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The Dual-Functioning Fumarate Reductase Is the Sole Succinate:Quinone Reductase in *Campylobacter jejuni* and Is Required for Full Host Colonization[▽]

Rebecca A. Weingarten,† Michael E. Taveirne, and Jonathan W. Olson*

Department of Microbiology, North Carolina State University, Raleigh, North Carolina

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Campylobacter jejuni **encodes all the enzymes necessary for a complete oxidative tricarboxylic acid (TCA) cycle. Because of its inability to utilize glucose, *C. jejuni* relies exclusively on amino acids as the source of reduced carbon, and they are incorporated into central carbon metabolism. The oxidation of succinate to fumarate is a key step in the oxidative TCA cycle. *C. jejuni* encodes enzymes annotated as a fumarate reductase (Cj0408 to Cj0410) and a succinate dehydrogenase (Cj0437 to Cj0439). Null alleles in the genes encoding each enzyme were constructed. Both enzymes contributed to the total fumarate reductase activity in vitro. The *frdA*::*cat*$^+$ strain was completely deficient in succinate dehydrogenase activity in vitro and was unable to perform whole-cell succinate-dependent respiration. The *sdhA*::*cat*$^+$ strain exhibited wild-type levels of succinate dehydrogenase activity both in vivo and in vitro. These data indicate that Frd is the only succinate dehydrogenase in *C. jejuni* and that the protein annotated as a succinate dehydrogenase has been misannotated. The *frdA*::*cat*$^+$ strain was also unable to grow with the characteristic wild-type biphasic growth pattern and exhibited only the first growth phase, which is 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 not catabolized by the the *frdA*::*cat*$^+$ strain, indicating that the oxidation of succinate is a crucial step in metabolism of these substrates. Chicken colonization trials confirmed the in vivo importance of succinate oxidation, as the *frdA*::*cat*$^+$ strain colonized chickens at significantly lower levels than the wild type, while the *sdhA*::*cat*$^+$ strain colonized chickens at wild-type levels.**

Campylobacter jejuni causes approximately two million cases of bacterial gastroenteritis in the United States annually (34). Humans are most often infected due to cross-contamination resulting from improper handling of poultry (27), which is the natural habitat of *C. jejuni* (28). 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 catabolism of small organic acids and amino acids as a carbon and energy source, and the products of this catabolism are used for glycolysis and the tricarboxylic acid (TCA) cycle (15, 29). Fumarate and succinate are key intermediates in the TCA cycle, and the interconversion of these compounds is a vital process in organisms that use the TCA cycle for central carbon metabolism. *C. jejuni* encodes a complete oxidative TCA cycle, which converts TCA intermediates (carboxylic acids) to CO_2, ATP, and reducing equivalents. One of the conversion steps, oxidation of succinate to fumarate, forms a reducing equivalent and is required for a complete cycle. Reduction of fumarate to succinate also occurs as part of the reductive TCA cycle, and this carbon fixation pathway has been proposed to be utilized by ε-proteobacteria found in deep-sea hydrothermal vents (3). *C. jejuni* encodes 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*) (29); however, *C. jejuni* does not encode an ATP citrate lyase, which is required for full cyclic reductive carboxylation (3). The fumarate-succinate interconversion is also involved in respiration (11), and fumarate has specifically been implicated as an electron acceptor that is an alternative to oxygen in other ε-proteobacteria (5, 17).

C. jejuni encodes an enzyme which is annotated as a fumarate reductase (Cj0408 to Cj0410) and an enzyme which is annotated as a succinate dehydrogenase (Cj0437 to Cj0439) (29). Both of these enzymes are part of a large family of proteins called the succinate:quinone oxidoreductases (SQRs). These compounds are membrane-bound enzymes that either catalyze the two-electron oxidation of succinate to the two-electron reduction of quinone/quinol or, in the reverse direction, couple the oxidation of quinol/quinone to the reduction of fumarate to succinate. The amino acid sequence, however, does not dictate the in vivo function (18), and in characterized organisms like *Escherichia coli* both enzymes are able to reduce fumarate and oxidize succinate, albeit with a preference for one substrate (6, 21).

The SQRs can be divided into three distinct classes based on function, all of which have similar subunit compositions and primary amino acid sequences. Class 1 SQRs couple the oxidation of succinate to the reduction of a high-redox-potential quinone like ubiquinone in vivo. Class 2 SQRs are the quinol:fumarate reductases, which couple the oxidation of menaquinol to the reduction of fumarate. And class 3 SQRs couple the oxidation of succinate to the reduction of a low-potential qui-

* Corresponding author. Mailing address: Department of Microbiology, North Carolina State University, Campus Box 7615, Raleigh, NC 27695. Phone: (919) 515-7860. Fax: (919) 515-7867. E-mail: jwolson@ncsu.edu.

† Present address: Department of Microbiology and Immunology, The Uniformed Services University of the Health Sciences, Bethesda, MD.

