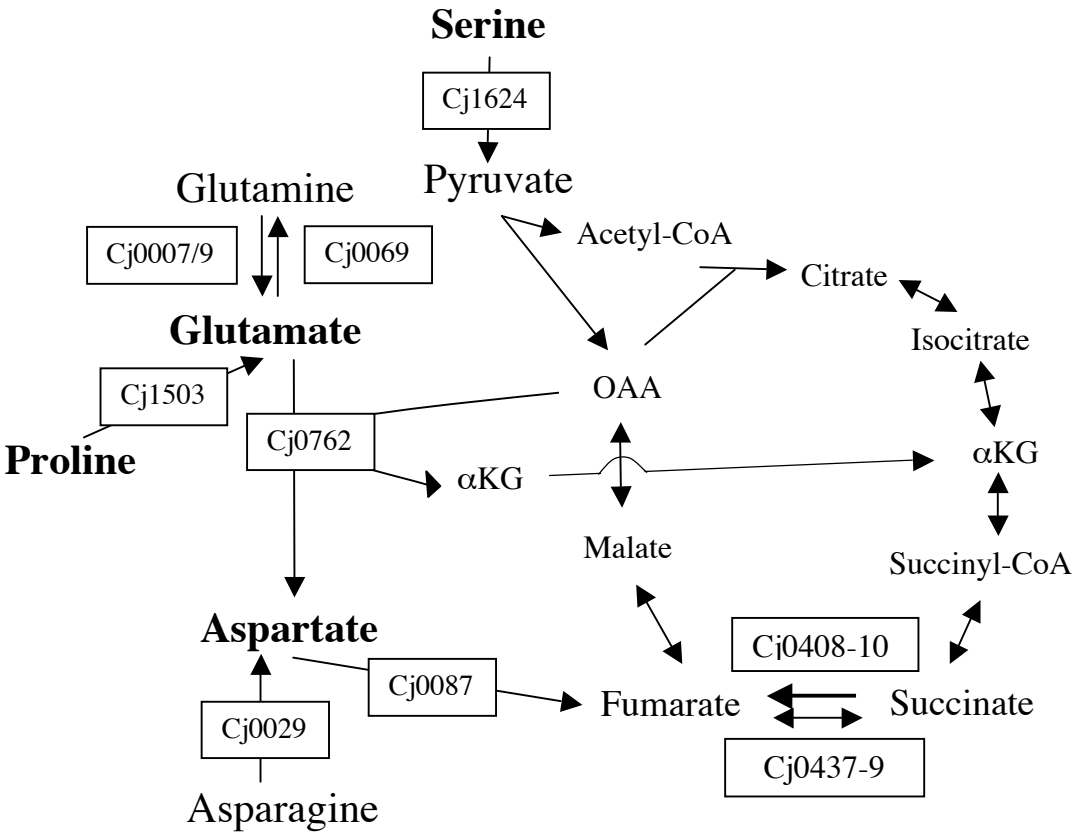

Figure 1: Amino acid catabolism of *C. jejuni* adapted from (46). *C. jejuni* only has the capability to transport and metabolize the six amino acids shown. Substrates in bold are the amino acids that were depleted in Mueller Hinton Broth by *C. jejuni* as measured by GC-MS (33).

Dicarboxylate transport

TCA intermediates can be transported through C_4-dicarboxylate transporters. *C. jejuni* encodes for three: DctA (Cj1192), DcuA (Cj0088), and DcuB (Cj0671) (72). The C_4-dicarboxylate transporters have homologues in *E. coli* and *W. succinogenes* that have been studied extensively (41, 98). DctA is annotated as a C_4-dicarboxylate transporter. In studied systems, DctA works as a symporter, and catalyzes H^+ or Na^+ symport with C_4-dicarboxylates and dicarboxylic amino acids (41). DcuA is annotated as an anaerobic C_4-dicarboxylate transporter, and is considered a redundant transporter with DcuB, which is also annotated as an anaerobic C_4-dicarboxlyate transporter (33). DcuA and DcuB function as antiporters in the transport of fumarate, succinate, malate and aspartate in anaerobic or facultative anaerobic systems (87, 98).

Respiration metabolism

C. jejuni is a respiratory microorganism. In 1982, Hoffman and Goodman characterized the electron transport chain of *C. jejuni* (36). They discovered that *C. jejuni* has high respiration rates with formate and hydrogen, and less so with lactate, succinate and malate. NADH was not an efficient donor (36). In 2000, the published genome of *C. jejuni* predicted a highly branched chain with multiple donors and acceptors (Figure 2) (72). A formate dehydrogenase and hydrogenase were annotated (72), as well as the NADH:ubiquinone oxidoreductase (Cj1566c-1579c) known as complex I that is found in all domains of life: bacteria, archaea and the mitochondria of eukaryotes (28). However, it has been shown that

C. jejuni Complex I uses flavodoxin as an electron donor instead of NADH (102). Sulfite and gluconate have also been implicated as donors to the electron transport chain of *C. jejuni*.

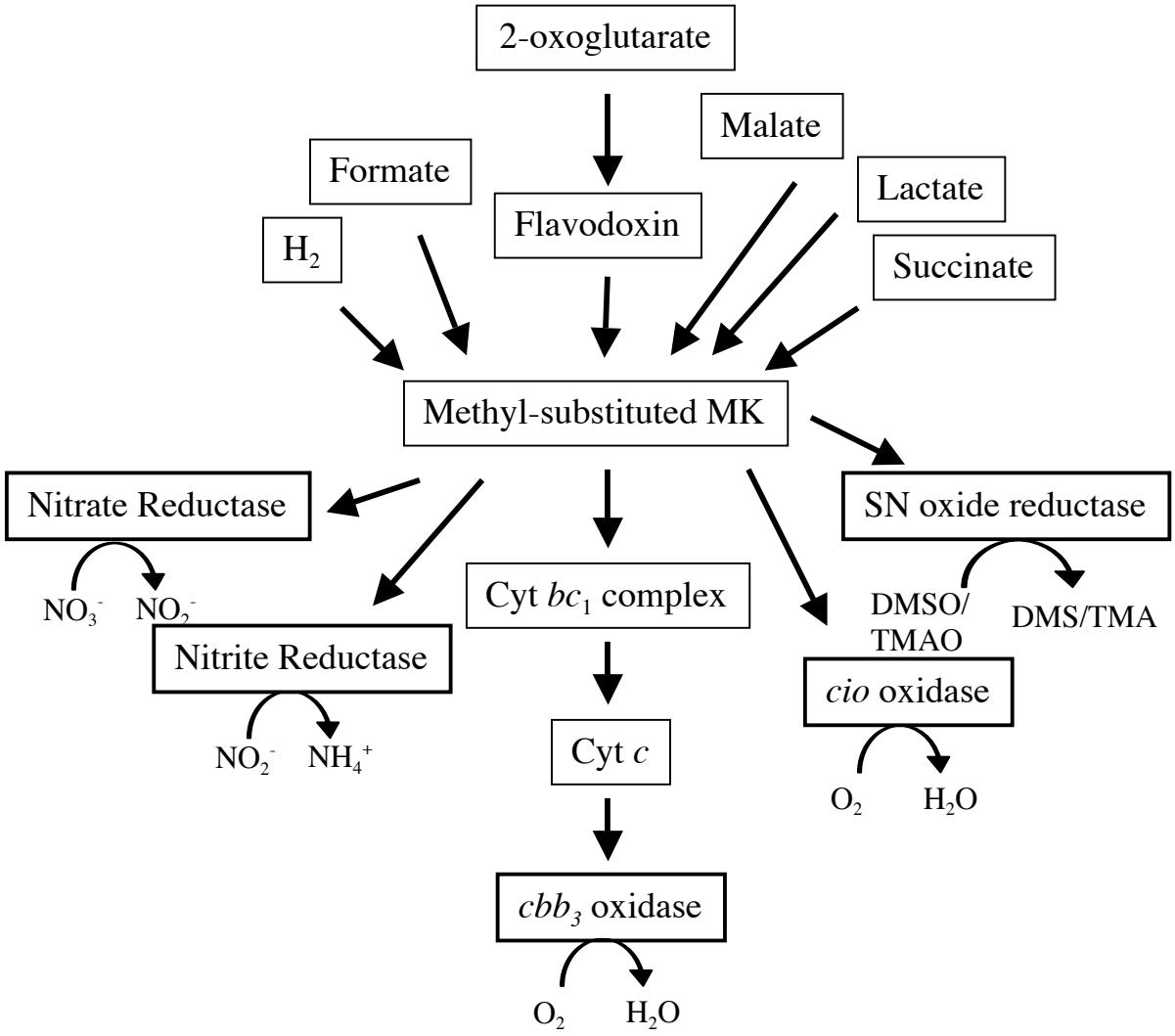

Figure 2: Proposed respiratory pathways of *C. jejuni* including confirmed electron donors and acceptors. MK, menaquinone.

Gluconate dehydrogenase (Cj0414/0415) is required for gluconate respiration and full chicken colonization (70) and the sulfite oxidase (Cj0005c/4c) can utilize sulfite as a donor to the electron transport chain (66). Malate and lactate are also confirmed donors via the malate dehydrogenase (Cj0532) and lactate dehydrogenase (Cj1167), respectively (62, 72).

The electrons enter the electron transport chain through the donors, and are then normally passed to an isoprenoid quinone pool. There are two types of quinones: benzoquinones (which include ubiquinones) and napthoquinones (which include menaquinones) (64). Quinones are lipids that transfer electrons in the electron transport chain and can aid in oxidative phosphorylation. Mass spectrometry and NMR revealed that *C. jejuni* has two respiratory quinones; menaquinone-6 and methyl-substituted menaquinone-6 (17). The authors theorize that the methyl group of the methyl substituted menaquinone may stabilize the menaquinone, thus lowering the redox potential (17). The redox potential of these two types of quinones has not been assessed in *C. jejuni*, but in *W. succinogenes* the methyl-substituted menaquinone is assumed to have a redox potential of $E_0' = -90mV$ (24).

Terminal electron acceptors

C. jejuni has the potential to utilize multiple electron acceptors including oxygen and anaerobic alternatives. In reference to its microaerophilic nature, *C. jejuni* is predicted to encode a bc_1 cytochrome complex and two terminal oxidases: a cbb_3-type oxidase and a cytochrome *bd* type oxidase (72). The cytochrome cbb_3-type oxidase operon consists of four genes (*Cj*1490c-*Cj*1487c) annotated as ccoN-O-Q-P (72). The cbb_3 oxidase couples the

reduction of oxygen to proton translocation across the periplasmic membrane (89). There are three groups of the heme-copper oxidase superfamily found in bacteria: quinol oxidases, which directly accept electrons from quinol and cytochrome *c* oxidases, which contain a purple copper metal center and accept electrons from cytochrome *c* (75). The more recently discovered third type of oxidase is called the cytochrome cbb_3 oxidases found mainly throughout the Proteobacteria phylum, but have been found elsewhere (20). The cbb_3-type can be distinguished from the other oxidases by the unique heme prosthetic group in the active site, the high oxygen affinity, and how they receive electrons (75). The oxygen affinity for cbb_3-type oxidases has only been calculated for *Bradyrhizobium japonicum* (7 nM) and *C. jejuni* (40 nM) (40, 78). However, because the sequence of the catalytic subunit (CcoN) is highly conserved and the expression of these oxidases is only seen under microaerobic conditions, it is believed that all cbb_3-type cytochrome *c* oxidases have a high affinity for oxygen (75). CcoN is predicted to contain 12 transmembrane helices and an active site of a high-spin heme B and copper ion (20). CcoO is a membrane anchor protein that contains a single *c*-type heme. CcoP, also a membrane anchor, contains two *c*-type hemes, which can pass the electrons from the cytochrome *c* to CcoN (20). The function of CcoQ has not been determined, but it does not seem to be required for the assembly of the enzyme nor the reduction of oxygen (68, 108). There is evidence that CcoQ may help protect the oxidase under oxic conditions by acting as a redox center in *Rhodobacter sphaeroides* (68). It has previously been reported that the cbb_3-type oxidase of *C. jejuni* is an essential enzyme (77), but recent work has shown that while a *C. jejuni* mutant in the cbb_3-type oxidase can be isolated, the resulting strain is extremely oxygen sensitive and required for

poultry colonization (103). The high affinity *cbb*$_3$-type cytochrome *c* oxidase of *Brucella suis*, which causes acute brucellosis in humans, is necessary for persistence in mice spleen and livers (43).

The cytochrome *bd* oxidase is a two gene operon (*Cj*0081/0082) annotated as *cydAB* (72). However, no *d*-type cytochromes can be visualized in membrane difference spectra, suggesting a lack of *d* cytochromes in *C. jejuni* (36, 40). Hoffman and Goodman first reported cyanide-resistant cytochromes, and the cytochrome *bd* type oxidase has recently been renamed a cyanide-resistant, low-affinity oxidase annotated as *cioAB* (40). *Pseudomonas aeruginosa* encodes for subunits similar to *bd*-type cytochromes without the *d*-type cytochrome characteristics, which is similar to the *C. jejuni* oxidase (22). In both *E. coli* and *Azotobacter*, cyanide insensitivity has been attributed to cytochrome *bd* (44, 79), but the *d* type cytochrome is not required (1). The *P. aeruginosa* CioAB is required when copper is severely limited in the environment (26), although this requirement has not been tested in a *cioAB C. jejuni* mutant. The oxygen affinity of the two terminal oxidases of *C. jejuni* were measured by oxygen affinity studies using leghemoglobin and myoglobin that are converted to their oxy forms. The cyanide-resistant oxidase is of low-oxygen affinity with a K_m value = 0.8 μM and the cytochrome *cbb*$_3$ terminal oxidase (inferred from the subtraction of wild type minus *cioAB* mutant oxygen affinity levels) has a high oxygen affinity (K_m = 40 nM) (40).

The genome sequence also reveals the presence of alternative terminal reductases to oxygen, allowing for the potential to grow by anaerobic respiration (72). *C. jejuni* has both a nitrate

reductase (*nap* operon) and nitrite reductase (*nrf* operon), which are located in the periplasm. The *nap* operon consists of six *nap* genes, arranged in order as A-G-H-B-L-D (72). The nitrate reductase is a two protein complex encoded by the *napA* (*Cj*0780) and *napB* (*Cj*0783) (Figure 3). NapA is the catalytic subunit of nitrate reductase and binds a [4Fe4S] cluster and bis-molybdenum guanosine dinucleoside (MGD) cofactor. The second subunit is NapB (Cj0783), which is a di-heme cytochrome *c* and functions with NapA to form a NapAB complex. The NapAB complex is normally coupled to quinol oxidation through a tetra-heme cytochrome NapC (80). *C. jejuni* does not contain a protein annotated as a NapC. Instead, electrons are passed between quinol and the NapAB complex through the iron-sulfur proteins encoded by *napG* (*Cj*0781), which is annotated as a quinol dehydrogenase periplasmic component, and *napH* (*Cj*0782), which is annotated as a quinol dehydrogenase membrane anchor component. A secondary pathway of electrons to the NapAB complex occurs through a NapC-like homologue (NrfH/Cj1358c), a cytochrome *c*-containing membrane anchor that is part of the nitrite reductase operon (77). NapG can be disrupted and nitrate reductase activity is similar to wild type, suggesting the ability of *C. jejuni* to use an alternate route for electron donation, but the disruption of both *napG* and *nrfH*, resulted in abolition of nitrate reductase activity. While NapG is the primary route for electrons to be passed to NapAB, NrfH seems to play an alternative route for the nitrate reductase. NapD is considered a "private chaperone", which is involved in the export and maturation of NapA (71). NapL is a soluble periplasmic protein with unknown function.

