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Genetic Characterization of a Single Bifunctional Enzyme for Fumarate Reduction and Succinate Oxidation in *Geobacter sulfurreducens* and Engineering of Fumarate Reduction in *Geobacter metallireducens*

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The mechanism of fumarate reduction in *Geobacter sulfurreducens* was investigated. The genome contained genes encoding a heterotrimeric fumarate reductase, FrdCAB, with homology to the fumarate reductase of *Wolinella succinogenes* and the succinate dehydrogenase of *Bacillus subtilis*. Mutation of the putative catalytic subunit of the enzyme resulted in a strain that lacked fumarate reductase activity and was unable to grow with fumarate as the terminal electron acceptor. The mutant strain also lacked succinate dehydrogenase activity and did not grow with acetate as the electron donor and Fe(III) as the electron acceptor. The mutant strain could grow with acetate as the electron donor and Fe(III) as the electron acceptor if fumarate was provided to alleviate the need for succinate dehydrogenase activity in the tricarboxylic acid cycle. The growth rate of the mutant strain under these conditions was faster and the cell yields were higher than for wild type grown under conditions requiring succinate dehydrogenase activity, suggesting that the succinate dehydrogenase reaction consumes energy. An orthologous *frdCAB* operon was present in *Geobacter metallireducens*, which cannot grow with fumarate as the terminal electron acceptor. When a putative dicarboxylic acid transporter from *G. sulfurreducens* was expressed in *G. metallireducens*, growth with fumarate as the sole electron acceptor was possible. These results demonstrate that, unlike previously described organisms, *G. sulfurreducens* and possibly *G. metallireducens* use the same enzyme for both fumarate reduction and succinate oxidation in vivo.

Geobacter species are environmentally significant, in part because of their ability to anaerobically oxidize acetate to carbon dioxide with the reduction of extracellular electron acceptors such as Fe(III) and Mn(IV) oxides (27, 29), humic substances (24), U(VI) (28), and graphite electrodes (2). Some *Geobacter* species, including *Geobacter sulfurreducens*, are also able to use the tricarboxylic acid (TCA) cycle intermediate fumarate as an electron acceptor, catalyzing the two-electron reduction of fumarate to succinate (26), a process that is well understood for other organisms (15). It has previously been shown that the fumarate reductase activity of *G. sulfurreducens* is membrane bound and is sensitive to the menaquinol analog HOQNO (2-*n*-heptyl-4-hydroxyquinoline-*N*-oxide) (8), suggesting that the fumarate reductase might be more like those found in *Wolinella succinogenes* and *Escherichia coli* than to the soluble periplasmic enzyme found in the other well-studied Fe(III)-reducing organism *Shewanella oneidensis* (4, 14, 30).

To completely oxidize acetate with Fe(III) as the electron acceptor, *Geobacter* species require the membrane-bound TCA cycle enzyme that catalyzes the reverse reaction, succinate dehydrogenase (5, 8). The redox potential of the succinate/fumarate couple (+30 mV) is such that ubiquinone (+110 mV) is the energetically favorable electron acceptor for succinate oxidation, whereas menaquinol (−80 mV) is the favorable electron donor for fumarate reduction (15). In *E. coli*, two separate enzymes are expressed with two different quinones: succinate dehydrogenase and ubiquinone during aerobic growth and fumarate reductase and menaquinone during anaerobic growth (4). In *Geobacter* species, the mechanisms and energetics of the fumarate reductase and succinate dehydrogenase reactions are less clear. Depending on the electron acceptor, fumarate reductase and succinate dehydrogenase can be required during anaerobic oxidation of acetate, and *Geobacter* species have been shown to contain only menaquinone, not ubiquinone (3, 25).

It is demonstrated here that *G. sulfurreducens* has only one enzyme, FrdCAB, that functions in vivo as both the fumarate reductase and the succinate dehydrogenase, with an apparent energetic cost when catalyzing succinate oxidation. *G. metallireducens* is also shown to contain orthologous *frdCAB* genes, and evidence is presented that suggests that the presence of a dicarboxylic acid transporter is the key adaptation which allows *G. sulfurreducens* to use fumarate as a terminal electron acceptor, compared to *G. metallireducens*, which cannot.