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none, such as menaquinone, in vivo (11). Although each class has shared motifs, the in vivo function of an SQR enzyme cannot be resolved based on the primary sequence and must be determined experimentally. Fumarate reductase (Frd) activity has been reported to occur in the particulate fraction of *C. jejuni* cell lysates, and addition of formate to whole cells increased Frd activity (38), which implies that there is an active electron transport pathway. However, *C. jejuni* is unable to utilize fumarate as an alternative electron acceptor under anaerobic conditions (37, 41). *C. jejuni* can also use succinate as an electron donor to a respiratory quinone (12), which has been 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 ($E_m = -80$ mV). Although succinate oxidation coupled to menaquinone reduction would be an "uphill" reaction, class 3 SQRs can catalyze this reaction. Studies of gram-positive bacteria belonging to the genus *Bacillus*, as well as studies of sulfate-reducing bacteria, have shown that oxidation of succinate through menaquinone is driven by reverse transmembrane electron transport (18, 36, 45), and it is hypothesized that *C. jejuni* behaves similarly. The *C. jejuni* Frd enzyme contains three subunits, FrdC, FrdA, and FrdB, and the gene order in the operon is similar to that in *Wolinella succinogenes* (16, 19) and *Helicobacter pylori* (1, 9, 40). Based on Frd enzymes of other bacteria, FrdC (Cj0408) is the membrane anchor and diheme cytochrome *b*, FrdA (Cj0409) is a flavoprotein where the reduction of fumarate to succinate occurs, and FrdB (Cj0410) is an Fe-S protein (29). The succinate dehydrogenase of *C. jejuni* is also composed of three subunits, SdhABC encoded by Cj0437 to Cj0439 (29). 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, and FrdC and SdhC share 13% identity.

A better understanding of the *C. jejuni* 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 contribute to the total 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 colonization of chickens by *C. jejuni*. The *sdh* operon has been misannotated as the enzyme that it encodes exhibits no succinate dehydrogenase activity, as has recently been reported to be the case for the annotated succinate dehydrogenase of *W. succinogenes* (14).

MATERIALS AND METHODS

Bacterial strains and growth conditions. Table 1 lists the strains of *C. jejuni* and *E. coli* and all of the plasmids and primers utilized in this study. Tryptic soy agar (Difco, Sparks, MD) supplemented with 10% defibrinated sheep blood (Gibson Laboratories, Inc., Lexington, KY) (TSA-B) was used for growth of *C. jejuni*. *C. jejuni* was routinely cultured at 37°C microaerobically in a tri-gas incubator (model 550D; Fisher Scientific) maintained with 5% CO_2, 12% O_2, and 83% N_2. Liquid cultures were grown in Mueller-Hinton broth (MHB) (Difco, Sparks, MD) or modified Eagle's medium (MEM) lacking glucose, glutamine, phenol red, and sodium pyruvate (Sigma, St. Louis, MO). Liquid cultures were incubated at 37°C with shaking under a microaerobic atmosphere. Chloramphenicol (25 μg/ml) was added as indicated below. Genetic manipulations were performed with *E. coli* strain DH5α (laboratory stock). Luria-Bertani broth and agar were supplemented with ampicillin (100 μg/ml) or chloramphenicol (25 μg/ml) as noted below.

Succinate dehydrogenase assay. Succinate dehydrogenase activity was measured by measuring 2,6-dichlorophenol-indophenol (DCPIP)-dependent reduction by succinate, as described by Schirawski and Unden (35). 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 extract was made anoxic by sparging it with N_2 gas. The reaction was started by addition of 20 mM sodium succinate (pH 7.4), and the DCPIP-dependent reduction kinetics were recorded with a spectrophotometer at 600 nm. The rate of reduction was expressed in μmol DCPIP reduced min^{-1} mg $protein^{-1}$ using a molar extinction coefficient for DCPIP of 2.1×10^4 cm^{-1}.

Fumarate reductase assay. Benzyl viologen-linked reductase assays were carried out with sonicated cell extracts using a 1-ml assay mixture as described previously (37). Reagents were added with a syringe through the stopper, while N_2 gas was flushed through the cuvette. The reaction mixture in 1-ml quartz cuvettes with stoppers (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 to 5 μg of cell extract. Freshly made 20 mM sodium dithionite was then injected into each cuvette until the absorbance at 585 nm reached 0.8 to 0.9, which represented half-reduced benzyl viologen. An anaerobic solution of sodium fumarate (final concentration, 5 mM) was added, and the benzyl viologen (extinction coefficient, 8.65 cm^{-1} mM^{-1}) oxidation kinetics were recorded with a spectrophotometer at 585 nm. Fumarate reductase activity was expressed in nmol of benzyl viologen oxidized min^{-1} mg^{-1}.

O_2 uptake assay. O_2 uptake experiments were performed using a Clarke-type electrode and a YSI model 5300 oxygen monitor (YSI, Yellow Springs, OH) as described previously (44). Briefly, washed whole cells were resuspended in phosphate-buffered saline (PBS), 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, 5 mM) was added to the chamber through a capillary tube via a Hamilton syringe, a chart recorder documented the rate of oxygen consumption, and the slope of the line showed the rate of oxygen uptake. The rate was expressed in nmol of O_2 consumed min^{-1} mg^{-1}.