In addition to the nitrate reductase, the annotated sequence of *C. jejuni* contains a gene (*Cj*1357c, annotated as encoding a probable periplasmic cytochrome *c*) that has significant

homology to the nitrite reductase of *E. coli, nrfA* (72). NrfA is the catalytic subunit of the enzyme, which is encoded by a seven gene operon required for nitrite reduction that involves the six-electron dissimilatory reduction of nitrite to ammonia (39). The NrfA of *C. jejuni* is required for nitrite reductase activity as measured by benzyl viologen linked nitrite reductase activity. The concentration of nitrite was depleted in wild type grown cultures, but not in the *nrfA* mutant cultures (77). As stated previously, *Cj*1358c (*nrfH*) encodes a membrane anchor tetra-heme cytochrome. A NrfH mutant has wild type levels of nitrite reductase activity, however under "oxygen-limiting conditions," the NrfH mutant was unable to grow with nitrite, thus proving NrfH is the sole electron donor to NrfA (77).

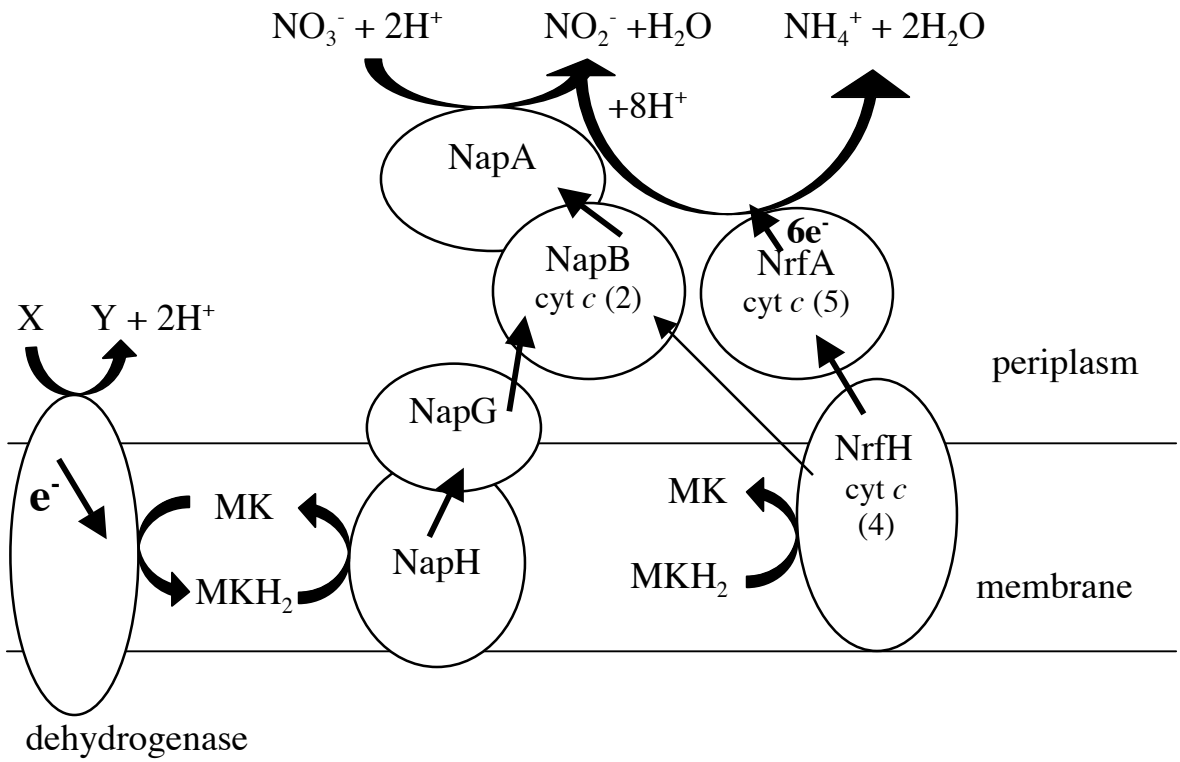

Figure 3: Denitrification pathway of *C. jejuni* adapted from (77). Nap proteins represent nitrate reductase and Nrf proteins represent nitrite reductase. Electrons are donated from a dehydrogenase to the menaquinone pool of *C. jejuni*, which can then be passed to the Nap complex to reduce nitrate to nitrite. Electrons can also be passed to the Nrf complex to reduce nitrite to ammonium. NrfH is also able to directly donate electrons to nitrate reductase. MK/MKH_2, menaquinone/menaquinol pool. Numbers in parentheses represent the number of cytochromes in a given protein.

Dimethyl sulfoxide (DMSO) and trimethylamine oxide (TMAO) are both alternative electron acceptors to oxygen. *C. jejuni* encodes two proteins that together function as a heterodimeric DMSO/TMAO reductase termed an SN oxide reductase because it can reduce both organic sulfoxides (S-oxides) and amine oxides (N-oxides). *Cj*0264c and *Cj*0265c are annotated as a molybdopterin-containing oxidoreductase and putative cytochrome *c*-type heme-binding periplasmic protein, which together make an SN oxide reductase (72, 85). The SN oxide reductase of *Rhodobacter sphaeroides* is able to reduce both TMAO and DMSO (49). A *Cj*0264c mutant was constructed and benzyl viologen linked DMSO and TMAO reductase activity confirmed it was the sole SN oxide reductase (85). TMA accumulation can also be measured through NMR, and the SN oxide mutant produced negligible amounts of TMA (85). There is no *in vivo* data to show levels of DMSO or TMAO in the gut of chickens. Both *E. coli* and *Salmonella* species have an active SN oxide reductase; however, the function remains unknown. It is known that neither TMA nor TMAO are used as a source of carbon or nitrogen (9).

Utilizing fumarate as an alternative electron acceptor is a common form of anaerobic respiration (48). The *C. jejuni* genome encodes for a fumarate reductase, with an operon containing three genes. FrdC (Cj0408) is the membrane anchor and diheme cytochrome B, FrdA (Cj0409) is where the reduction of fumarate to succinate occurs and contains an FAD flavoprotein and FrdB (Cj0410) is an Fe-S protein (72). The order of annotated genes for the subunits of fumarate reductase are similar to *H. pylori* and *W. succinogenes*. *W. succinogenes* is a facultative anaerobe that can grow by fumarate respiration (48). The

FrdCAB complex of *W. succinogenes* is the sole fumarate reductase and is required for anaerobic growth with fumarate as a terminal acceptor (86). *C. jejuni*, however, is unable to respire anaerobically with fumarate as an electron acceptor (101), but fumarate will increase growth under "oxygen-limited" conditions (85). *C. jejuni* is able to metabolize fumarate and this activity is found only in the particulate fraction with a K_m value = 1.9 ± 0.2 mM (90). Additionally, the fumarate reductase enzyme of *C. jejuni* can complement the ΔfrdCAB *W. succinogenes* (61). Strains of *W. succinogenes* were constructed that had a complete deletion of the *frd* operon. *C. jejuni frdCAB* was cloned into the deletion strain and expressed under the control of the *W. succinogenes frd* promoter. The *W. succinogenes* containing a sole *C. jejuni* fumarate reductase was able to respire anaerobically with fumarate as a terminal electron acceptor. Specific fumarate reductase activity could be measured by the oxidation of 2,3-dimethyl-1,4-naphthoquinone (menaquinone analogue) (61).

Fumarate reductase and succinate dehydrogenase can interconvert fumarate and succinate in *E. coli* (34, 55). However, it is not possible to determine the function of each enzyme by amino acid sequence alone (50). *C. jejuni* encodes for a succinate dehydrogenase, which is composed of three subunits: SdhABC (Cj0437-0439) (72). SdhA is annotated as a succinate dehydrogenase flavoprotein subunit, SdhB is a putative succinate dehydrogenase iron-sulfur protein and SdhC is a putative succinate dehydrogenase subunit C. The A and B subunits of fumarate reductase and succinate dehydrogenase are similar based on amino acid sequence, but SdhC of *C. jejuni* is not similar to the FrdC protein. SdhC belongs to the family *Sulfolobus acidocalarius* and *Acidianus ambivalens* because it is not a transmembrane

subunit and is hydrophilic in nature (32, 42). *W. succinogenes* encodes for a succinate dehydrogenase (6), but it is lacking in *H. pylori*'s genome (96). Instead of a cyclic oxidative TCA cycle, *H. pylori* has a branched TCA, and is missing the key enzymes: 2-oxoglutarate dehydrogenase, succinyl-CoA synthetase, and succinate dehydrogenase (76). Succinate dehydrogenase activity in *H. pylori* was not detected using NMR or spectrophotometrically with benzyl viologen, FAD^+, NAD^+ or $NADP^+$ as electron acceptors (76). *C. jejuni* is able to oxidize succinate as a potential respiratory donor (36), but menaquinone is a very poor electron acceptor (E_m = -80mV) from succinate (E_m = +30mV) making succinate oxidation energetically unfavorable. Menaquinol is able to donate electrons to fumarate reductase to reduce fumarate to succinate. *C. jejuni* is not the only bacteria that performs succinate oxidation coupled to menaquinone reduction. *Desulfovibrio vulgaris, Geobacter sulfurreducens* and *Bacillus subtilis* have all been assessed for succinate oxidation by menaquinone reduction (14, 52, 106). The donation of electrons to menaquinone through succinate oxidation requires an electrochemical proton potential Δp (84). According to structural predictions and location of the heme-B molecules of the membrane anchor protein, it is assumed that the site of menaquinone reduction and the site of accession of the protons is on the outer side of the membrane (84). The electrons are delivered from the inner side of the membrane and move along the electrical gradient, and the Δp is required for driving this flow of electrons across the membrane.

The tricarboxylic acid cycle (TCA) is often investigated for drug target research. Specifically, fumarate reductase has been implicated in virulence. *Salmonella enterica*

Serovar *Typhimurium* (SR-11) requires a full operating TCA cycle for BALB/c mice infection (95), for the disruption of genes throughout the cycle leads to varying degrees of avirulence. The disruption of both succinate dehydrogenase (SDH) and fumarate reductase (FRD) of SR-11 leads to complete avirulence in BALB/c mice infection (60). The authors attribute the conversion of succinate to fumarate, of which both enzymes can function, as a requirement for virulence. Even more interesting is that mice infected with the SDH/FRD double mutant, and then challenged with wild-type SR-11, remained healthy 30 days post-challenge (60). The FRD of *Helicobacter pylori* is required for the colonization of the mouse stomach (31). The FRD has been studied as a drug target for *H. pylori* (30, 59), and there has been recent success with nafuredin in treating sheep infected with the parasite *Haemonchus contorus* (69, 97). Also, upon deletion of the fumarate reductase gene, *Actinobacillus pleuropneumoniae*, a porcine pathogen, became attenuated in virulence, with less severe lung lesions associated with the disease (12).

C. jejuni uses oxidative phosphorylation for its energy requirements, and encodes for a highly branched respiratory chain with the potential to utilize multiple acceptors. Here we characterize the terminal electron acceptors of *C. jejuni* by mutating the catalytic subunit of each enzyme. Each mutated enzyme prevents *C. jejuni* growth when provided with the corresponding substrate as a respiratory acceptor with the exception of fumarate. The fumarate reductase of *C. jejuni* is the sole succinate dehydrogenase and, in fact, acts as an electron donor to the electron transport chain.

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CHAPTER 2

Role of *Campylobacter jejuni* respiratory oxidases and reductases in host colonization

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Citation: Weingarten, RA, Grimes, JL, and Olson, JW. 2008. Role of *Campylobacter jejuni* respiratory oxidases and reductases in host colonization. *Appl. Env. Microbiol.* 74(5):1367-1375.

ABSTRACT

Campylobacter jejuni is the leading cause of human foodborne bacterial gastroenteritis. The *C. jejuni* genome sequence predicts a branched electron transport chain capable of utilizing multiple electron acceptors. Mutants were constructed by disrupting the coding regions of the respiratory enzymes nitrate reductase (*napA*::Cm), nitrite reductase (*nrfA*::Cm), DMSO and TMAO reductase, (termed Cj0264::Cm), and the two terminal oxidases: a cyanide-insensitive oxidase (*cydA*::Cm) and cbb_3-type oxidase (*ccoN*::Cm). Each strain was characterized for the loss of the associated enzymatic function *in vitro*. Strains were then inoculated into one-week old chicks and the cecal contents were assayed for the presence of *C. jejuni* two weeks post-inoculation. *cydA*::Cm and Cj0264c::Cm colonized as well as wild type; *napA*::Cm and *nrfA*::Cm colonized at significantly lower than wild type levels. *ccoN*::Cm was unable to colonize the chicken; no colonies were recovered at the end of the experiment. While there appears to be a role for anaerobic respiration in host colonization, oxygen is the most important respiratory acceptor for *C. jejuni* in the chicken cecum.

INTRODUCTION

Campylobacter jejuni is the primary agent of campylobacteriosis, the most frequent form of human bacterial gastroenteritis worldwide (2, 13). An estimated 2 million cases per year of campylobacteriosis occur in the United States alone (37). *C. jejuni*'s natural host appears to be avian species (30), and contaminated poultry meat is a major route of transmission into the human population (2, 23). In one recent study, it was found that 71% of packaged chicken taken from 59 retail grocery stores contained *Campylobacter* species, and 91% of the stores sold contaminated chicken (46). *C. jejuni* is non-pathogenic to poultry and is primarily an enteric bacterium, preferentially localizing in the cecum in experimentally inoculated chicks (7). The chicken cecum is a highly developed organ located at the junction of the large and small intestines that contains more than 10^{11} bacteria per gram (6, 29). 16S rRNA sequencing reveals that the cecum tends to be dominated by the anaerobic *Clostridiaceae*-related bacteria (65%) followed by the anaerobic bacteria of the genera *Fusobacterium*, *Lactobacillus*, and *Bacteroides* (26), which would imply that *C. jejuni* is exposed to an anaerobic environment in its normal host. A paradox exists, however, in that *C. jejuni* has long been considered a microaerophile, meaning it is an obligate aerobe that cannot survive fully aerobic conditions (18). *C. jejuni* is unable to grow fermentatively and must rely on oxidative phosphorylation for all its energy requirements. The genome sequence of strain *C. jejuni* NCTC 11168 encodes two different terminal oxidases, a cbb_3-type cytochrome *c* oxidoreductase and a *bd*-type quinol oxidase (33). Recently, it has been demonstrated that *cydAB* encodes a cyanide-insensitive, low-affinity oxidase rather than the *bd*-type quinol oxidase that is annotated in the genome (19). In the same report, the cbb_3-type cytochrome *c*

oxidoreductase was considered an essential enzyme due to the inability to obtain mutants after repeated transformations (19). Consistent with the anaerobic environment of *C. jejuni*, the genome sequence also reveals the presence of alternative terminal acceptors to oxygen, allowing for the potential to grow by anaerobic respiration (33). These enzymes include a periplasmic nitrate reductase (Cj0780-0785), nitrite reductase (Cj1357c-1358c) and an enzyme responsible for both dimethyl sulfoxide (DMSO) and trimethylamine N-oxide (TMAO) reduction (Cj0264c-0265c), which we term an SN oxide reductase. Recently the respiration of nitrate and nitrite, DMSO and TMAO by *C. jejuni* has been reported (35, 38). However, the importance of these respiratory pathways in the avian host has not been addressed.

We set out to determine the individual roles of the respiratory acceptor enzymes of *C. jejuni* and their importance in the colonization of chickens. Mutants in all five terminal acceptor enzymes of *C. jejuni* were constructed and characterized. Insight into the *in vivo* physiology of *Campylobacter* will provide a better understanding of how the organism thrives in the intestinal tract of its host. This is especially important for the ultimate goal is removing *C. jejuni* from poultry flocks and preventing transmission to humans.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Table 1 lists strains of *C. jejuni* and *E. coli* along with all plasmids and primers utilized in this study. The NCTC 11168 strain used in this study is helical in morphology, fully motile, and is not impaired in poultry colonization. Tryptic soy agar (Difco, Sparks, MD) plates supplemented with 10% defibrinated sheep blood (Gibson Laboratories, Inc. Lexington, KY) are called BA plates and used for growth of *C. jejuni*. *C. jejuni* was routinely cultured at 37°C microaerobically in a tri-gas incubator (model 550D; Fisher Scientific) constantly maintained at 5% CO_2 and 12% O_2, balance N_2. The O_2 sensitive *ccoN*::Cm mutant was routinely cultured in an atmosphere generated by the Anaerobic BBL Gas Pak Plus system. Mueller Hinton broth (Difco) with the addition of 50 mM sodium formate (MHF) was used for *C. jejuni* cultures and 10 mM sodium nitrate, 5 mM DMSO or 20 mM TMAO was added as indicated; liquid cultures were incubated at 37°C with shaking. Chloramphenicol (25 μg/ml) was added as indicated. Genetic manipulations were performed with *E. coli* strain DH5α. Luria-Bertani broth and agar was supplemented with ampicillin (100 μg/ml) or chloramphenicol (25 μg/ml), as noted.

Anaerobic growth of *C. jejuni*. Anaerobic conditions were achieved by the following protocol: MHF was sparged with a glass sintered gas dispersion tube for 15 minutes with anaerobic gas mix (5% CO_2, 10% H_2, balance N_2). Twenty ml of MHF was placed in a 150 ml serum bottle, and sealed with a gas-impermeable butyl rubber septum type stopper (Bellco Glass Inc, Vinland, NJ) secured with an aluminum seal (Wheaton Scientific, Milleville, NJ). The serum bottle was made anaerobic by evacuating the headspace to 30" Hg for one minute

and then flushed for one minute with argon that had passed through a heated copper pellet oxygen scrubber. The process was repeated three times and the bottle was removed after the last argon flush. Bottles prepared in this way contain argon at atmospheric pressure and are referred to as vacuum sparged.

Gas analysis. An OmniStar™ Gas Analyzer (Pfeiffer Vacuum, Asslar, Germany) was utilized to measure the gas composition of the headspace above the cultures. The gas analyzer uses a turbo-molecular pump coupled to a quadrapole mass spectrometer to identify gasses based on molecular mass.

Cloning and construction of *C. jejuni* mutants. Oligonucleotide primers for cloning genes of interest were designed from the sequenced strain NCTC 11168 (33) and are listed in Table 1. PCR amplification was performed with *Taq* DNA polymerase (Promega, Madison, WI) using chromosomal DNA isolated from *C. jejuni* NCTC 11168 as a template. The PCR products (~1 kb) were inserted into the appropriate vector (either pKS:ΔXbaI or pCR®TOPO2.1®) and confirmed by restriction analysis. pKS:Δ*Xba*I is a version of pBluescript II KS^+ in which the *Xba* I site has been destroyed by digestion with *Xba* I, treated with T4 DNA polymerase (Promega) and dNTPs, and religation. The coding region of the gene of interest was disrupted by insertion of an antibiotic resistance cassette into an appropriate restriction endonuclease recognition sequence within the DNA fragment (Table 1). The chloramphenicol resistance gene (*cat*) was originally isolated from *Campylobacter coli* (41). pJMA-001 contains the chloramphenicol resistance cassette from plasmid pRY111

(44) cloned into the *Pvu* II site of pGEM-T Easy (Promega). Electrocompetent *C. jejuni* cells were transformed with each construct to yield the corresponding *C. jejuni* mutant. Briefly, 1-5 μg of plasmid DNA was incubated on ice ten minutes with 50 μl *C. jejuni* cells that had been previously washed four times with an ice-cold 9% sucrose and 15% glycerol solution. The cells and DNA were then placed in a 2 mm electroporation cuvette and pulsed with 2500V in an ECM399 electroporator (BTX, San Diego, CA). Immediately after the pulse, 50 μl of Mueller-Hinton broth (Difco) was added to the cuvette containing the competent cells and the cuvette remained on ice for ten minutes. Cells (100 μl) were spotted onto cold nonselective BA plates, and the plates were incubated microaerobically for 24 hours. The cells were then transferred to BA plates containing chloramphenicol. In obtaining *ccoN*::Cm, however, anaerobic jars containing an Anaerobic BBL™ GasPak Plus™ with palladium catalyst were utilized and BA plates were supplemented with 50 mM formate and 10 mM nitrate. Resistant colonies were passed on selective BA plates and the correct insertion of the cassette was confirmed by isolation of chromosomal DNA from the mutant strain and PCR amplification of the gene. Agarose gel electrophoresis of the PCR product was used to monitor the size increase of the gene of interest with the antibiotic cassette insertion (data not shown).

Quantitative Reverse-Transcriptase PCR. PCR primers used in this study are listed in Table 1 and were designed to amplify 100-150 nucleotide fragments of genes of interest. Total RNA was isolated from the parent strain of *C. jejuni*, as well as mutants *ccoN*::Cm, *napA*::Cm and *nrfA*::Cm using a MasterPure™ Complete RNA Purification Kit (Epicentre®

Biotechnologies, Madison, WI). qRT-PCR was performed by using the Quantitect™ SYBR® Green RT-PCR kit (QIAGEN, Valencia, CA). The PCR mixture (20 μl) contained 40 ng RNA, 10 μl 2x QuantiTect SYBR Green RT-PCR Master Mix, and 0.2 μl QuantiTect RT mix. The reverse transcriptase cycle was 50°C for 30 minutes, followed by a PCR initial activation step of 95°C for 15 minutes. The mixtures were then amplified in 30 cycles of 94°C for 15 seconds, 60°C for 30 seconds, and 72°C for 30 seconds in an automated thermal cycler (BIORAD iCycler™, 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 using the internal control *gyrA* (*Cj*1027c).

Nitrite production assay. Cells were removed by centrifugation and 10 μl of supernatant was added to a reaction mixture containing 500 μl of 1% (wt/vol) sulfanilamide dissolved in 2.5 N HCl and 500 μl of 0.02% (wt/vol) naphthylethylenediamine. Samples were incubated at room temperature for 10 minutes, and the absorbance at 540 nm was measured and nitrite concentrations were determined by reference to a standard curve.

Sonication. Cells were disrupted by sonication. Sonication was performed on ice in a W-370 horn cup sonicator (Heat Systems—Ultrasonics, Inc, Farmingdale, NY) for four 45-second pulses at 60% power and 7.0 output control setting.

Protein assay. Protein concentration was determined using the BCA™ Protein Assay Kit (Pierce, Rockford, IL) using bovine serum albumin as a standard.

Nitrate reductase activity. Nitrate reductase activity was determined by the production of nitrite by whole cell extracts incubated with nitrate as substrate and reduced benzyl viologen as electron donor (27, 31). One to two μg protein of sonicated vacuum-sparged cell extracts were added to a vacuum-sparged assay mixture containing 0.1 mM benzyl viologen, 20 mM sodium dithionite solution, 54 mM sodium phosphate buffer (pH 6.8) and 10 mM sodium nitrate. The reaction was incubated for two minutes at room temperature and then stopped by removing the stopper and vortexing the mixture until the benzyl viologen was completely oxidized. One percent (wt/vol) sulfanilamide (500 μl) dissolved in 2.5 N HCl and 500 μl of 0.02% (wt/vol) naphthylethylenediamine were added to the reaction mixture, and after 10 minutes the absorbance at 540 nm was measured. Nitrite concentrations were then determined by reference to a standard curve. Activity was expressed as nmol nitrite produced min^{-1} mg^{-1}.

Nitrite reductase activity. Benzyl viologen-linked reductase assays were carried out with sonicated cell extracts in a 1 ml assay volume as described in (38). Reagents were added by syringe through the stopper, while argon gas was flushed through the cuvette. The reaction mixture (reagents were kept anaerobic during the course of the assay) contained 75 mM sodium phosphate buffer (pH 6.8), 0.2 mM benzyl viologen and 1-5 μg of cell extract in 1 ml stoppered quartz cuvette. Freshly made 20 mM sodium dithionite was then injected into the

cuvette until the absorbance at 585 nm reached 0.9 to 1.3, which represents half reduced benzyl viologen. An anaerobic solution of sodium nitrite to a final concentration of 2.5 mM was added and the benzyl viologen oxidation kinetics were recorded by a spectrophotometer at 585 nm. Nitrite reductase activity was expressed as μmol benzyl viologen oxidized min^{-1} mg^{-1}.

DMSO reductase assay. Cells were harvested by centrifugation and washed once with sterile PBS (pH 7.4). The cell pellet was resuspended in 10 ml PBS and 50 mM formate to a final optical density of approximately 0.2 at 600 nm and the mixture was vacuum sparged. DMSO was added to a final concentration of 10 mM to start the reaction and cultures were shaken at 37°C. An OmniStar™ Gas Analyzer (Pfeiffer Vacuum, Asslar, Germany) was utilized to measure the dimethyl sulfide (DMS) evolution into the headspace. A DMS standard curve was generated using >99% pure DMS (Sigma-Aldrich, St Louis, MO), and DMS concentrations were determined by reference to the standard curve. DMSO reductase activity was expressed as nmol DMS produced min^{-1} mg^{-1}.

Oxygen uptake assay. O_2 uptake experiments were performed using a Clarke-type electrode and YSI Model 5300 oxygen monitor (Yellow Springs, OH). Washed whole cells were resuspended in PBS and added to the constantly stirred chamber and allowed to equilibrate until no change in O_2 consumption was noticed. Formate (final concentration of 5 mM) was added through a capillary tube via a Hamilton syringe into the chamber and the chart

recorder measured the oxygen consumption, and the slope of the line determined the rate of oxygen uptake. Rates were expressed as nmol O_2 consumed min^{-1} mg^1.

Cytochrome reduction assay. Cells were harvested by swab into cold PBS (pH 7.4) and washed once. Cells were broken by passage through a French pressure cell (American Instruments Co. Silver Springs, MD) twice at 20,000 lb/in^2. Broken cells were centrifuged at 12,000 X *g* for 15 minutes to remove debris. The supernatant was collected and spun by ultra-centrifugation at 100,000 X *g* for 90 minutes. Membranes were collected and resuspended in cold PBS (pH 7.4). One ml membrane suspensions were placed into matched quartz cuvettes and left exposed to air to become fully oxidized. A few grains of sodium dithionite were added to one of the cuvettes and capped with a rubber stopper. Spectra (300-600 nm) were collected of the reduced minus oxidized samples using a Shimadzu UV-1650PC spectrophotometer at 300 to 600 nm.

Chicken colonization. Lake Wheeler Poultry Facility operated by the NCSU Poultry Department supplied *Campylobacter*-free day old broiler chicks. For 21 days, birds were housed in isolation rooms at the Dearstyne Avian Health Center (Department of Poultry Science, NCSU) in isolation brooder batteries (10 chicks per battery). Chicks were fed Purina Mills® Start & Grow® SunFresh® Recipe (Purina Mills LLC, St. Louis, MO) feed and water *ad libidum*. One week old chicks were inoculated by oral gavage with 0.1 ml of ~10^8 *C. jejuni* cells/ml, which had been grown for approximately 16 hours on BA plates and cultured at 37°C microaerobically, with the exception of *ccoN*::Cm, which was grown at 2%

O_2, 5% CO_2, balance N_2. Control chicks were inoculated with 0.1 ml sterile PBS (pH 7.4). Two weeks post-inoculation the chickens were humanely sacrificed by CO_2 asphyxiation. Approximately one g of cecal contents were collected by necropsy, serially diluted (in PBS) and plated on selective BA media containing: 40 μg/ml Cefoperazone, 40 μg/ml Vancomycin, 10 μg/ml Trimethoprim and 100 μg/ml Cyclohexamide. Samples from the *ccoN*::Cm inoculated chickens and 8 out of 20 wild type samples were incubated at 2% O_2, 5% CO_2, balance N_2 at 37°C. All other samples were incubated microaerobically at 37°C. After two days incubation, colonies, if any, were counted and the CFU/g cecal content was calculated. Data was analyzed by one-tailed Mann-Whitney test, using a 95% confidence interval.

RESULTS

Mutant construction. Mutants of *C. jejuni* constructed in this study include: *napA*::Cm (nitrate reductase), *nrfA*::Cm (nitrite reductase), *cydA*::Cm (cyanide-insensitive oxidase), *ccoN*::Cm (cbb_3-terminal oxidase) and Cj0264c::Cm (SN oxide reductase) (see Figure 1). All mutants were constructed by insertional mutagenesis using a chloramphenicol acetyltransferase (*cat*) cassette through allelic replacement of a disrupted copy of the genome. The use of the resistance cassette for mutagenesis can interfere with the proper transcription of genes "downstream" of the cassette insertion site (polar effects). To measure polar effects of downstream genes, quantitative RT-PCR (qRT-PCR) was employed to measure transcript levels between mutant and parent strain genes. *napA*::Cm (nitrate reductase) contains the *cat* cassette in the first gene of the nitrate reductase operon, encoded by *napAGHBLD* (Figure 1). All six of these genes are involved in the reduction of nitrate; *napA* is the catalytic subunit and the other genes function in electron transfer and/or assembly of the enzyme (15). Downstream of the *nap* operon are two genes *Cj*0786 and *Cj*0787 (which encode 57 aa hydrophobic protein and a hypothetical protein, respectively). The transcription of these genes were shown to not be affected by the upstream *cat* insertion, having a transcript abundance of 0.8 ± 0.17 times that of the parent strain. *nrfA*::Cm (nitrite reductase) also contains the cassette within the catalytic subunit of the enzyme (Figure 1). Downstream of *nrfA* is *Cj*1356c (which encodes an integral membrane protein). Transcript abundance of *Cj*1356c downstream of *nrfA*::Cm was 1.1 ± 0.27 fold that of wild type. *ccoN*::Cm contains the *cat* cassette that is inserted into the first gene of the four-gene *cco* operon (Figure 1), which together make up the cbb_3-type terminal oxidase (34). Downstream

of the *ccoNOPQ* operon (*Cj*1490c-*Cj*1487c) lies *Cj*1486c, which encodes a putative periplasmic protein. The relative transcript levels of *Cj*1486c in *ccoN*::Cm was 0.41 ± 0.14 fold changed in comparison to its parent strain, which would indicate that this gene is mildly affected by the *cat* insertion within *ccoN*. Polar effects are not a concern for Cj0264c::Cm or *cydA*::Cm. The *cat* cassette in *cydA*::Cm is the first gene of the *cydAB* operon (*Cj*0081 and *Cj*0082). The gene downstream of *cydAB* (*Cj*0085c, which encodes for a putative amino acid racemase) is encoded on the opposite DNA strand. The *cat* cassette in Cj0264c::Cm is located in the second gene of the two gene operon. Directly downstream of *Cj*0264c is *zupT* (*Cj*0263), which is also encoded on the opposite DNA strand (Figure 1).

Growth. Under microaerobic conditions (5% CO_2, 12% O_2, balance N_2) all mutants had similar generation times to wild type (data not shown), with the exception of *ccoN*::Cm, which is oxygen sensitive (Table 4). In wild type cultures, the addition of the respiratory donor formate and/or the acceptor nitrate promoted growth as shown by a decrease in generation time (Table 2). Despite the presence of oxygen-independent terminal acceptors in the electron transport pathway (22, 33), it has proved difficult to grow *C. jejuni* anaerobically (38). Under our conditions, we found that the addition of formate to the media supported anaerobic growth of *C. jejuni* using the acceptors nitrate, DMSO or TMAO (Table 2). Either sulfite or hydrogen could replace formate as a potential donor (data not shown).

O_2 analysis of anaerobic growth conditions. Three different anaerobic systems were first compared using mass spectrum gas analysis: vacuum sparged serum bottles, the Coy

anaerobic chamber, and an anaerobic jar containing an Anaerobic BBL™ GasPak Plus™ with palladium catalyst. The Coy chamber contains 85% N_2, 5% CO_2, and 10% H_2 and a palladium catalyst that reacts with hydrogen to promote the reduction of any residual oxygen to H_2O. The anaerobic jars had the lowest O_2 partial pressure followed by our serum bottles, and the Coy chamber had the highest O_2 partial pressure. Overall, it has been shown that the anaerobic chamber (Coy Laboratories, Grass Lake, MI) has the best efficiency in the recovery of fastidious anaerobes when compared to the anaerobic jars and GasPak Plus™ (9-11). The O_2 partial pressure above anaerobically growing *C. jejuni* cells was measured. Three samples of the parent strain were grown ± nitrate in vacuum sparged bottles and the O_2 content measured every four hours for 24 hours. O_2 concentrations in the headspace of our anaerobic cultures hovered around zero and never exceeded 0.03%, which is lower than the O_2 measured in the Coy anaerobic chamber. In order to determine what this concentration of O_2 means physiologically for the cells, dissolved O_2 in the media was calculated using Henry's Law. At the highest O_2 concentration measured (0.03% O_2), the media would contain 0.36 nM O_2.