Cloning and construction of *C. jejuni* mutants. Oligonucleotide primers for cloning genes of interest were designed using sequenced strain NCTC 11168 (29) 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 the template. The PCR products (1,154 bp of *frdA*, 904 bp of *sdhA*, and 2,327 bp of Cj0411) were cloned into pCR2.1-TOPO vectors (Invitrogen, Carlsbad, CA) and confirmed by restriction analysis. The coding region of a gene of interest was disrupted by insertion of a chloramphenicol resistance gene (*cat*), which was originally isolated from *Campylobacter coli* (Fig. 1) (43). The restriction endonuclease recognition sequences utilized were Eco47III for *frdA*, SspI for *sdhA*, and Eco47III for Cj0411 (Table 1). Electrocompetent *C. jejuni* cells were transformed with each construct to obtain the corresponding *C. jejuni* mutant as described previously (44). Transformed cells were spotted onto cold nonselective TSA-B plates, and the plates were incubated microaerobically for 24 h, after which the cells were transferred to TSA-B plates containing chloramphenicol. Resistant colonies that appeared after 2 to 5 days were passed on selective TSA-B plates, and correct insertion of the cassette was confirmed by isolation of chromosomal DNA from the candidate strains and by PCR amplification of the gene. Agarose gel electrophoresis of the PCR product was used to monitor the increase in the size of the gene of interest with the antibiotic cassette insertion (data not shown).

Quantitative RT-PCR. PCR primers used in this study are listed in Table 1 and were designed to amplify gene fragments that were the following sizes: 115 nucleotides for *gyrA*, and 136 nucleotides for Cj0411. Total RNA was isolated from the *C. jejuni* parent strain and the *frdA*::*cat*$^+$ strain using a MasterPure Complete RNA purification kit (Epicentre Biotechnologies, Madison, WI). Quantitative reverse transcriptase PCR (RT-PCR) was performed by using a Quantitect SYBR green RT-PCR kit (Qiagen, Valencia, CA). The PCR mixture (20 μl) contained 40 ng RNA, 10 μl 2× QuantiTect SYBR green RT-PCR master mixture, and 0.2 μl QuantiTect RT mixture. The RT cycle step consisted of 50°C for 30 min, and this was followed by an initial PCR activation step of 95°C for 15 min. The mixtures were then amplified using 30 cycles of 94°C for 15 s, 60°C for 30 s, and 72°C for 30 s in an automated thermal cycler (iCycler; Bio-Rad, Hercules, CA). The iCycler software was used to determine the thresh-

TABLE 1. Strains, plasmids, and primers

Strain, plasmid, or primer	Description or sequence	Source[a]
C. jejuni strains		
NCTC 11168	Parent of all *C. jejuni* strains	NCTC
frdA::*cat*$^+$	*cat* insertion in *frdA*	This study
sdhA::*cat*$^+$	*cat* insertion in *sdhA*	This study
Cj0411::*cat*$^+$	*cat* insertion in Cj0411	This study
E. coli strains		
DH5α	*fhuA2* Δ*(argF-lacZ)U169 phoA glnV44* ϕ80Δ*lacZ*M15 *gyrA96 recA1 relA1 endA1 thi-1 hsdR17*	Lab stock
One Shot Top10	F$^-$ *mcrA* (*mrr-hsdRMS-mcrBC*) ϕ80Δ*lacZ*M15 *lacX74 recA1 ara139* (*ara-leu*)*7697 galU galK rpsL* (Str^r) *endA1 nupG*; chemically competent cells	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 into Eco47III site of pFrdA:TOPO	This study
pSdhA:TOPO	*sdhA* cloned from NCTC 11168 into pCRTOPO2.1	This study
pSdhA:cm	*cat* inserted into SspI site of pSdhA:TOPO	This study
pCj0411:TOPO	Cj0411 cloned from NCTC 11168 into pCRTOPO2.1	This study
pCj0411:cm	*cat* inserted into Eco47III site of p*Cj*0411: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
RTFrdAF	5′-GCAGCCATCGCTCTTGAAACAG-3′	IDT
RTFrdAR	5′-CGACAGCCCTCAGTAAGCAAAATAC-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.

old cycle at which each transcript could be detected. Threshold cycles were then compared to a standard curve, which was generated independently for each gene, to determine the number of RNA molecules at the start. The total RNA in each sample was normalized using the internal control gene *gyrA* (Cj1027c).

Sonication. Cells in PBS (resuspended to an optical density of 5.0) were disrupted by sonication. Sonication was performed on ice with a W-370 horn cup sonicator (Heat Systems-Ultrasonics, Inc., Farmingdale, NY) using four 45-s pulses at 60% power and an output control setting of 7.0. Cell debris was removed by centrifugation at 1,000 × *g* for 10 min at 4°C, and the supernatant was removed. The resulting preparation is referred to below as the cell extract.

Protein assay. Protein concentrations were determined using a bicinchoninic acid protein assay kit (Pierce, Rockford, IL) with bovine serum albumin as a standard.

Chicken colonization. *Campylobacter*-free 1-day-old broiler chicks were supplied by the Lake Wheeler Poultry Facility operated by the North Carolina State University Poultry Department. Birds were housed in isolation rooms at the Dearstyne Avian Health Center (Department of Poultry Science, North Carolina State University) in isolation brooder batteries (10 chicks per battery). The chicks were fed Purina Mills Start & Grow SunFresh Recipe (Purina Mills LLC, St. Louis, MO) and given water ad libidum. One-week-old chicks were inoculated by oral gavage with 0.1 ml of *C. jejuni* in PBS harvested from TSA-B plates which had been incubated at 37°C microaerobically. Control chicks were inoculated with 0.1 ml sterile PBS (pH 7.4). Two weeks postinoculation the chickens were humanely sacrificed by CO_2 asphyxiation, and approximately 1 g of cecal contents was collected, serially diluted (in PBS), and plated on selective TSA-B containing 40 μg/ml cefoperazone, 40 μg/ml vancomycin, 10 μg/ml trimethoprim, and 100 μg/ml cycloheximide. All samples were incubated microaerobically at 37°C. After 2 days of incubation, colonies were counted, and the number of CFU/g of cecal contents was calculated. Data were analyzed by using a one-tailed Mann-Whitney test and a 95% confidence interval.