Nitrate and nitrite reductase activity. Nitrate reductase activity was readily detectable in both wild type strain and *nrfA*::Cm. Due to an active nitrite reductase in wild type however, a specific activity (NO_2 produced min^{-1} mg^{-1}) was only reported for *nrfA*::Cm and *napA*::Cm (Table 3). *napA*::Cm showed negligible activity when grown in microaerobic conditions, but the lack of growth in anaerobic conditions precluded determining a percentage O_2 activity. *nrfA*::Cm showed nitrate reductase activity under both anaerobic and microaerobic

conditions, with activity for cells grown in anaerobic conditions approximately three fold higher than microaerobic conditions (Table 3).

Wild type cells were able to oxidize benzyl viologen using sodium nitrite as the terminal acceptor (Table 3). Nitrite reductase activities of *C. jejuni* cells grown under anaerobic conditions was 3-fold higher than cells grown microaerobically, and *nrfA*::Cm is devoid in activity (Table 3).

Nitrate and nitrite-dependent growth. Figure 2 shows the growth of nitrate dependent anaerobic growth of *C. jejuni* (all growth curves are shown in linear scale in order to visualize the increased absorbance; however, the data was log transformed to determine generation times). The addition of nitrate provides the wild type strain a generation time of 2.7 ± 0.2 hours (Figure 2A, open circles). When nitrate is omitted from the media, very little growth is detected (Figure 2A, closed circles). *napA*::Cm failed to grow either with (Figure 2A, open triangles) or without added nitrate (Figure 2A, closed triangles), which would be predicted as this strain is unable to reduce nitrate and growth of the parent strain is nitrate dependent. The nitrite reductase mutant *nrfA*::Cm was able to grow anaerobically in the plus nitrate condition (Figure 2A, open squares), although with a slower generation time (3.2 ± 0.07 hours), and a lower terminal optical density reading at 600 nm. In order to track the fate of the added nitrate in anaerobically grown cells, nitrite was measured in the supernatant of cultures of wild type and *nrfA*::Cm grown under anaerobic conditions. In both cultures, nitrite accumulates for the first 16 hours of growth (Figure 2B), indicating an active nitrate

reductase. At 16 hours, the nitrite concentration in the supernatant of *nrfA*::Cm has accumulated to 10 mM, indicating that the nitrate (initial concentration of 10 mM) has been stoichiometrically converted to nitrite. After exhausting the nitrate, growth stops (Figure 2A) and the nitrite concentration remains 10 mM until the experiment was terminated at 30 hours (Figure 2B black bars). The wild type culture, however, keeps growing after the nitrate is consumed (Figure 2A), and at 30 hours the accumulated nitrite has also been consumed (Figure 2B white bars). These data confirm that wild type *C. jejuni* can utilize both nitrate and nitrite as terminal electron acceptors, and the *nrfA*::Cm can use nitrate but not nitrite as an electron acceptor.

SN oxide reductase mutant. *Cj*0264c and *Cj*0265c are annotated in the genome sequence as a probable molybdopterin-containing oxidoreductase, and a probable cytochrome *c*-type heme-binding periplasmic protein, which together encode an SN oxide reductase (33). DMSO is reduced to dimethyl sulfide (DMS) and TMAO is reduced to trimethylamine (TMA). DMSO reduction was quantified by measuring DMS accumulation in the headspace of serum bottles containing whole cells incubated with sodium formate and DMSO (see materials and methods). Wild type cultures grown under both microaerobic and anaerobic conditions showed highest DMSO reductase activity during mid-log phase (data not shown). This is in contrast to previous reports that TMA production is highest during stationary phase of microaerobic and "oxygen-limited" grown *C. jejuni* cells (38). Cj0264c::Cm was unable to reduce DMSO (0.61 ± 0.93 nmol DMS min^{-1} mg^{-1}).

DMSO and TMAO can also serve as alternative electron acceptors (28). Wild type *C. jejuni* will grow anaerobically with a generation time of 2.9 ± 0.2 hours with the addition of DMSO (Figure 3). Wild type *C. jejuni* was also able to respire with TMAO as an electron acceptor with a generation time of 3.7 ± 0.2 hours. Cj0264c::Cm was unable to utilize either DMSO or TMAO as electron acceptors and therefore was unable to grow under these anaerobic conditions, but was able to grow anaerobically when supplemented with nitrate and had a generation time of 2.3 ± 0.15 hours (Figure 3).

Terminal oxidase mutants. Mutants were constructed in both terminal oxidases: a cyanide-insensitive oxidase (*cydA*::Cm) and a cbb_3-type cytochrome *c* oxidoreductase (*ccoN*::Cm). Formate dependent respiration rates of *C. jejuni* whole cells were measured. Wild type whole cells consumed oxygen at a rate of 123 ± 4 nmol O_2 min^{-1} mg^{-1}, *cydA*::Cm had a slower rate of 102 ± 2 nmol O_2 min^{-1} mg^{-1}. *ccoN*::Cm exhibited a negligible rate of oxygen consumption with a rate of 5.2 ± 3.6 nmol O_2 min^{-1} mg^{-1}. Dithionite-reduced minus air-oxidized spectra on isolated membrane particles of all three strains revealed no gross changes in the cytochrome composition; however the soret peak (421 nm) was 75% reduced in *ccoN*::Cm compared to both wild type and *cydA*::Cm (data not shown). The intensity of the soret peak is correlated to total cytochrome content, as each type of cytochrome contributes to this value, thus *ccoN*::Cm contains fewer total cytochromes on a per milligram basis. No cytochrome *d* peaks were detected, consistent with recent work that concludes *C. jejuni* does not contain the *d*-type cytochrome (19).

cydA::Cm grew anaerobically when supplemented with formate and nitrate (Figure 4), and had similar generation times to its parent strain under microaerobic conditions (data not shown). *ccoN*::Cm failed to grow at oxygen tensions above 8% O_2 on BA plates (Table 4). Under anaerobic conditions, *ccoN*::Cm grew similarly to wild type in the anaerobic jar with an Anaerobic BBL™ GasPak Plus™ with palladium catalyst, with single, isolated colonies of the tertiary streak (Table 4). In O_2 tensions from 2-6%, isolated colonies were still present; however, these colonies were smaller than wild type. At 7% O_2, no isolated colonies were formed, but some growth was seen on the primary streak. No growth was observed at 8% or above. Generation times for *ccoN*::Cm were similar to wild type in anaerobic cultures (2.35 ± 0.22 hr), but the terminal optical density was lower than wild type (Figure 4).

Chicken colonization. Wild type and each strain (10^7 CFU) were inoculated into 8 to 20 birds by oral gavage. Two weeks post-inoculation, birds were sacrificed by CO_2 asphyxiation and the cecal contents were removed and assayed for viable *C. jejuni*. *cydA*::Cm and Cj0264c::Cm colonized as well as wild type (Figure 5B). *napA*::Cm colonized at significantly lower than wild type (*p* value = 0.022). *nrfA*::Cm colonized at significantly lower than wild type levels (*p* value = 0.015) (Figure 5A). No *ccoN*::Cm was recovered from the chicken ceca. Care was taken to make sure the cecal samples were not exposed to O_2 during recovery of colonies. PBS was sparged with N_2 before dilution of cells, and cells were incubated in a 2% O_2 incubator. Wild type *C. jejuni* (ranging from 8.7 X 10^7 to 4.9 X 10^8 CFU/gram cecal content) was recovered from the 8 wild type samples incubated at 2%

O_2. No *C. jejuni* was found in the control birds that were given PBS for inoculum (data not shown).

DISCUSSION

The respiratory chain of *C. jejuni* is surprisingly flexible. The branched pathways allow for *C. jejuni* to thrive in its natural environment, utilizing multiple respiratory donors and acceptors. Besides encoding two separate terminal oxidases, the genome sequence predicts a nitrate reductase, nitrite reductase, and an SN oxide reductase (33). Anaerobic growth of *C. jejuni* is reasonable in light of its normal ecological niche. *C. jejuni* preferentially colonizes the cecum of experimentally inoculated chickens (7, 30). The avian cecum is a specialized organ used in the anaerobic fermentation of undigested polysaccharides, especially cellulose (8). Not surprisingly, molecular 16S rRNA analysis of the chicken cecum reveals it is dominated by obligate anaerobic bacteria (3, 26), suggesting that *C. jejuni* often encounters anaerobic conditions despite its microaerophilic designation (18). We set out to determine the importance of the various respiratory acceptors in *C. jejuni* host colonization using strains with mutations in each of the respiratory acceptor enzymes.

Under specific *in vitro* conditions, nitrate was able to support anaerobic growth of *C. jejuni* (Figure 2A). For optimal growth under anaerobic conditions, both the nitrate and nitrite reductases must be intact (Figure 2B). Mutants deficient in either reductase were unable to reduce their respective substrates (Table 3). Denitrification seems to play an important role in the chicken cecum; cultured chicken cecal isolates have been shown to reduce nitrate (36). Our chicken colonization experiments showed the importance of the nitrate and nitrite reductases of *C. jejuni* in the ceca of chickens. *napA*::Cm and *nrfA*::Cm both colonized the ceca of chickens at significantly lower levels when compared to wild type (Figure 5A).

Recently, a *nrfA* mutant in *C. jejuni* strain 81-176 was shown to not be impaired in host colonization when compared to the parent strain (35). This discrepancy may arise from differences in the colonization assay protocol, in which these authors used an inoculating dose 100 fold higher than we used (1×10^9 versus 1×10^7 CFU). *C. jejuni* chick colonization has been shown to be dose dependent (1, 45), and thus subtle differences in colonization potential may only be observed at the lower inoculating dose. *C. jejuni* can also utilize DMSO and TMAO as respiratory acceptors. Both these activities can be attributed to a single heterodimeric enzyme (encoded by *Cj*0264c and *Cj*0265c), a property that has been shown in other SN oxide reductases (25). Growth with DMSO or TMAO was slightly slower than with nitrate (Table 2) and anaerobic cultures had a lower terminal optical density when compared to nitrate grown cells (Figure 3). This could be because DMSO and TMAO are poor electron acceptors when compared to nitrate. The standard redox potential for the DMS/DMSO and TMA/TMAO couple is +160 mV and +130 mV, respectively (14, 42), compared to +420 mV for nitrate/nitrite. Cj0264c::Cm colonized the chicken at similar levels to wild type (Figure 5B). There has been very little published data on *in vivo* S- or N-oxide concentrations. The DMSO reductase of *Actinobacillus pleuropneumoniae* has been identified as a virulence factor in the porcine pathogen (which cause pleuroneumonia), as mutants are attenuated for pyrogenicity and have lower endoscopy scores for inflammation. Colonization and persistence, however, was not affected in this strain (5).

Wild type cultures fail to grow in anaerobic serum bottles without the addition of an alternative respiratory acceptor (Table 2). This clearly indicates these cultures are not using

O_2 as a respiratory electron acceptor. Furthermore, our O_2 analysis of anaerobically grown cells confirm that the sub-nanomolar dissolved O_2 levels is far below the recently calculated K_m values for either of the terminal oxidases of *C. jejuni* (19). It has been postulated that *C. jejuni* requires O_2 for production of deoxynucleotides via the enzyme ribonucleotide reductase (RNR) (38). *C. jejuni* encodes a single I-type RNR (33). I-type RNR requires molecular oxygen for generation of the tyrosyl radical for catalysis, however other facultative anaerobes also only encode I-type ribonucleotide reductases (16, 40). It is not known if RNR could function at the O_2 levels detected in our anaerobic cultures, as the O_2 affinity of this enzyme has not been determined.

C. jejuni encodes for two terminal oxidases: a cbb_3-type cytochrome *c* oxidoreductase (encoded by *ccoNOQP)* and an enzyme annotated in the genome sequence as a *bd*-type quinol oxidase, encoded by *cydAB* (33). It has been shown that no *d*-type cytochromes can be detected in *C. jejuni* using multiple spectrometric techniques, which leaves the original annotation of *cydAB* in doubt (19). This same group used a *cydAB* mutant to assign this oxidase to the low O_2 affinity, cyanide insensitive component of the respiratory chain (19). In our hands, *cydA*::Cm grew as well as the wild type in both microaerobic and anaerobic growth (Figure 4) and retained over 80% of the wild type respiratory activity. *cydA*::Cm also colonized the chicken as well as the wild type (Figure 5B). In both the laboratory setting and the host, the cbb_3-type terminal oxidase appears to predominate. *in vitro ccoN*::Cm exhibits less than 5% of the wild type formate-dependent O_2 uptake, and is severely affected in microaerobic growth, no colonies could be recovered above 7% O_2 (Table 4). There are two

possible explanations for the observed oxygen toxicity in *ccoN*::Cm. (i) It is possible that the cells are susceptible to reactive oxygen species. Cyanide-insensitive oxidases such as the one encoded by *cydAB* produce H_2O_2 (18, 19, 24). If large amounts of H_2O_2 are produced when the cyanide insensitive enzyme is the sole oxidase, the oxidative protection enzymes (specifically catalase and alkyl-hydroperoxide reductase) could be overwhelmed. (ii) The cbb_3-type oxidase keeps cytoplasmic O_2 tensions low enough to allow O_2 sensitive metabolically important enzymes functioning. This phenomenon (termed respiratory protection) was first described in *Azotobacter vinelandii* as a way of using respiration to protect the O_2 sensitive nitrogenase from inactivation (20). While either or both of these explanations could contribute to the O_2 sensitive phenotype of *ccoN*::Cm, we think the second (respiratory protection) is more likely to be the cause. O_2 sensitive enzymes have long been considered to be the cause of *C. jejuni*'s microaerophilic requirement (24). Of particular interest is the enzyme pyruvate:ferredoxin oxidoreductase (PFOR), which has been implicated in conferring oxygen sensitivity to *C. jejuni* as well as other species (32, 39). No colonies of *ccoN*::Cm were recovered from the cecum of inoculated chickens. While we can conclude that an active cbb_3-type oxidase is important for colonization of the host, we cannot confirm that *ccoN*::Cm ever reached the cecum. It is entirely possible that cells of *ccoN*::Cm did not survive passage through the more oxygen rich areas of the upper GI tract.