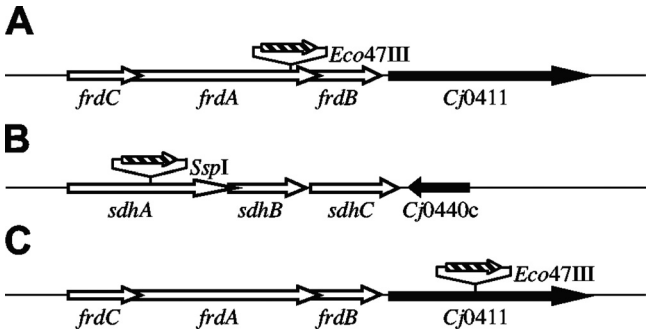

FIG. 1. (A) Gene organization in the fumarate reductase operon (*frdCAB*) of *C. jejuni*. (B) Gene organization in the succinate dehydrogenase operon (*sdhABC*) of *C. jejuni*. (C) Directly downstream of the *frdCAB* operons is Cj0411, which encodes a putative GTPase. The insertion sites of the chloramphenicol cassette (striped arrows) used for mutagenesis are indicated. The directions of the arrows indicate the transcriptional orientations of the genes. Unrelated genes directly downstream of the genes of interest are indicated by black arrows.

RESULTS

Fumarate reductase mutants do not enter the second *C. jejuni* growth phase. Typical growth curves for the wild type and the *frdA*::*cat*$^+$ strain are shown in Fig. 2. The wild type exhibits a characteristic *C. jejuni* biphasic growth pattern when it is grown in MHB under microaerobic conditions. The *frdA*::*cat*$^+$ strain behaves like the wild type during the first

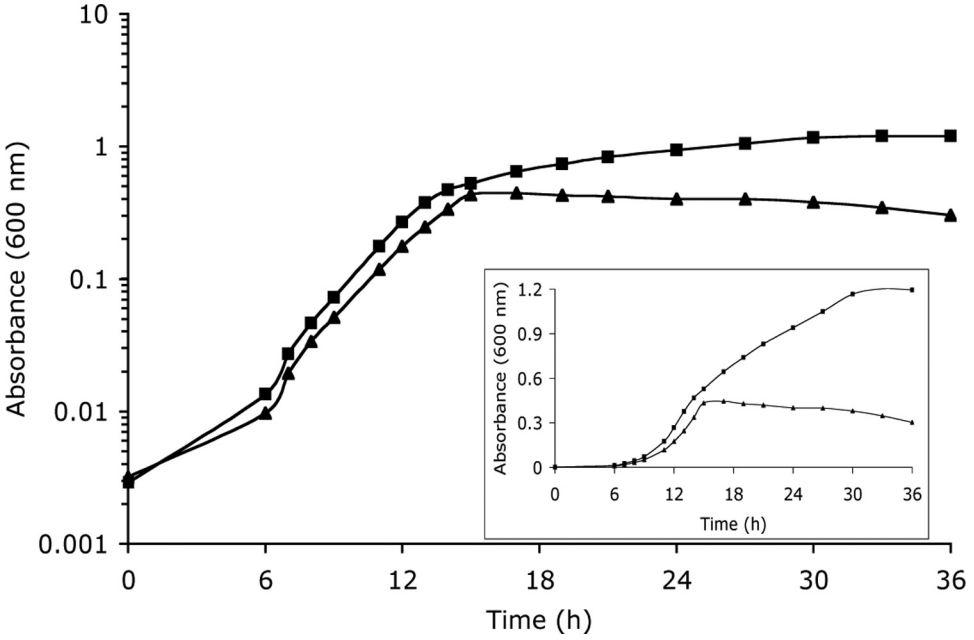

FIG. 2. Microaerobic growth of *C. jejuni* NCTC 11168 (■) and the *frdA*::*cat*$^+$ strain (▲). Cultures grown in MHB were incubated at 37°C. (Inset) Growth curves with a linear scale to better visualize the marked difference in growth characteristics. The growth curves shown are examples of five independent growth curves.

growth phase, and the generation time of this strain is 2.2 ± 0.7 h, compared to a growth rate of 2.0 ± 0.4 h for the wild type. When wild-type *C. jejuni* reaches the mid-log phase (absorbance at 600 nm of approximately 0.3 in MHB), there is a shift to a second growth phase and a longer generation time, 10.3 ± 1.2 h. The *frdA*::*cat*$^+$ strain never enters the second growth phase and stops growing after the first growth phase. The inset in Fig. 2 shows the growth curve with a linear scale to better visualize the marked difference in growth characteristics between the wild type and the *frdA*::*cat*$^+$ strain. The *sdhA*::*cat*$^+$ strain grows like the wild type, with two distinct growth phases with generation times of 2.2 ± 0.05 h and 12 ± 1 h.

The *frdA*::*cat*$^+$ strain does not utilize substrates that precede succinate oxidation in the TCA cycle. Although fumarate reductase can be used as an alternative electron acceptor in place of oxygen by other *Campylobacter* species and the ε-proteobacterium *W. succinogenes* (5, 17), *C. jejuni* is unable to grow anaerobically with fumarate (37, 38). Fumarate reductase is also an essential enzyme in the reverse (reductive) TCA cycle. TCA cycle intermediates were added to MHB to see if the wild-type growth phenotype could be restored to the *frdA*::*cat*$^+$ strain. Addition of several metabolic intermediates increased the length in the first growth phase (and thus the terminal optical density) of the *frdA*::*cat*$^+$ strain; however, no substrate produced the second growth phase typically seen with wild-type *C. jejuni* (data not shown). Table 2 shows the terminal optical density at 600 nm (OD_{600}) of the *frdA*::*cat*$^+$ strain after 26 h of microaerobic growth after addition of TCA substrates. The TCA intermediates fumarate, malate, oxaloacetate, and pyruvate (which can be converted to oxaloacetate via oxaloacetate decarboxylase) did extend the first growth phase and thus increased the terminal optical density of the *frdA*::*cat*$^+$ strain. However, the terminal optical density of the *frdA*::*cat*$^+$ strain was not affected by addition of acetate, citrate, 2-oxoglutarate, and succinate, which fall in the first half of the TCA cycle (Fig. 3). When grown in media containing amino acids, *C. jejuni* utilizes aspartate, glutamate, proline, and serine until they are depleted (10, 20). Both aspartate (a precursor of fumarate via AspA) and serine (via serine dehydratase, which deaminates serine to pyruvate) increased the growth of the *frdA*::*cat*$^+$ strain (Table 2). Glutamate (a precursor of 2-oxoglutarate) and proline (proline dehydrogenase oxidizes proline to glutamate) had no effect on the *frdA*::*cat*$^+$ strain. The substrates were also tested using the defined medium MEM, which has been shown to support growth when it is supplemented with amino acids (23). The results were es-

TABLE 2. Final absorbance at 600 nm of *frdA*::*cat*$^+$ strain cultures grown in MHB or MEM (with or without substrate) for 26 h

Substrate (28 mM)	Absorbance at 600 nm in MHB	Absorbance at 600 nm in MEM	Growth stimulus[a]
None	0.24 ± 0.01	0.01 ± 0.00	
Citrate	0.30 ± 0.02	0.01 ± 0.00	N
α-Ketoglutarate	0.28 ± 0.01	0.01 ± 0.00	N
Succinate	0.29 ± 0.01	0.01 ± 0.00	N
Glutamate	0.24 ± 0.01	0.01 ± 0.00	N
Proline	0.24 ± 0.01	0.01 ± 0.00	N
Acetate	0.27 ± 0.01	0.01 ± 0.00	N
Pyruvate	0.56 ± 0.02	0.26 ± 0.02	Y
Oxaloacetate	0.52 ± 0.01	0.31 ± 0.01	Y
Malate	0.69 ± 0.04	0.49 ± 0.02	Y
Fumarate	0.67 ± 0.03	0.57 ± 0.02	Y
Aspartate	0.92 ± 0.05	0.41 ± 0.01	Y
Serine	0.51 ± 0.02	0.01 ± 0.00	Y or N

[a] Y, yes; N, no.

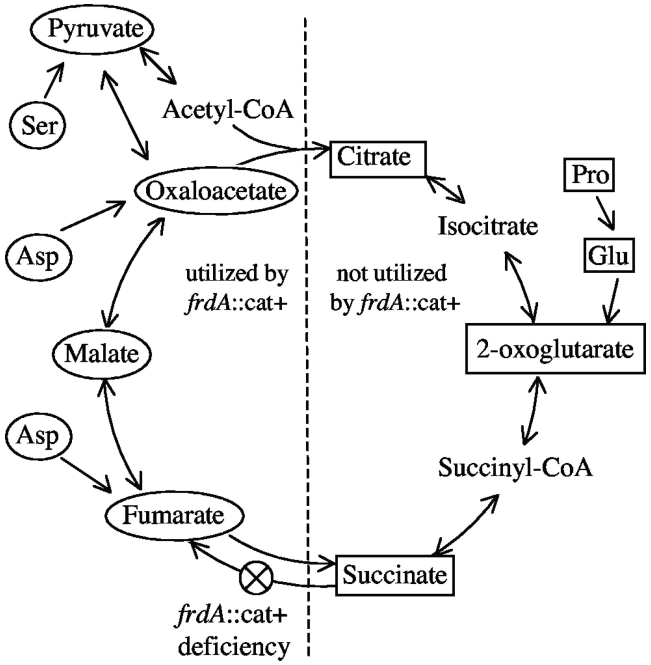

FIG. 3. TCA cycle of *C. jejuni*. Substrates in rectangles are intermediates that did not have an effect on the growth of the *frdA*::*cat*$^+$ strain. Substrates in ellipses are intermediates that increased the terminal optical density of the *frdA*::*cat*$^+$ strain. CoA, coenzyme A.

sentially the same; none of the non-growth-promoting substrates were utilized by the *frdA*::*cat*$^+$ strain, while all of the substrates (except serine) that enhanced growth in MHB supported growth (Table 2). These results are summarized in Fig. 3. TCA intermediates that are utilized by the *frdA*::*cat*$^+$ strain group together immediately following the fumarate-succinate interconversion. The intermediates that had no effect on the *frdA*::*cat*$^+$ strain group together immediately prior to the fumarate and succinate enzymatic reactions (Fig. 3).