There is clearly a role for both anaerobic and aerobic respiration in *C. jejuni* host colonization. A high-affinity terminal oxidase mutant was unable to colonize and nitrate and nitrite reductase mutants were impaired in this ability using a chicken model. These studies

agree with transcriptional profiling of *C. jejuni* during cecal colonization, in which the genes for the three enzymes (*ccoNOPQ*, *napAGB*, and *nrfAH*) were all shown to be up-regulated *in vivo* (43). This work also parallels recent studies of *E. coli*'s ability to colonize the mouse intestine. It was shown that while the anaerobic respiration pathways are important in colonization, it is the high affinity oxidase that is crucial of *E. coli*'s ability to be maintained in the mouse intestine (21). While this might seem surprising that O_2 plays such an important role in what is typically characterized as an anaerobic niche (4), it now appears there may be more O_2 in the animal intestine than previously thought. New imaging techniques have made it possible to measure oxygen concentrations in the GI tract of living mice (17). When the dissolved O_2 levels are converted to percent oxygen tension, it was shown that O_2 levels in the gut fall from a high of 7% in the stomach, to 4.2% in the mid duodenum, 1.4% in the mid small intestine and mid colon, to a low of 0.4% in the distal sigmoid colon-rectal junction (17). In experimentally inoculated chickens *C. jejuni* localized to the cecum, which projects from the proximal colon at the junction with the small intestine (7, 30). Although the cecum would likely be at the low range of O_2 concentration, *C. jejuni* preferentially localizes to the mucosal crypts in the cecum, where oxygen could diffuse into from adjacent epithelial cells (7, 24). The bacteria do not physically attach to the microvilli, however, and could easily be displaced to more anaerobic regions via cecal mixing (24). The cecal wall is constantly contracting, which causes the mixing of the contents required for the filling and evacuation of the organ (8, 12). The shifting environment of the ceca is likely the impetus behind the redundant respiratory pathways. The ability of the non-fermentative *C. jejuni* to use alternative respiratory acceptors gives the bacterium an advantage when exposed to

anaerobic conditions, even transiently. The single disruption of the nitrate or nitrite reductase creates a significant decrease in colonization, but colonization persists (albeit at lower levels). The ability to use multiple alternative acceptors (nitrate, nitrite, DMSO and TMAO) for anaerobic growth may compensate for the loss of any one enzyme. The role of the cyanide insensitive oxidase encoded by *cydAB* remains elusive. The low affinity of this enzyme for O_2 (19) would seem to rule out a role in the host, and our colonization data bear this out. There is a clear role for the cbb_3-type terminal oxidase both *in vitro* and *in vivo*. The disruption of this oxidase (*ccoN*::Cm) abolishes *C. jejuni*'s ability to colonize the chicken ceca, and mutants are unable to grow at elevated O_2 levels *in vitro*. This stresses the importance of the high-affinity terminal oxidase of *C. jejuni*, thereby implying that oxygen is a key factor in chicken colonization despite the anaerobic conditions in which *C. jejuni* is consistently exposed.

ACKNOWLEDGEMENTS

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

We are grateful to Jason Andrus and Debbie Threadgill of the University of North Carolina, Chapel Hill, for the kind gift of pJMA-001 and helpful discussions.

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Table 1. Strains, plasmids, and primers

	Description	Source
C. jejuni		
NCTC 11168	Parent strain for all *C. jejuni* strains	NCTC[a]
napA::Cm	*cat* insertion within *napA*	This study
nrfA::Cm	*cat* insertion within *nrfA*	This study
ccoN::Cm	*cat* insertion within *ccoN*	This study
cydA::Cm	*cat* insertion within *cydA*	This study
Cj0264c::Cm	*cat* insertion within *Cj*0264c	This study
E. coli DH5α	Cloning strain	Lab stock
One Shot® Top10 Chemically Competent cells	Cloning strain	Invitrogen™
Plasmids		
pCR®TOPO2.1®	Cloning vector	Invitrogen™
pJMA-001	PGEM-T containing *cat* insert	Jay Andrus
pNapA:TOPO	*napA* cloned from NCTC 11168 into PCR®TOPO2.1	This study
pNapA:cm	*cat* inserted in *Ssp* I site of pNapA:TOPO	This study
pBluescriptII® KS^+	Cloning vector	Stratagene®
pKS:ΔXbaI	pBluescriptII$^+$ KS with destroyed *Xba* I site	This study
pNrfA:pKSΔXbaI	*nrfA* cloned from NCTC 11168 into *Eco*R V of pKSΔXba	This study
pNrfA:cm	*cat* inserted in *Xba* I site of pNrfA:pKSΔXba	This study
pCcoN:TOPO	*ccoN* cloned from NCTC 11168 into PCR®TOPO2.1	This study
pCcoN:cm	*cat* inserted in *Nru* I site of pCcoN:TOPO	This study
pCydA:TOPO	*cydA* cloned from NCTC 11168 into PCR®TOPO2.1	This study
pCydA:cm	*cat* inserted in *Eco*47 III site of pCydA:TOPO	This study
p*Cj*0264c:TOPO	*Cj*0264c cloned from NCTC 11168 into PCR®TOPO2.1	This study
p*Cj*0264c:cm	*cat* inserted in *Cla* I site of p*Cj*0264c:TOPO	This study

Table 1 Continued

Primers		
NapAF	5’-TGGGCAATACACTATCCAAGAAGG-3’	IDT[b]
NapAR	5’-TAAAGAAAAGGCTCCACTACCTGG-3’	IDT
NrfAF	5’-GAGGGCATTTTTGGGTTC-3’	IDT
NrfAR	5’-AACGCTGTTTTGTATGTCAAGCAC-3’	IDT
CcoNF	5’-GCAAGTTACCATACTCATCTGTCGC-3’	IDT
CcoNR	5’-GCTTTCAGGGATTTGGGCAAC-3’	IDT
CydAF	5’-TTTAGTGGCAAATGGTTGGATG-3’	IDT
CydAR	5’-TGGAATGGATAACAAACACACCC-3’	IDT
Cj0264cF	5’-TTATGTGCGTAAATCCTACCTTG-3’	IDT
Cj0264cR	5’-AACGGTGCGTGGTTTTCTCC-3’	IDT
RTCj1356cF	5’-TGGTATTTTTCTGTGCTTAGCG-3’	IDT
RTCj1356cR	5’-TTACAACAACCCCTACTCCAAG-3’	IDT
RTCj1486cF	5’-GTTACAGGTATGTTAATTGCTACGG-3’	IDT
RTCj486cR	5’-GACTATATGGCTTTTGCATCACTTC-3’	IDT
RTCj0787F	5’-GCTTCTATCTATGAGGCTCAAGGG-3’	IDT
RTCj0787R	5’-ACTAAGACGACGAACCGCATC-3’	IDT

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

Table 2: Growth rates of *C. jejuni* NCTC 11168.

Addition	Growth Rate[a]	
	Microaerobic	Anaerobic
None	1.8 ± 0.1	No Growth[b]
Formate	1.0 ± 0.1	No Growth
Nitrate	1.4 ± 0.1	No Growth
DMSO	ND	No Growth
TMAO	ND	No Growth
Formate/Nitrate	1.0 ± 0.2	2.7 ± 0.2
Formate/DMSO	1.5 ± 0.1	2.9 ± 0.2
Formate/TMAO	1.9 ± 0.1	3.7 ± 0.2

[a] = Generation time in hours. Results are expressed as the mean of at least three independent growth curves ± the standard deviation.
[b] = No growth is defined as less than two generations during log phase.

Table 3. Nitrate and nitrite reductase activity of *C. jejuni*.

	Nitrate Reductase Activity[a]		Nitrite Reductase Activity[b]	
Strains	0% O_2	12% O_2	0% O_2	12% O_2
11168	ND[c]	ND	205 ± 45.1	73.4 ± 25.6
napA*::Cm*	ND	7 ± 4	ND	ND
nrfA*::Cm*	2335 ± 769	757 ± 81	22.5 ± 23.9	8.9 ± 6.6

[a] expressed as nmol NO_2 produced min^{-1} mg^{-1} ± SD from three independent experiments.
[b] expressed as μmol benzyl viologen oxidized min^{-1} mg^{-1} ± SD from three independent experiments.
[c] Not Determined.

Table 4: Oxygen effect on growth of *ccoN*::Cm on BA plates compared to wild type.

	WT	*ccoN*::Cm
0% O_2	+++	+++
2% O_2	+++	++
4% O_2	+++	++
6% O_2	+++	++
7% O_2	+++	+
8% O_2	+++	-
12% O_2	+++	-

+++ = Large (> 1mm) single, isolated colonies from tertiary streak.
++ = Small (<1 mm) single, isolated colonies from tertiary streak.
+ = Growth primary streak, no isolated colonies.
- = No growth.

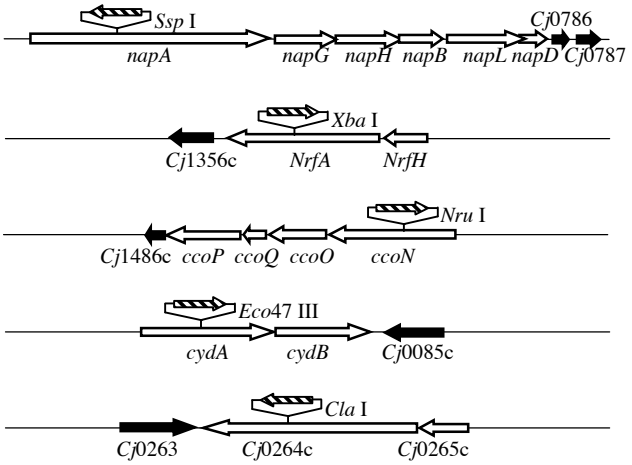

Figure 1: Gene organization of the alternative respiratory pathway operons of *C. jejuni*. Also shown are the insertion sites of the chloramphenicol cassette (dashed arrow) used for mutagenesis. The direction of the arrow indicates the transcriptional orientation of the genes. Unrelated genes directly downstream of the genes of interest are indicated with black arrows.

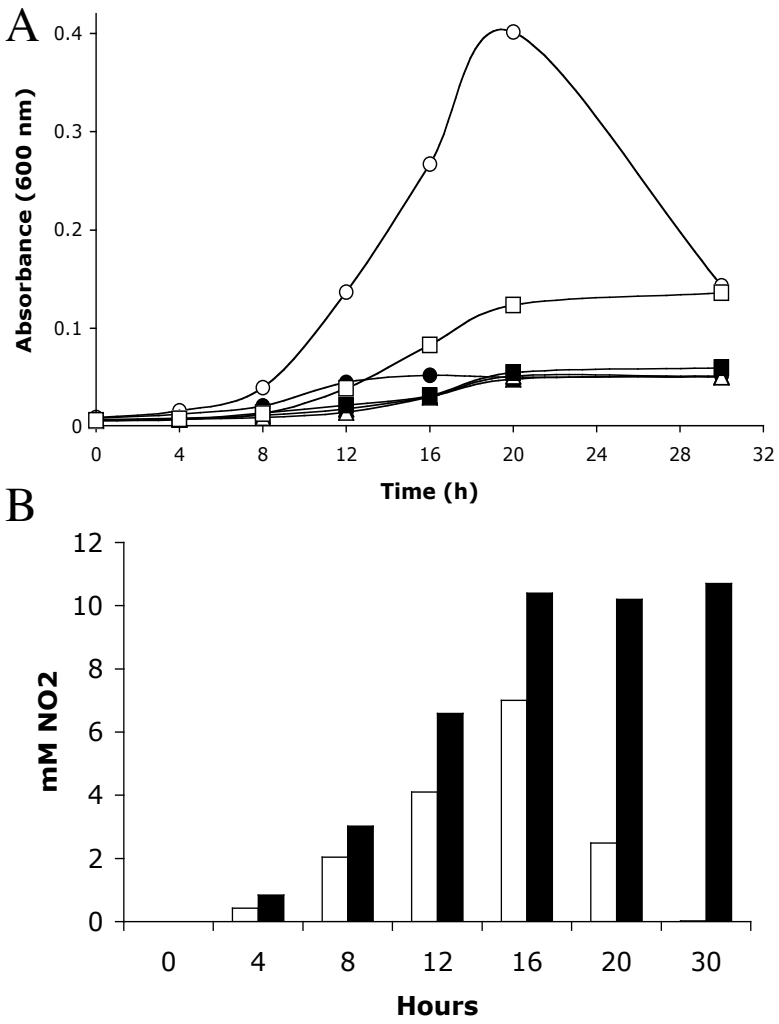

Figure 2: (A): Anaerobic growth of *C. jejuni* NCTC 11168 (circle), *napA*::Cm (triangle), and *nrfA*::Cm (square). Cultures grown in MHF (solid symbols) or MHF plus 10 mM $NaNO_3$ (open symbols) were incubated at 37°C under anaerobic conditions. This growth curve is representative of three independent growth curves. (B): Nitrite concentrations in the supernatant from cultures of wild type (white bars) and *nrfA*::Cm (black bars).

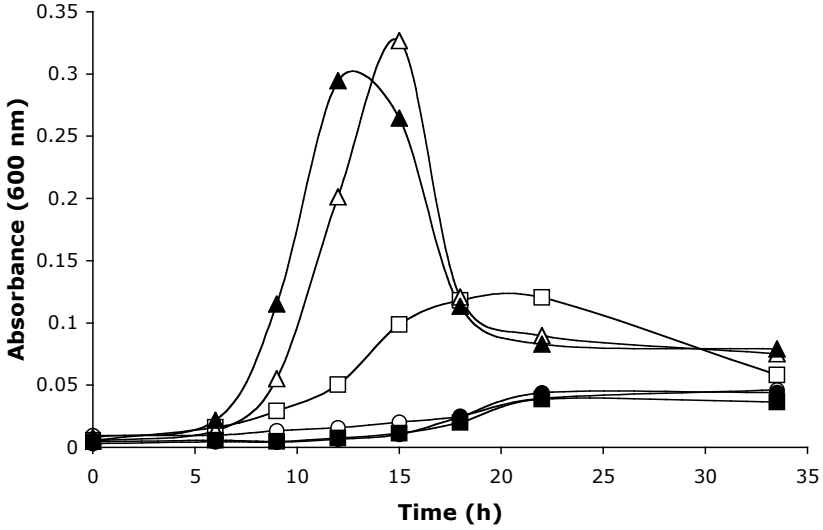

Figure 3: Anaerobic growth of *C. jejuni* NCTC 11168 (open symbol) and Cj0264c::Cm (closed symbol). Cultures grown in MHF (circle) or MHF plus 10 mM $NaNO_3$ (triangle) or MHF plus 5 mM DMSO (square) were incubated at 37°C under anaerobic conditions. This growth curve is representative of three independent growth curves.

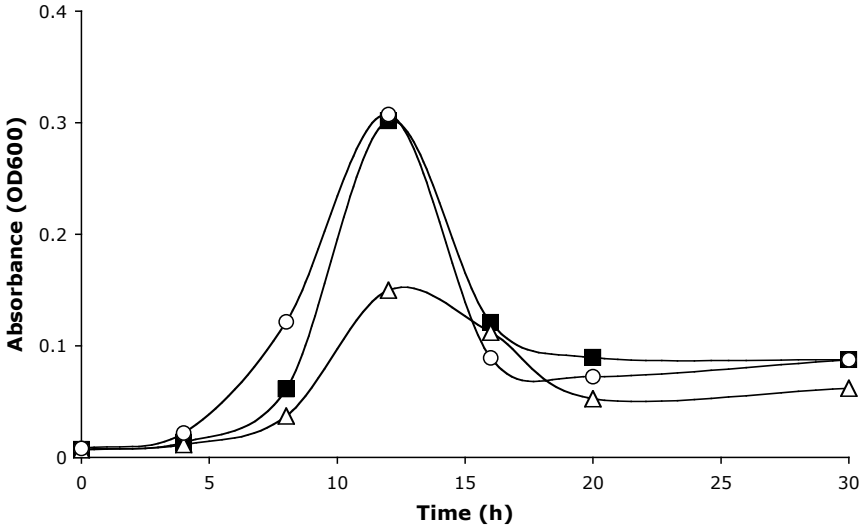

Figure 4: Anaerobic growth of *C. jejuni* NCTC 11168 (closed square), *cydA*::Cm (open circle) and *ccoN*::Cm (open triangle). Cultures grown in MHF plus 10 mM $NaNO_3$ were incubated at 37°C under anaerobic conditions.