Investigation of possible polar effects. The insertion of an antibiotic cassette into the chromosome can affect the transcription of downstream genes (a "polar effect") (Fig. 1). Immediately 3′ of the *sdh* operon is Cj0440c, which encodes a TenA/Thi-4 family protein; however, this gene is on the opposite DNA coding strand, and transcription should not be affected (29). 3′ of the *frd* operon is Cj0411, which encodes a putative GTP-binding protein. Quantitative RT-PCR was employed to measure the transcription of Cj0411 in both the wild type and the *frdA*::*cat*$^+$ strain. Polar effects were seen as the relative levels of transcription of Cj0411 in the *frdA*::*cat*$^+$ strain were 0.15 ± 0.08 times those in the wild-type strain. To confirm that the phenotype of the *frdA*::*cat*$^+$ strain was a result of fumarate reductase disruption and not due to reduced transcription of the GTP-binding protein, a strain with a mutation in this gene was constructed. The Cj0411::*cat*$^+$ strain behaved like the wild type, with two distinct growth phases and with growth rates in both phases similar to those of the wild type (data not shown), and it exhibited 94% of the succinate dehydrogenase activity (DCPIP reduction) of the wild-type strain. The growth phenotype associated with the *frdA*::*cat*$^+$ strain can be attributed solely to the disruption of the *frdA* gene.

TABLE 3. Fumarate reductase activity of *C. jejuni* cell extracts

OD_{600}	Fumarate reductase activity (nmol benzyl viologen reduced min^{-1} mg^{-1})		
	Wild type	*frdA*::*cat*$^+$ strain	*sdhA*::*cat*$^+$ strain
0.1	61 ± 21	4 ± 3	63 ± 24
0.2	37 ± 13	2 ± 0.4	40 ± 17
0.3	347 ± 158	67 ± 12	139 ± 76
0.5	1265 ± 248	ND[a]	386 ± 156
0.5 (with pyruvate)	911 ± 254	434 ± 236	570 ± 188

[a] ND, not done.

Fumarate reductase and succinate dehydrogenase activities. Fumarate reductase activity was measured in wild-type, *frdA*::*cat*$^+$, and *sdhA*::*cat*$^+$ strain cell extracts. In all three extracts reduced benzyl viologen was used to reduce fumarate (Table 3). The activity in cultures was measured at different growth phases (Table 3). The *frdA*::*cat*$^+$ strain required addition of pyruvate to the medium for the terminal optical density of the culture to reach an OD_{600} of 0.5; the greatest OD_{600} of the unsupplemented cultures was 0.3 (Table 2). The wild type and the *sdhA*::*cat*$^+$ strain were grown with pyruvate as controls. Under all of the conditions tested, the *frdA*::*cat*$^+$ strain and the *sdhA*::*cat*$^+$ strain exhibited fumarate reductase activity, and the sum of these activities equaled the fumarate reductase activity of wild-type *C. jejuni* (Table 3).

Succinate dehydrogenase activity was measured for the wild type and the two mutants at different growth phases. Cell extracts were utilized to measure the reduction of the artificial acceptor DCPIP coupled to succinate oxidation. The wild type and the *sdhA*::*cat*$^+$ strain had similar succinate dehydrogenase activities under all of the conditions tested (Table 4). The *frdA*::*cat*$^+$ strain, however, was unable to oxidize succinate using DCPIP as an artificial electron acceptor under all of the conditions tested (Table 4). Pyruvate was added to the medium so the OD_{600} of the *frdA*::*cat*$^+$ strain cultures could reach 0.5.

Succinate-dependent oxygen uptake is not affected in the *sdhA*::*cat*$^+$ strain. Whole cells of the wild type, the *frdA*::*cat*$^+$ strain, and the *sdhA*::*cat*$^+$ strain were assayed to determine succinate oxidation as measured by oxygen uptake. Cultures were grown to an OD_{600} of approximately 0.5. Pyruvate was added to the medium for the *frdA*::*cat*$^+$ strain. Addition of pyruvate to the medium had no effect on the wild-type respiration rate (data not shown). The wild type and the *sdhA*::*cat*$^+$ strain consumed oxygen at similar rates. The succinate-dependent respiration rate of the wild type was 28 ± 7 nmol O_2 consumed/min/10^8 cells, and the *sdhA*::*cat*$^+$ strain respired at

TABLE 4. Succinate dehydrogenase activity of *C. jejuni* cell extracts

OD_{600}	Succinate dehydrogenase activity (μmol DCPIP reduced min^{-1} mg^{-1})		
	Wild type	*frdA*::*cat*$^+$ strain	*sdhA*::*cat*$^+$ strain
0.1	11 ± 4	2 ± 1	18 ± 2
0.2	14 ± 4	2 ± 1	24 ± 5
0.3	56 ± 17	2 ± 1	59 ± 18
0.5	87 ± 34	ND[a]	55 ± 21
0.5 (with pyruvate)	56 ± 20	4 ± 4	69 ± 19

[a] ND, not done.

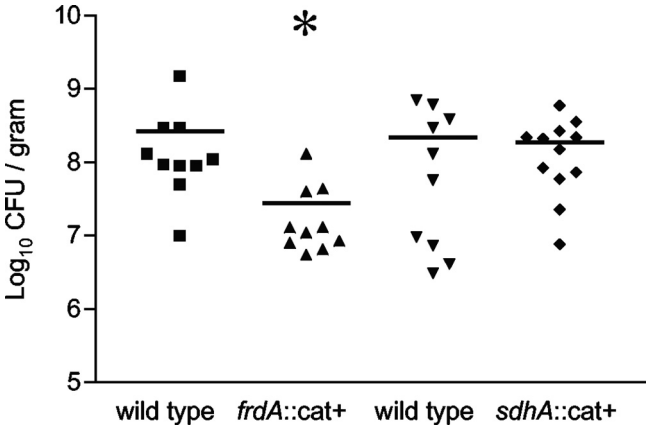

FIG. 4. Abilities of *C. jejuni* strains to colonize chickens. ■ and ▼, CFU of wild-type *C. jejuni*/g cecal contents; ▲, CFU of *frdA*::*cat*$^+$ strain/g cecal contents for one colonization trial; ◆, CFU of *sdhA*::*cat*$^+$ strain/g cecal contents for second independent colonization trial. The horizontal bars indicate the median values. *, $P = 0.001$ compared to the wild type.

a rate of 37 ± 15 nmol O_2 consumed/min/10^8 cells. The *frdA*::*cat*$^+$ strain was unable to respire with succinate as the sole electron donor (0.6 ± 1 nmol O_2 consumed/min/10^8 cells). The three strains exhibited similar rates of lactate-dependent respiration, which was used as a positive control.

The *frdA*::*cat*$^+$ strain is deficient in host colonization. At 1 week of age, groups of 10 birds were inoculated with 6.1×10^6 CFU of the wild type or 1.1×10^7 CFU of the *frdA*::*cat*$^+$ strain by oral gavage. Two weeks postinoculation, the birds were sacrificed by CO_2 asphyxiation, and the cecal contents were collected for enumeration of viable *C. jejuni* cells. All birds were colonized with *C. jejuni* at the conclusion of the experiment. The *frdA*::*cat*$^+$ strain colonized chickens at significantly lower levels than the wild type ($P = 0.001$) (Fig. 4). A second host colonization trial was performed with groups of 1-week-old birds inoculated with 2.7×10^6 CFU of the wild type or 1.8×10^6 CFU of the *sdhA*::*cat*$^+$ strain by oral gavage. Two weeks postinoculation the cecal contents were examined to enumerate the viable *C. jejuni* cells. The *sdhA*::*cat*$^+$ strain and the wild-type strain colonized at similar levels (Fig. 4). No *C. jejuni* was recovered from the negative control birds inoculated with PBS in both colonization trials (data not shown).

DISCUSSION

The enzyme annotated as a fumarate reductase is the sole succinate dehydrogenase of *C. jejuni*. Mutations in this enzyme have serious and previously unsuspected implications for the growth and metabolic flexibility of this important pathogen. Although fumarate reductase activity has been measured in *C. jejuni* (37, 38), this organism is unable to respire anaerobically using fumarate as a terminal electron acceptor (37, 41), leaving the physiological role of the fumarate reductase in doubt. We constructed mutants with mutations in both the fumarate reductase and succinate dehydrogenase in order determine the in vivo functions of these two enzymes.

Our first indication that the fumarate reductase has a central role in the microaerobic physiology of *C. jejuni* came from the unusual growth characteristics of the fumarate reductase mutant. The *frdA*::*cat*$^+$ strain grew like the wild type until mid-log phase (OD_{600}, approximately 0.3) and then stopped growing (Fig. 2). Wild-type cultures, on the other hand, continued to grow, but the growth rate was lower than the initial growth rate until the terminal optical density was approximately 1.0 (Fig. 2). The *sdhA*::*cat*$^+$ strain grew like the wild type, with two distinct growth phases (data not shown).

A possible explanation for the aborted growth of the *frdA*::*cat*$^+$ strain is that this mutant is unable to utilize a particular class of substrates for carbon and energy. *C. jejuni* does not encode a complete glycolytic pathway (29) and must rely on the catabolism of small organic and amino acids for its carbon requirement (10). These acids are incorporated into the TCA cycle through a variety of transport systems and enzymes (15). We added TCA cycle intermediates to test whether such additions could rescue growth. Although none of the added substrates were able to restore biphasic growth to the *frdA*::*cat*$^+$ strain (data not shown), addition of certain TCA cycle intermediates did extend the first growth phase and increase the terminal optical density compared to unsupplemented cultures (Table 2). The intermediates that did not extend the primary growth phase of the *frdA*::*cat*$^+$ strain include citrate 2-oxoglutarate and succinate, which group together in the TCA cycle immediately preceding (in the oxidative cycle) the succinate-fumarate interconversion. The substrates that were growth stimuli for the *frdA*::*cat*$^+$ strain included pyruvate, oxaloacetate, malate, and fumarate, which occur after the succinate-fumarate interconversion. Disruption of Frd results in an inability of *C. jejuni* to incorporate one-half of the TCA cycle intermediates into biomass, and these intermediates all occur before succinate oxidation in the oxidative TCA cycle (Fig. 3). This is especially detrimental to *C. jejuni*, which lacks sugar transporters and therefore relies on gluconeogenesis for its carbohydrate requirement (29). Neither oxaloacetate nor pyruvate (starting material for gluconeogenesis) could be produced with the block in the oxidative TCA cycle in the *frdA*::*cat*$^+$ strain (Fig. 3).