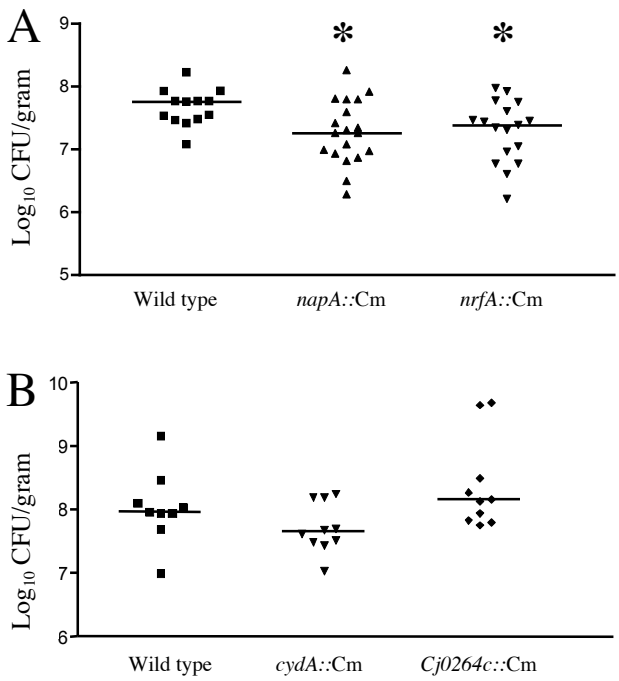

Figure 5: Chicken colonization ability of various strains. A: CFU/gram cecal contents of *C. jejuni* wild type (square), *napA*::Cm (triangle), and *nrfA*::Cm (inverted triangle). B: CFU/gram cecal contents of wild type (square), *cydA*::Cm (inverted triangle), Cj0264c::Cm (diamond). Horizontal bars represent median value for each group. * = $p < 0.05$ when compared to wild type.

CHAPTER 3

The dual functioning fumarate reductase of *Campylobacter jejuni* is the sole succinate:quinone reductase and is required for full host colonization

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To be submitted to the Journal of Bacteriology

ABSTRACT

Campylobacter jejuni encodes all the enzymes for a complete oxidative tricarboxylic (TCA) cycle. Because of its inability to utilize glucose, *C. jejuni* relies exclusively on amino acids as the reduced carbon source, which are incorporated into central carbon metabolism. The oxidation of succinate to fumarate is a key step in the oxidative TCA cycle. *C. jejuni* encodes for enzymes annotated as a fumarate reductase (*Cj*0408-0410) and succinate dehydrogenase (*Cj*0437-0439). Mutants were constructed in each enzyme: *frdA*::Cm (fumarate reductase) and *sdhA*::Cm (succinate dehydrogenase). Both enzymes contributed equal fumarate reductase activity *in vitro*. *frdA*::Cm was completely deficient in succinate dehydrogenase activity *in vitro* and was unable to perform whole cell succinate-dependent respiration. *sdhA*::Cm exhibited wild type rates of succinate dehydrogenase both *in vivo* and *in vitro*. These data indicate that Frd operates as the exclusive succinate dehydrogenase in *C. jejuni*, and the enzyme annotated as a succinate dehydrogenase has been misannotated. *frdA*::Cm was also unable to grow with the characteristic wild type biphasic growth pattern, only exhibiting the first growth phase marked by the consumption of aspartate, serine, and associated organic acids. Substrates consumed in the second growth phase (glutamate, proline, and associated organic acids) were unavailable to *frdA*::Cm, indicating that the oxidation of succinate is a crucial step in metabolism of these substrates. Chicken colonization studies confirmed the *in vivo* importance of succinate oxidation as *frdA*::Cm colonized at significantly lower numbers than wild type, while *sdhA*::Cm was unaffected.

INTRODUCTION

Campylobacter jejuni causes approximately two million cases of bacterial gastroenteritis annually (33). Humans are most often infected from cross-contamination brought on by improper food handling of poultry (25), which is the natural habitat of *C. jejuni* (26). The eradication of *C. jejuni* from poultry flocks is an important goal in reducing the number of campylobacteriosis cases.

C. jejuni can rely solely on amino acid catabolism for its carbon source, the products of which are incorporated into glycolysis and the TCA cycle (14, 27). Fumarate and succinate are key intermediates in the TCA cycle (Figure 1). Succinate:quinone oxidoreductases (SQR) are membrane bound enzymes that catalyze either the two-electron oxidation of succinate to the two-electron reduction of quinone/quinol, or in the reverse direction coupling the oxidation of quinol/quinone to the reduction of fumarate to succinate. SQRs can be divided into three distinct classes based on function, all of which have similar subunit makeup and primary amino acid sequence. Class one SQRs couple the oxidation of succinate to the reduction of a high redox potential quinone like ubiquinone *in vivo*. Class two is the quinol:fumarate reductases (QFR), which couple the oxidation of menaquinol to the reduction of fumarate. The third class of SQRs couple the oxidation of succinate to the reduction of a low potential quinone such as menaquinone *in vivo* (11). Although each class has shared motif's, the *in vivo* function of an SQR enzyme can not be resolved based on primary sequence, and must be determined experimentally. The *C. jejuni* genome encodes for two enzymes that fall into the succinate:quinone oxidoreductase family, one annotated as

a fumarate reductase (*Cj*0408-0410) and the other a succinate dehydrogenase (*Cj*0437-0439) (27). Fumarate reductase (Frd) activity as measured by NMR spectroscopy has been reported in the particulate fraction of *C. jejuni* cell lysates (37). The addition of formate to whole cells increased Frd activity (37), which implies an active electron transport pathway. However, *C. jejuni* is unable to utilize fumarate as an alternative electron acceptor under anaerobic conditions (36, 40). *C. jejuni* can also use succinate as an electron donor to a respiratory quinone (12), identified as either a menaquinone-6 or methylmenaquinone-6 (4). Yet, succinate oxidation via menaquinone is an endergonic reaction; succinate has a redox midpoint potential (E_m) of +30 mV and menaquinone is more electronegative at E_m = –80 mV. Although succinate oxidation coupled to menaquinone reduction would be an "uphill" reaction, Class three SQRs can catalyze this reaction. Studies on gram-positive bacteria from the genus *Bacillus*, as well sulfate-reducing bacteria have shown that oxidation of succinate through menaquinone is driven by reversed, transmembrane electron transport (17, 35, 44) and it is hypothesized that *C. jejuni* behaves similarly.

The Frd enzyme contains three subunits: FrdCAB, the gene order in the operon is similar to *Wolinella succinogenes* (15, 18) and *Helicobacter pylori* (1, 9, 39). FrdC (Cj0408) is the membrane anchor and diheme cytochrome B, FrdA (Cj0409) is where the reduction of fumarate to succinate occurs and contains an FAD flavoprotein and FrdB (Cj0410) is an Fe-S protein (27). The succinate dehydrogenase of *C. jejuni* is also composed of three subunits: SdhABC encoded by *Cj*0437-0439 (27). SdhA is annotated as a succinate dehydrogenase flavoprotein subunit, SdhB is a putative succinate dehydrogenase Fe-S protein and SdhC is a

putative succinate dehydrogenase subunit C. According to ClustalW pairwise alignment, FrdA and SdhA of *C. jejuni* share 29% identity; FrdB and SdhB share 18% identity. FrdC and SdhC share 13% identity based on BLOSUM matrix, with an open gap penalty of 10.0 and extend gap penalty of 0.1.

A better understanding of *C. jejuni* tricarboxylic acid (TCA) cycle may help identify metabolic pathways that are crucial to *C. jejuni*'s ability to thrive in poultry. The roles of the *C. jejuni* fumarate reductase and succinate dehydrogenase in the TCA cycle and respiration were investigated. Both enzymes equally contribute to fumarate reductase activity. We determined that the protein annotated as the fumarate reductase functions as the sole succinate dehydrogenase, and that this enzyme is required for full chicken colonization ability by *C. jejuni*. The *sdh* operon has been misannotated as the enzyme plays has no role in succinate dehydrogenase activity.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Table 1 lists strains of *C. jejuni* and *E. coli* along with all plasmids and primers utilized in this study. Tryptic soy agar (Difco, Sparks, MD) plates supplemented with 10% defibrinated sheep blood (Gibson Laboratories, Inc. Lexington, KY) are called BA plates and used for growth of *C. jejuni*. *C. jejuni* was routinely cultured at 37°C microaerobically in a tri-gas incubator (model 550D; Fisher Scientific) constantly maintained at 5% CO_2 and 12% O_2, balance N_2. Mueller Hinton broth (MHB) (Difco, Sparks, MD) was used for *C. jejuni* cultures and liquid cultures were incubated at 37°C with shaking. Chloramphenicol (25 μg/ml) was added as indicated. Genetic manipulations were performed with *Escherichia coli* strain DH5α (laboratory stock). Luria-Bertani broth and agar was supplemented with ampicillin (100 μg/ml) or chloramphenicol (25 μg/ml), as noted.

Succinate dehydrogenase assay: Succinate dehydrogenase activity was measured by 2,6-dichlorophenol-indophenol (DCPIP)-dependent reduction by succinate as adapted by (34). Briefly, a closed quartz cuvette containing 50 mM $NaPO_4$ buffer pH 7.2, 0.25 mM DCPIP, 0.4 mM phenazine methosulfate, and 100 μl of cell-free extract was made anoxic by sparging with N_2 gas. The reaction was started by the addition of 20 mM succinate pH 7.4 and the DCPIP-dependent reduction kinetics were recorded by a spectrophotometer at 600 nm. The rate was calculated as μmol DCPIP reduced min^{-1} mg^{-1} protein using a molar extinction coefficient for DCPIP of 21 x 10^3 cm^{-1}.

Fumarate reductase assay: Benzyl viologen-linked reductase assays were carried out with sonicated cell extracts in a 1 ml assay volume as described in (36). Reagents were added by syringe through the stopper, while argon gas was flushed through the cuvette. The reaction mixture (reagents were kept anaerobic during the course of the assay) contained 75 mM sodium phosphate buffer (pH 6.8), 0.2 mM benzyl viologen and 1-5 μg of cell extract in 1 ml stoppered quartz cuvettes. Freshly made 20 mM sodium dithionite was then injected into the cuvette until the absorbance at 585 nm reached 0.8-0.9, which represents half reduced benzyl viologen. An anaerobic solution of sodium fumarate to a final concentration of 5 mM was added and the benzyl viologen oxidation kinetics were recorded by a spectrophotometer at 585 nm. Fumarate reductase activity was expressed as nmol benzyl viologen oxidized min^{-1} mg^{-1}.

O_2 uptake assay. O_2 uptake experiments were performed using a Clarke-type electrode and YSI Model 5300 oxygen monitor (Yellow Spring, OH) as described previously (43). Briefly, washed whole cells were resuspended in PBS and added to the constantly stirred chamber and allowed to equilibrate until no change in O_2 consumption was seen for several minutes. Lactate or succinate (final concentration of 5 mM) was added through a capillary tube via a Hamilton syringe into the chamber and the chart recorder documented the rate of oxygen consumption, and the slope of the line determined the rate of oxygen uptake. Rates were expressed as nmol O_2 consumed min^{-1} mg^{1}.

Cloning and construction of *C. jejuni* mutants. Oligonucleotide primers for cloning genes of interest were designed from the sequenced strain NCTC 11168 (27) and are listed in Table 1. Polymerase chain reaction (PCR) amplification was performed with *Taq* DNA polymerase (Promega, Madison, WI) using chromosomal DNA isolated from *C. jejuni* NCTC 11168 as a template. The PCR products (1154 bp of *frdA*, 904 bp of *sdhA* and 2327 bp of *Cj*0411) were cloned into pCR®2.1-TOPO® vectors (Invitrogen, Carlsbad, CA) and confirmed by restriction analysis. The coding region of the gene of interest was disrupted by insertion of a chloramphenicol resistance gene (*cat*), which was originally isolated from *Campylobacter coli* (Figure 2) (42). The restriction endonuclease recognition sequences utilized were *Eco*47III in *frdA*, *Ssp*I for *sdhA* and *Eco*47III for *Cj*0411 (Table 1). Electrocompetent *C. jejuni* cells were transformed with each construct to yield the corresponding *C. jejuni* mutant as described previously (43). 100 μl of transformed cells were spotted onto cold nonselective BA plates, and the plates were incubated microaerobically for 24 hours. The cells were then transferred to BA plates containing chloramphenicol. Resistant colonies that appeared after two to five days were passed on selective BA plates and the correct insertion of the cassette was confirmed by isolation of chromosomal DNA from the candidate strains and PCR amplification of the gene. Agarose gel electrophoresis of the PCR product was used to monitor the size increase of the gene of interest with the antibiotic cassette insertion (data not shown).

Quantitative Reverse-Transcriptase PCR (qRT-PCR). PCR primers used in this study are listed in Table 1 and were designed to amplify gene fragments of the following sizes: 115 nt

of *gyrA*, and 136 nt of *Cj*0411. Total RNA was isolated from the parent strain of *C. jejuni* and *frdA*::Cm using a MasterPure™ Complete RNA Purification Kit (Epicentre® Biotechnologies, Madison, WI). qRT-PCR was performed by using the Quantitect™ SYBR® Green RT-PCR kit (QIAGEN, Valencia, CA). The PCR mixture (20 μl) contained 40 ng RNA, 10 μl 2x QuantiTect SYBR Green RT-PCR Master Mix, and 0.2 μl QuantiTect RT mix. The reverse transcriptase cycle step was performed at 50°C for 30 minutes, followed by a PCR initial activation step of 95°C for 15 minutes. The mixtures were then amplified in 30 cycles of 94°C for 15 seconds, 60°C for 30 seconds, and 72°C for 30 seconds in an automated thermal cycler (BIORAD iCycler™, 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 using the internal control *gyrA* (*Cj*1027c).

Sonication. Cells were disrupted by sonication. Sonication was performed on ice in a W-370 horn cup sonicator (Heat Systems—Ultrasonics, Inc, Farmingdale, NY) for four 45-second pulses at 60% power and 7.0 output control setting.

Protein assay. Protein concentration was determined using the BCA™ Protein Assay Kit (Pierce, Rockford, IL) using bovine serum albumin as a standard.

Chicken colonization. *Campylobacter*-free day old broiler chicks were supplied by the Lake Wheeler Poultry Facility operated by the NCSU Poultry Department. Birds were housed in isolation rooms at the Dearstyne Avian Health Center (Department of Poultry Science, NCSU) in isolation brooder batteries (10 chicks per battery). Chicks were fed Purina Mills® Start & Grow® SunFresh® Recipe (Purina Mills LLC, St. Louis, MO) feed and water *ad libidum*. Week old chicks were inoculated by oral gavage with 0.1 ml of either ~10^7 or ~10^8 *C. jejuni* cells/ml, which had been grown for approximately 16 hours on BA plates and cultured at 37°C microaerobically. Control chicks were inoculated with 0.1 ml sterile PBS (pH 7.4). Two weeks post-inoculation the chickens were humanely sacrificed by CO_2 affixation and approximately 1 g of cecal contents were collected, serially diluted (in PBS) and plated on selective BA media containing: 40 μg/ml Cefoperazone, 40 μg/ml Vancomycin, 10 μg/ml Trimethoprim and 100 μg/ml Cyclohexamide. All samples were incubated microaerobically at 37°C. After two days incubation, colonies were counted and the CFU/g cecal content was calculated. Data was analyzed by one-tailed Mann-Whitney test, using a 95% confidence interval.

RESULTS

Growth. Figure 3 shows a typical growth pattern by *frdA*::Cm. Wild type *C. jejuni* has a characteristic biphasic growth pattern when grown in MHB under microaerobic conditions. *frdA*::Cm behaves similarly to wild type during the first growth phase with an *frdA*::Cm growth rate 2.23 ± 0.7 hours compared to wild type growth rate 2.02 ± 0.4. When wild type *C. jejuni* reaches mid-log (an absorbance at 600 nm of approximately 0.3 in MHB), there is a shift to a second growth phase and exhibits a second, slower growth rate of 10.3 ± 1.2 hours. *frdA*::Cm never enters the second growth phase, and stops growing after the first growth phase. The inset of Figure 3 shows the growth curve in linear scale to better visualize the marked difference in growth characteristics between wild type and *frdA*::Cm. *sdhA*::Cm grows similarly to wild type with two distinct growth phases, growth rates of 2.23 ± 0.05 hours and 12 ± 1 hours, respectively.

Although the fumarate reductase can be used as an alternative electron acceptor in place of oxygen by other *Campylobacter* species and the ε-proteobacteria *W. succinogenes*, an (5, 16), *C. jejuni* is unable to grow anaerobically with fumarate (36, 37). Fumarate reductase is also an essential enzyme in the reverse (reductive) TCA cycle. TCA cycle intermediates were added to MHB to see if *frdA*::Cm growth phenotype could be rescued. Table 2 shows the terminal optical density (600 nm) of *frdA*::Cm after 26 hours of microaerobic growth with the addition of TCA substrates. Several intermediates increased the first growth phase, (and thus the terminal optical density) of *frdA*::Cm; however, no substrate produced the second growth phase that is normally seen with wild type *C. jejuni* (data not shown). The TCA

intermediates fumarate, malate, oxaloacetate, and pyruvate (which can be a precursor to oxaloacetate via oxaloacetate decarboxylase) did extend the first growth phase, and thus raised the terminal optical density of *frdA*::Cm. However, the terminal optical density of *frdA*::Cm was not affected by the addition of acetate, citrate, 2-oxoglutarate, and succinate, which group into half of the TCA cycle (Figure 1). Citrate and acetate did not effect wild *C. jejuni* growth (data not shown). When grown in media containing amino acids, *C. jejuni* utilizes aspartate, glutamate, proline and serine to depletion (10, 19). Both aspartate (a precursor to fumarate via AspA) and serine (serine dehydratase (SdaA) deaminates serine to pyruvate) increased *frdA*::Cm growth (Table 2). Glutamate (a precursor to 2-oxoglutarate) and proline (proline dehydrogenase oxidizes proline to glutamate) had no effect on *frdA*::Cm. Figure 1 shows the TCA intermediates that are utilized by *frdA*::Cm (circled) group together immediately following the fumarate and succinate interconversion. Those intermediates that had no effect on *frdA*::Cm (squared) group immediately prior to the fumarate and succinate enzymatics (Figure 1).

Polar effects. Mutants were constructed as described previously (43). The insertion of an antibiotic cassette into the chromosome can effect the transcription of downstream genes ("polar effect") (Figure 2). Immediately downstream of the *sdh* operon, is *Cj*0440c, which encodes a TenA/Thi-4 family protein; however, this gene is on the opposite DNA coding strand and transcription should not be affected (27). Downstream of the *frd* operon is *Cj*0411, which encodes a putative GTP-binding protein. qRT-PCR was employed to measure the transcription of *Cj*0411 in both the wild type and *frdA*::Cm. Polar effects were seen as

the relative transcription levels of *Cj*0411 in *frdA*::Cm was 0.15 ± 0.08 fold that of wild type. To confirm that the phenotype of *frdA*::Cm was a result of the fumarate reductase disruption and not from the reduced transcription of the GTP-binding protein, a mutant was constructed. Cj0411::Cm behaved similarly to the wild type with two distinct growth phases with similar growth rates in both phases as wild type (data not shown). The growth phenotype associated with *frdA*::Cm can be attributed to the disruption of the fumarate reductase.

Fumarate reductase and succinate dehydrogenase activities. Fumarate reductase activity was measured in wild type, *frdA*::Cm and *sdhA*::Cm cell-free extracts. All three were able to oxidize benzyl viologen using fumarate as a terminal acceptor (Table 3). Activity was measured utilizing cultures at varying growth phases (Table 3). Fumarate reductase activity in *frdA*::Cm cultures greater than 0.3 absorbance (600 nm) required the addition of pyruvate to the media to increase the terminal optical density; unsupplemented cultures did not grow past 0.3 OD_{600} nm (Table 2). Wild type and *sdhA*::Cm were grown with the addition of pyruvate as controls. Under all conditions tested, *frdA*::Cm and *sdhA*::Cm each contributed approximately one half the fumarate reductase activity of wild type *C. jejuni* (Table 3).

Succinate dehydrogenase activity was measured for wild type and the two mutants at different growth phases. Cell-free extracts were utilized to measure the reduction of the artificial acceptor DCPIP coupled to succinate oxidation. Wild type and *sdhA*::Cm had similar succinate dehydrogenase activities under all conditions tested (Table 4). *frdA*::Cm, however, was unable to oxidize succinate using DCPIP as an artificial terminal acceptor

under all conditions tested (Table 4). Pyruvate was added to the media for *frdA*::Cm cultures to reach 0.5 OD_{600}.

Oxygen uptake by succinate oxidation. Wild type, *frdA*::Cm and *sdhA*::Cm whole cells were assayed for succinate oxidation as measured by oxygen uptake. Cultures were grown to an optical density of approximately 0.5. Pyruvate was supplemented in the media for *frdA*::Cm. The addition of pyruvate to the media had no effect on wild type respiration rates (data not shown). Wild type and *sdhA*::Cm consumed oxygen at similar rates. Wild type produced a succinate-dependent respiration rate of 28 ± 7 nmol O_2 consumed/min/10^8 cells and *sdhA*::Cm respired at a rate of 37 ± 15 nmol O_2 consumed/min/10^8 cells. *frdA*::Cm was unable to respire with succinate as the sole electron donor (0.6 ± 1 nmol O_2 consumed/min/10^8 cells). All three strains produced similar rates of lactate-dependent respiration, which was used as a positive control.

Chicken Colonization. At one week of age, groups of ten birds were inoculated with $6.1x10^6$ CFU of wild type or $1.1x10^7$ *frdA*::Cm by oral gavage. Two weeks post-inoculation, the birds were sacrificed by CO_2 asphyxiation and the cecal contents were collected for enumeration of viable *C. jejuni*. All birds were colonized with *C. jejuni* at the conclusion of the experiment. *frdA*::Cm colonized at significantly lower levels than wild type (*p* value = 0.001) (Figure 4). A second host colonization trial was performed and groups of week old birds were inoculated with $2.7x10^6$ CFU of wild type or $1.8x10^6$ *sdhA*::Cm by oral gavage. Two weeks post-inoculation the cecal contents were enumerated for viable *C. jejuni*.

sdhA::Cm and wild type colonized at similar levels (Figure 4). No *C. jejuni* was recovered from the negative control birds inoculated with PBS in either colonization trial (data not shown).

DISCUSSION

The interconversion of fumarate and succinate is a vital process for organisms that use the TCA cycle for central carbon metabolism. *C. jejuni* encodes for a complete oxidative TCA cycle, which converts TCA intermediates (carboxylic acids) to produce CO_2, ATP, and reducing equivalents. One of the conversion steps, succinate oxidation to fumarate, forms a reducing equivalent and is required for a complete cycle. Fumarate reduction to succinate also occurs as part of the reductive TCA cycle; a carbon fixation pathway that has been proposed to be utilized by ε-proteobacteria found in deep-sea hydrothermal vents (3). *C. jejuni* does encode for many of the reversible enzymes necessary for the reductive TCA cycle, including: 2-oxoglutarate ferredoxin oxidoreductase (encoded by *oorDABC*) and pyruvate carboxylase (encoded by *pycA* and *pycB*) (27), however, *C. jejuni* does not encode for an ATP citrate lyase, which is required for a full cyclic reductive carboxylation (3). The fumarate-succinate interconversion is also involved in respiration (11), and fumarate has specifically been implicated as an alternative electron acceptor to oxygen in other ε–proteobacteria (5, 16). *C. jejuni* encodes for two enzymes: *Cj*0408-0410, which is annotated as a fumarate reductase and *Cj*0437-0439, which is annotated as a succinate dehydrogenase (27). In *E. coli*, both enzymes are able to reduce fumarate and oxidize succinate, albeit with a preference for one substrate (6, 20), the amino acid sequence, however, does not dictate *in vivo* function (17). So, we set out to determine the function of each enzyme in *C. jejuni* and their role, if any, in poultry colonization.

C. jejuni is unable to respire anaerobically using fumarate as a terminal electron acceptor (36, 40). Fumarate reductase activity has been measured previously, but no fumarate reductase mutants were constructed (36, 37). A fumarate reductase mutant was constructed containing a chloramphenicol cassette disrupting *frdA* (*Cj*0409). *frdA*::Cm grows similarly to wild type until mid-log (an optical density approximately 0.3 at an absorbance of 600 nm) and then ceased growing (see Figure 3). Wild type cultures, on the other hand, continued to grow, but slower than the initial growth rate until a terminal optical density of approximately 1.0 was obtained (Figure 3). *sdhA*::Cm grew similarly to wild type with two distinct growth phases (data not shown).

A possible explanation for the aborted growth of *frdA*::Cm is that this mutant is unable to utilize a particular class of substrates for carbon and energy. To test this hypothesis, carbon substrates were added to try and rescue the mutant phenotype. *C. jejuni* is unable to use glycolysis (27), and must rely on amino acid biosynthesis for its carbon requirement (10). Amino acids are incorporated into the TCA cycle through a variety of transport systems and enzymes (14). We chose to add TCA intermediates to test whether the addition could rescue growth. Although none of the added substrates were able to restore the biphasic growth to *frdA*::Cm (data not shown), addition of certain TCA intermediates did extend the first growth phase and increase the terminal optical density when compared to unsupplemented cultures (Table 2). MHB with no addition of substrate resulted in a terminal optical density less than 0.3 (mid-log in MHB). Substrates that did not increase the terminal optical density beyond 0.3 were not considered stimuli for *frdA*::Cm. The intermediates that did not extend the

primary growth phase of *frdA*::Cm include citrate, 2-oxoglutarate, and succinate, which group together in the TCA cycle immediately preceding (in the oxidative cycle) the succinate and fumarate interconversion (Figure 1). Those substrates that raised the terminal optical density to above 0.5 were considered growth stimuli for *frdA*::Cm. Pyruvate, oxaloacetate, malate and fumarate extended the growth of *frdA*::Cm, and these intermediates group directly after the succinate and fumarate interconversion (Figure 1). The disruption of Frd results in the inability of *C. jejuni* to incorporate substrates from half its TCA cycle into biomass.

C. jejuni has preference for specific amino acid utilization (10, 19). The amino acid content of MHB was measured before *C. jejuni* inoculation and after 48 hours of growth (spent media) (10). The analysis of spent media showed aspartate, glutamate, serine, and proline were reduced over 50% of their original concentration (10). Therefore, we tested whether these four key amino acids were helpful in extending *frdA*::Cm growth. Aspartate and serine were able to increase the terminal optical density of *frdA*::Cm, while glutamate and proline had no effect (Table 2). Taken together, this data suggests that *C. jejuni* preferentially uses the amino acids and TCA cycle substrates immediately after the oxidation of succinate in the first growth phase, and at mid-log, it switches to a less preferred substrate and amino acid class, as shown in Figure 1.

Fumarate reductase and succinate dehydrogenase activities were determined for *frdA*::Cm and *sdhA*::Cm as well as the parent strain. All three strains exhibited fumarate reductase activity in all conditions tested, *frdA*::Cm and *sdhA*::Cm each contributing one half the

fumarate reductase activity of wild type (Table 3). Surprisingly, *sdhA*::Cm had similar succinate dehydrogenase activity to wild type under all conditions tested, and *frdA*::Cm had no succinate dehydrogenase activity (Table 4). Physiologically, succinate oxidation can also be measured through the uptake of oxygen by whole cells. The respiration rates of whole cells of wild type and *sdhA*::Cm cultures had similar oxygen uptake rates using succinate as the donor, but *frdA*::Cm was unable to respire with succinate. Lactate is an efficient respiratory donor for *C. jejuni* (12) and all three strains had similar lactate-dependent respiration rates (data not shown).

W. succinogenes is able to respire anaerobically using fumarate as an alternative acceptor (16). A *W. succinogenes* ΔFrdCAB strain containing a *C. jejuni* fumarate reductase under the control of *W. succinogenes frd* promoter was able to respire anaerobically with fumarate as a terminal electron acceptor (24). Fumarate reductase activity in complemented cells could be measured by the oxidation of 2,3-dimethyl-1,4-naphthoquinol, but activity levels were lower compared to the *W. succinogenes* parent strain (24). The isolated Frd protein produced both specific fumarate reductase activity and succinate dehydrogenase activity (24). Our data supports these findings; *C. jejuni* FrdCAB can both reduce fumarate and oxidize succinate.

The amino acid sequences of the fumarate reductase of *C. jejuni* align with the Frd proteins of *W. succinogenes* and *H. pylori*. Blast searches reveal that the Frd proteins of *C. jejuni* have higher homology to Frd of *W. succinogenes*. The *C. jejuni* subunits FrdA and FrdB

have 69% and 68% identity to *W. succinogenes*, respectively. FrdC shows only 52% homology to FrdC of *W. succinogenes*. The Sdh proteins of *C. jejuni* also align with *W. succinogenes*. The SdhABC subunits have 63%, 71%, and 69% homology to *W. succinogenes* SdhABC. The *H. pylori* genome does not encode for a succinate dehydrogenase; the incomplete TCA cycle is missing succinate dehydrogenase, as well as 2-oxoglutarate dehydrogenase and succinyl-CoA synthetase (31).

In *E. coli*, under aerobic conditions succinate is oxidized by ubiquinone, and under anaerobic conditions, fumarate is reduced by menaquinol oxidation (41). There is a third functional class of succinate:oxidoreductase, which catalyze the oxidation of succinate and the reduction of the low redox potential menaquinone (11). *C. jejuni* only contains menaquinone-6 and a methyl-substituted menaquinone (4). Succinate oxidation (E_m = +30 mV) coupled to menaquinone reduction (E_m = -80 mV), is an endergonic reaction that has been shown to require a Δp (35). *Desulfovibrio vulgaris, Geobacter sulfurreducens* and *Bacillus subtilis* are all able to couple succinate oxidation to menaquinone reduction via this mechanism (2, 21, 44).

The amino terminus of FrdC of *C. jejuni* is predicted to contain five transmembrane helices by TMHMM (www.cbs.dtu.dk/services/TMHMM/), which is also present in *W. succinogenes* FrdC and *B. subtilis* SdhC (35). These helices contain the four conserved His residues, which act as axial ligands for two heme B molecules required for transmembrane electron transfer (35). Also conserved are two glutamate residues, which are active sites for

menaquinol oxidation. All these elements are predicted to be required for menaquinone-dependent succinate dehydrogenase (44), the *sdhC* of *C. jejuni* does not encode for any of these elements. The fumarate reductase of *W. succinogenes*, which does contain these elements, produces both specific enzymatic activity of succinate dependent methylene blue reduction and benzyl viologen oxidation through fumarate reduction (24).

The enzyme annotated as a fumarate reductase is the sole succinate dehydrogenase enzyme of *C. jejuni*. Cell-free extracts of *frdA*::Cm have no succinate dehydrogenase activity as measured by DCPIP reduction coupled to succinate oxidation and whole cells are deficient in succinate-dependent respiration as measured by oxygen uptake assay. *frdA*::Cm is also unable to utilize half of the oxidative TCA cycle, and therefore fails to grow past mid-log. The FrdC subunit is a transmembrane anchor with four conserved histidines and contains sites of menaquinol oxidation, which are requirements for succinate:menaquinone dehydrogenases and the SdhC subunit of *C. jejuni* is lacking these traits. Taken together, these data indicate that FrdCAB is the sole succinate dehydrogenase of *C. jejuni* and SdhABC has been misannotated as it does not contribute to succinate dehydrogenase activity. *in vitro* studies indicate that FrdCAB and SdhABC equally contribute to fumarate reductase activity.

frdA::Cm showed significant decrease in poultry colonization ability compared to wild type and *sdhA*::Cm (Figure 4). The cecum of poultry contains fermentative byproducts including lactate, acetate, hydrogen and formate (32). Amino acids are abundant in the ceca of poultry

as a result of a high cellulose diet and from the biosynthesis of amino acids by microbes in the ceca (30). To study the *in vivo* availability of amino acids, one can compare the excreta from laying hens and caecectomized laying hens. The removal of ceca significantly reduces the microbes affect on digestion and it is believed that microbial metabolism of amino acids in the ceca is largely responsible for the differences between intact and caecectomized birds (28). Total amino acid excretion was greater in caecectomized laying hens than intact hens. Proline, threonine and isoleucine were increased significantly in the excreta of caecectomized laying hens, and it is inferred that the microbes of the ceca utilize these amino acids (29). Because *frdA*::Cm is unable to metabolize proline into the TCA cycle, it may be at a disadvantage in the ceca of poultry, thus explaining the decreased colonization ability.