In addition to direct incorporation of TCA intermediates, *C. jejuni* can grow on amino acids and has a preference for utilization of specific amino acids (10, 20). Analysis of spent medium from strains grown in MHB showed that the original concentrations of aspartate, glutamate, serine, and proline were reduced by over 50% (10). Therefore, we tested whether these four key amino acids could extend the first growth phase of the *frdA*::*cat*$^+$ strain. Both aspartate and serine increased the terminal optical density of the *frdA*::*cat*$^+$ strain, while glutamate and proline had no effect (Table 2). Taken together, these data suggest that *C. jejuni* preferentially uses the amino acids and TCA cycle substrates immediately after the oxidation of succinate in the first growth phase and switches to a less preferred substrate and amino acid class, which includes glutamate and proline, after depletion of the preferred substrates.

In an effort to explain the altered substrate utilization pattern of the the *frdA*::*cat*$^+$ strain, we tried to determine the biochemical activities available to each strain. All strains exhibited fumarate reductase activity in all conditions tested. The fumarate reductase activities of the *frdA*::*cat*$^+$ strain and the *sdhA*::*cat*$^+$ strain were similar, and each of these activities contributed to the total fumarate reductase activity of the wild

type (Table 3). Surprisingly, the *sdhA*::*cat*$^+$ strain had succinate dehydrogenase activity similar to that of the wild type under all conditions tested, and the *frdA*::*cat*$^+$ strain exhibited no succinate dehydrogenase activity (Table 4). Physiologically, succinate oxidation can also be measured using the uptake of oxygen by whole cells. The succinate-dependent respiration rates of whole cells of the wild-type and *sdhA*::*cat*$^+$ strain cultures were similar, but the *frdA*::*cat*$^+$ strain was unable to respire with succinate. Lactate is an efficient respiratory donor for *C. jejuni* (12), and the three strains had similar lactate-dependent respiration rates (data not shown), indicating that the respiratory capacity of these strains was not impaired.

The use of succinate as a respiratory chain donor in *C. jejuni* was initially surprising due to the demonstrated lack of a quinone pool in this organism (4). Typically in facultative anaerobes, succinate oxidation is coupled to ubiquinone, and under anaerobic conditions fumarate oxidation is coupled to menaquinol (42). There is, however, a third functional class of succinate oxidoreductases, which catalyze the oxidation of succinate and the reduction of the low-redox-potential menaquinone (11). *C. jejuni* contains only menaquinone-6 and a methyl-substituted menaquinone (4). In this class of enzymes the endergonic reaction of succinate oxidation (E_m = 30 mV) coupled to menaquinone reduction (E_m = −80 mV) has been shown to require a proton gradient (36). *Desulfovibrio vulgaris*, *Geobacter sulfurreducens*, and *Bacillus subtilis* have all been reported to couple succinate oxidation to menaquinone reduction via this mechanism (2, 22, 45).

The amino terminus of FrdC of *C. jejuni* is predicted to contain the five transmembrane helices predicted by TMHMM (www.cbs.dtu.dk/services/TMHMM/), and these helices contain the four conserved His residues that ligate the two heme B molecules required for transmembrane electron transfer (36). These helices and residues are also present in *W. succinogenes* FrdC and *B. subtilis* SdhC (36). 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 activity (45). The fumarate reductase of *W. succinogenes* is capable of both specific enzymatic activity during succinate-dependent methylene blue reduction and benzyl viologen oxidation through fumarate reduction (26).

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* lacks these traits. Taken together, these data indicate that FrdCAB is the sole succinate dehydrogenase of *C. jejuni* and that SdhABC has been misannotated as it does not contribute to succinate dehydrogenase activity. Very recently, this has been shown to be the case in *W. succinogenes* (14). The *W. succinogenes* enzyme previously annotated as SdhABC (having 63%, 71%, and 69% homology to *C. jejuni* SdhABC) was determined to instead be a novel methylmenaquinol:fumarate reductase (MFR). Our data indicate the Sdh is probably an MFR in *C. jejuni* as well. The in vivo role of MFR in both these organisms has yet to be determined; however, our in vitro studies indicate that FrdCAB and SdhABC each contribute to fumarate reductase activity.

The *frdA*::*cat*$^+$ strain showed a significant decrease in the ability to colonize poultry compared to both the wild type and the *sdhA*::*cat*$^+$ strain (Fig. 4). The cecum of poultry contains fermentative by-products, including lactate, acetate, hydrogen, and formate (33). Amino acids are abundant in the cecum of poultry as a result of a high-cellulose diet and the biosynthesis of amino acids by microbes in the cecum (32). To study the in vivo availability of amino acids, one can compare the excreta from laying hens and cecectomized laying hens. Removal of the cecum significantly reduces the effect of microbes on digestion, and it is believed that microbial metabolism of amino acids in the cecum is largely responsible for the differences between intact and cecectomized birds (30). The total amino acid excretion was greater for cecectomized laying hens than for intact hens. The levels of proline, threonine, and isoleucine were increased significantly in the excreta of cecectomized laying hens, and it was inferred that the microbes in the cecum utilize these amino acids (31). Because the *frdA*::*cat*$^+$ strain is unable to metabolize proline in the TCA cycle, it may be at a disadvantage in the cecum of poultry, thus explaining its decreased ability to colonize.

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 colonization of mice (8) and has been studied as a potential drug target (7, 24). The ability of an *E. coli* fumarate reductase mutant to colonize mice also was significantly decreased (13). In *Salmonella enterica* serovar Typhimurium, a complete TCA cycle is necessary for full virulence in mice (39), and a fumarate reductase-succinate dehydrogenase double mutant is avirulent in BALB/c mice (25).

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