Fumarate reductase and succinate dehydrogenase have been implicated in colonization and virulence studies in other systems. The fumarate reductase of *H. pylori* is required for mouse colonization (8) and has been studied as a potential drug target (7, 22). An *E. coli* fumarate reductase mutant also showed a significant decrease in colonization ability in mice (13). In *Salmonella enterica* serovar Typhimurium, a complete TCA cycle is necessary for full virulence in mice (38) and a fumarate reductase/succinate dehydrogenase double mutant is avirulent in BALB/c mice (23).

ACKOWLEDGEMENTS

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

We would like to thank Dr. Jesse L. Grimes, Department of Poultry Science, NCSU for his help with the poultry colonization trials and Dr. Dilan Weerakoon for providing us with the *sdhA*::Cm strain.

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Table 1: Strains, plasmids, and primers

	Description or Sequence	Source[a]
Strains		
C. jejuni NCTC 11168	Parent strain for all *C. jejuni* strains	NCTC
frdA::Cm	*cat* insertion within *frdA*	This study
sdhA::Cm	*cat* insertion within *sdhA*	This study
Cj0411::Cm	*cat* insertion within *Cj*0411	This study
E. coli DH5α	Cloning strain	Lab stock
One Shot® Top10 Cells	Cloning strain	Invitrogen
Plasmids		
PCRTOPO2.1	Cloning vector	Invitrogen
pJMA-001	PGEM-T containing *cat* insert	Jay Andrus
pFrdA:TOPO	*frdA* cloned from NCTC 11168 into PCRTOPO2.1	This study
pFrdA:cm	*cat* inserted in *Eco*47III site of pFrdA:TOPO	This study
pSdhA:TOPO	*sdhA* cloned from NCTC 11168 into PCRTOPO2.1	This study
pSdhA:cm	*cat* inserted in *Ssp*I site of pSdhA:TOPO	This study
pCj0411:TOPO	*Cj*0411 cloned from NCTC 11168 into PCRTOPO2.1	This study
pCj0411:cm	*cat* inserted in *Eco*47III site of pCj0411:TOPO	This study
Primers		
FrdAF	5'-GTTGTAGCAGGTATGATAGTGGGAG-3'	IDT
FrdAR	5'-AACTTCTGGCTCAATGCGTTC-3'	IDT
SdhAF	5'-TGATTGCTACAGGGGGATACAC-3'	IDT
SdhAR	5'-TCACTTCATTAGGATTTGGAATACG-3'	IDT
Cj0411F	5'-AAGCAAACTTGATACAGCCGAG-3'	IDT
Cj0411R	5'-GATTTACCACTTGAAAACTGCCC -3'	IDT
RTGyrAF	5'-TGGTTGTAACTATCACACATCGTGG-3'	IDT
RTGyrAR	5'-AATCATCATCATAAGTCGTAACGGC-3'	IDT
RTCj0411F	5'-CAGGGCTTGATGATGTTGTAGTTC -3'	IDT
RTCj0411R	5'-GACAATGCACCAAAAAGTCTGC-3'	IDT

[a] NCTC, National Collection of Type Cultures; IDT, Integrated DNA technologies, Coralville, IA.

Table 2: *frdA*::Cm cultures grown in MHB ± substrate for 26 hours

28 mM substrate	Absorbance (600 nm)	Growth Stimulus
No addition	0.236 ± 0.007	
Citrate	0.296 ± 0.015	N
2-oxoglutarate	0.283 ± 0.013	N
Succinate	0.294 ± 0.004	N
Glutamate	0.235 ± 0.012	N
Proline	0.239 ± 0.004	N
Acetate	0.270 ± 0.007	N
Pyruvate	0.564 ± 0.023	Y
Oxaloacetate	0.523 ± 0.002	Y
Malate	0.687 ± 0.040	Y
Fumarate	0.674 ± 0.032	Y
Aspartate	0.919 ± 0.048	Y
Serine	0.561 ± 0.023	Y

Table 3: Fumarate reductase activity* of *C. jejuni* cell-free extracts

Absorbance (600 nm)	Strains		
	Wild type	*frdA*::Cm	*sdhA*::Cm
0.1 OD	61 ± 21	4 ± 3	63 ± 24
0.2 OD	37 ± 13	2 ± 0.4	40 ± 17
0.3 OD	347 ± 158	67 ± 12	139 ± 76
0.5 OD	1265 ± 248	NA[a]	386 ± 156
0.5 +Pyruvate	911 ± 254	434 ± 236	570 ± 188

*nmol benzyl viologen reduced min^{-1} mg^{-1}
[a]NA, not applicable

Table 4: Succinate dehydrogenase activity* of *C. jejuni* cell-free extracts

Absorbance (600 nm)	Strains		
	Wild type	*frdA*::Cm	*sdhA*::Cm
0.1 OD	11 ± 4	2 ± 1	18 ± 2
0.2 OD	14 ± 4	2 ± 1	24 ± 5
0.3 OD	56 ± 17	2 ± 1	59 ± 18
0.5 OD	87 ± 34	NA[a]	55 ± 21
0.5 +Pyruvate	56 ± 20	4 ± 4	69 ± 19

*μmol DCPIP reduced min^{-1} mg^{-1}
[a]NA, not applicable

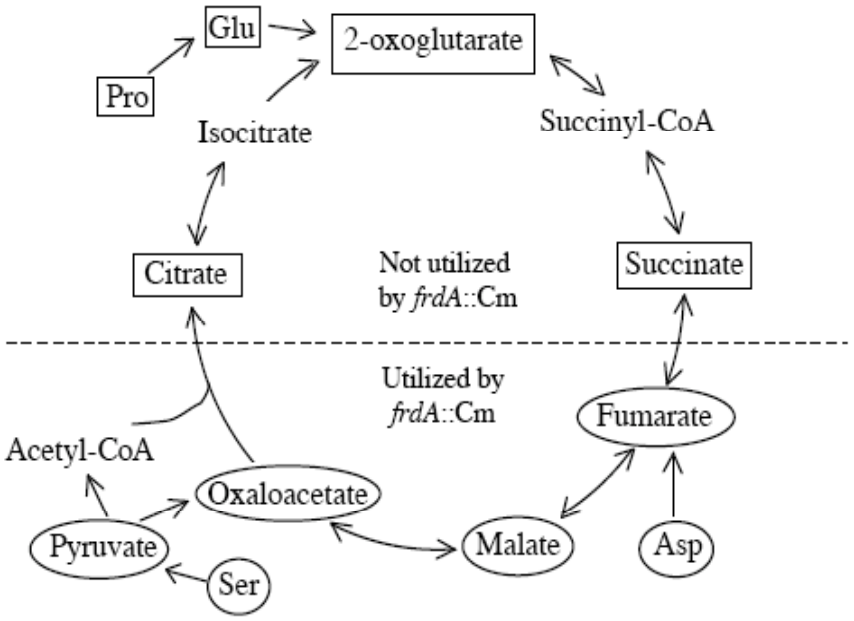

Figure 1: TCA cycle of *C. jejuni*. Boxed substrates represents intermediates that did not have an effect on *frdA*::Cm growth. Circled substrates represent intermediates that raised the terminal optical density of *frdA*::Cm.

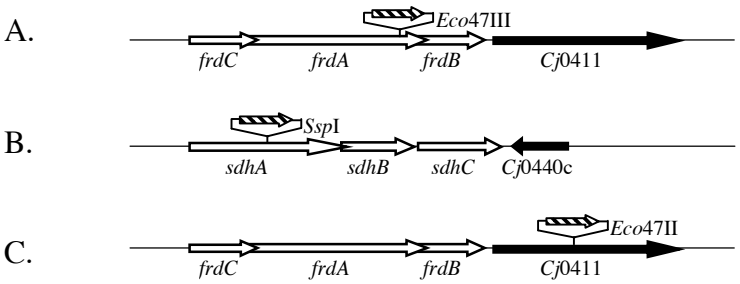

Figure 2: A. Gene organization of the fumarate reductase operon *frdCAB* of *C. jejuni*. B. Gene organization of the succinate dehydrogenase operon *sdhABC* of *C. jejuni*. C. Directly downstream of the *frdCAB* operons is *Cj*0411, which encodes a putative GTPase. The insertion sites of the chloramphenicol cassette (dashed arrow) used for mutagenesis are also shown. The direction of the arrow indicates the transcriptional orientation of the genes. Unrelated genes directly downstream of the genes of interest are indicated with black arrows.

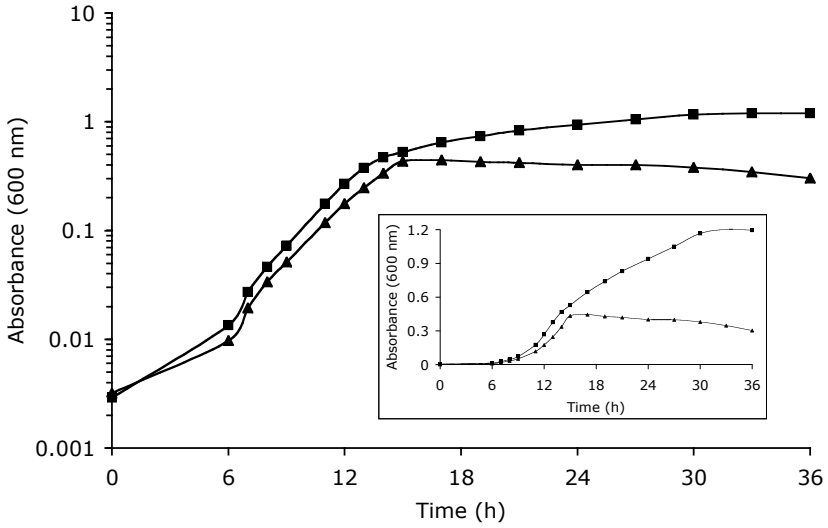

Figure 3: Microaerobic growth of *C. jejuni* NCTC 11168 (square), *frdA*::Cm (triangle). Cultures grown in MHB were incubated at 37°C. Inset shows growth curve in linear scale to better visualize marked difference in growth characteristics. This growth curve shown is an example of five independent growth curves.

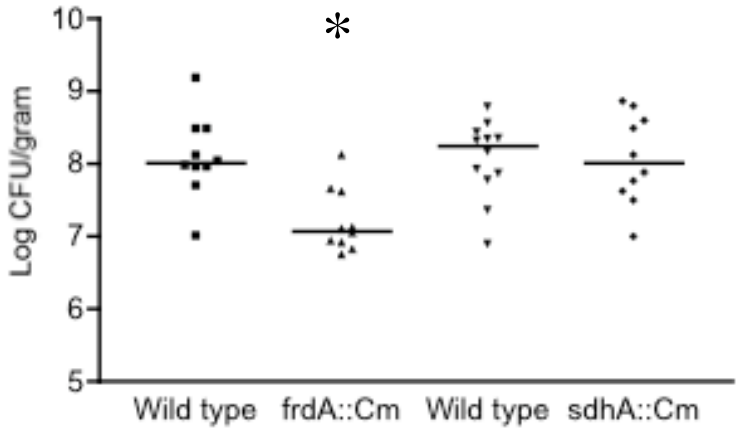

Figure 4: Chicken colonization ability of *C. jejuni* strains. CFU/gram cecal contents of *C. jejuni* wild type (square), *frdA*::Cm (triangle) from one colonization trial. CFU/gram cecal content of *C. jejuni* wild type (inverted triangle), and *sdhA*::Cm (diamond) of second independent colonization trial. Horizontal bars represent median value for each group. *p = 0.001 when compared to wild type.

CONCLUSIONS

Campylobacter jejuni relies on respiration to meet all of its energy demands. Dogma in the field has always stated that *C. jejuni* is a microaerophile meaning that although it cannot survive full aerobic conditions, it requires oxygen for growth. Several laboratories have tried and failed to grow *C. jejuni* anaerobically *in vitro* (4, 6). However, the natural habitat of *C. jejuni* is the ceca of birds, an organ dominated by obligate anaerobes (1). In 2000, the genome sequence revealed that in addition to expressing two terminal oxidases, *C. jejuni* also has the ability to express alternative respiratory acceptors enzymes allowing for the potential for *C. jejuni* to grow anaerobically (3). My project was to characterize the terminal acceptors of *C. jejuni* and identify their role in poultry colonization.

The two oxidases as well as four alternative electron acceptor enzymes were targeted for mutagenesis. *C. jejuni* encodes for two enzymes in the denitrification pathway. Nitrate reductase reduces nitrate to nitrite, and the catalytic subunit of the six gene operon encoding this enzyme was disrupted by the insertion of an antibiotic cassette marker. Nitrite reductase reduces nitrite to ammonia, and the catalytic subunit of the two gene operon was also disrupted. *C. jejuni* encodes for a putative SN oxide reductase, which has the capability to reduce S-oxides and N-oxides, and a mutation was made in this enzyme. Fumarate can also be used as an alternative respiratory acceptor in other *Campylobacter* species, and *C. jejuni* encodes a putative fumarate reductase (2, 3). The flavoprotein subunit was disrupted of the three gene operon encoding a fumarate reductase. The two oxidases targeted for mutation included the catalytic subunits of both a cbb_3-type terminal oxidase as well as a *bd*-type

quinol oxidase were disrupted.

C. jejuni is able to grow anaerobically *in vitro*. The addition of nitrate, TMAO, or DMSO mediated anaerobic growth. The addition of nitrite, even in low concentrations, proved toxic to *C. jejuni*. Nitrite was confirmed to be a viable anaerobic acceptor by measuring its consumption by wild type cultures grown with nitrate. While nitrite initially accumulates in wild type cultures, it is eventually fully consumed. The nitrite reductase mutant was unable to utilize the nitrite, and also did not reach the same optical density (600 nm) as the wild type cultures, proving that nitrite is an alternative respiratory acceptor for *C. jejuni.*

The disruption of both the nitrate and nitrite reductase reduced the ability of *C. jejuni* to colonize the ceca of poultry, however, the SN oxide reductase did not have a role in colonization for *C. jejuni* under our colonization procedures. The denitrification pathway has been implicated colonization for cultured cecal isolates are able to reduce nitrate (5). Anaerobic respiration does seem to play a limited role in *C. jejuni* colonization of the ceca.

Despite the ability of *C. jejuni* to grow anaerobically *in vitro* and its natural habitat being dominated by obligate anaerobes, oxygen is the most important acceptor both *in vitro* and *in vivo*. The cbb_3-type oxidase was absolutely required for colonization of the ceca. While the ceca is an anaerobic environment, *C. jejuni* is found near the epithelial cells where oxygen is present, but it does not physically attach. The ceca is constantly contracting resulting in the cecal contents being in constant motion, and therefore, *C. jejuni* is likely to encounter true

anaerobiosis periodically, which could explain why the nitrate and nitrite reductase mutants have a colonization defect.

Lastly, fumarate is not an alternative acceptor for *C. jejuni*. The addition of fumarate to media did not stimulate anaerobic growth. *C. jejuni* has a characteristic biphasic growth pattern under microaerobic conditions, the fumarate reductase (Frd) mutant only has a single growth phase. The disruption of Frd abolished succinate dehydrogenase (Sdh) activity both *in vivo* and *in vitro*, and the disruption of Sdh had no effect on Sdh activity. The protein annotated as an Frd is the sole Sdh despite another protein being annotated as an Sdh. It was determined that *C. jejuni* preferentially utilizes aspartate and serine in its first growth phase, and glutamate and proline in the second growth phase. The Frd mutant is unable to utilize glutamate and proline as the compounds would require an active succinate dehydrogenase to be incorporated into biomass.

C. jejuni has adapted well to compete in its favored ecological niche: the ceca of birds. *C. jejuni* has the capability for transportation and catabolism of the four dominant amino acids in the ceca. Its highly branched electron transport chain allows it to thrive in an environment with ever changing oxygen availability.

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