MATERIALS AND METHODS

Cell growth. *G. sulfurreducens* strain DL1 was obtained from our laboratory culture collection and cultivated anaerobically at 30°C in a freshwater fumarate

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or Fe(III) citrate medium as previously described (3, 27). Growth was monitored by optical density or epifluorescence microscopy, and Fe(III) reduction was assessed as accumulation of Fe(II) as previously described (19).

Northern analysis. Total RNA was isolated with the RNeasy kit (QIAGEN, Inc.), separated by 1.8% denaturing gel electrophoresis, and transferred to a charged nylon membrane with Turboblotter (Schleicher & Schuell, Dassel, Germany). The *frdA* probe was amplified with the primers FrdA1 (GAACTCGGT TACAACGTTG) and FrdA2 (GTCACCATGCTGCGGAATGC) and labeled with [α-^{32}P]dCTP (New England Nuclear, Boston, MA) by using the NEBlot kit (New England Biolabs).

Construction of an *frdA*-deficient mutant strain. Single-step gene replacement of *frdA* was performed as previously described (19, 22). The upstream region of the gene was amplified with primers FrdA01 (CAACGACAAGTCACTTC) and FrdA02 (GAGCTACGCACTGATCGATG) and the downstream region was amplified with primers FrdA05 (GCAAGAAATCGGTTGAGG) and FrdA06 (GACATGAAGGGTAAGAGTC). The kanamycin resistance cassette from pBBR1MCS-2 (13) was amplified with primers FrdA03 (CATCGATCAGTGC GTAGCTCACAGCAAGCGAACCGGAATTG) and FrdA04 (CCTCAACCG ATTTCTTGCATTTCGAACCCCAGAGTC). Recombinant PCR was carried out as previously described (22), except that the annealing temperature was 53°C. Electrocompetent cells were prepared, cells were electroporated, and mutants were isolated as previously described (6), with the exception that the recovery and plating media used Fe(III) citrate media supplemented with 0.2% yeast extract, 0.25 mM cysteine, and 400 μg/ml kanamycin. Gene disruption was confirmed with two PCR amplifications of the region using primers FrdA01/ FrdA06 and FrdA03/FrdA04, and one positive clone was chosen as the representative mutant strain.

Expression of *dcuB* in *G. metallireducens*. The fumarate transporter *dcuB* was cloned from *G. sulfurreducens* with primers RGBB1 (GCGATGAATTCAAGG GGAGGCAGTTATGATG) and RGBB2 (CGCTGCCCTTCTTTTACAGCAC GAACTG). The product was end filled, digested with EcoRI, and ligated into pRG5 (12) that had been digested with HindIII, end filled, and digested with EcoRI. Preparation of electrocompetent *G. metallireducens* was as previously described for *G. sulfurreducens* (6), except cells were grown and recovered in Fe(III) citrate media with 0.1% yeast extract, and the wash buffer contained 1.0 mM HEPES (pH 7.0), 1.5 mM $MgCl_2$, 225 mM sucrose, and 1% glycerol. A single colony of *G. metallireducens* carrying pRG5*dcuB* was recovered with the roll-tube method (10). The isolated strain was confirmed as *G. metallireducens* by sequencing the 16S rRNA gene PCR product amplified with primers 338F and 907R (1, 18), and the presence of pRG5*dcuB* was confirmed by sequencing *dcuB* amplified with primers RGG1 and RGG2 from plasmid DNA purified from the strain *G. sulfurreducens* carrying the pRG5*dcuB* plasmid, as previously described (6).

Enzyme assays. Cell extract preparation and enzyme assays were carried out anoxically on cultures that had been grown in acetate-Fe(III) citrate medium supplemented with 20 mM fumarate. Cells were washed twice with 50 mM HEPES (pH 7.5) containing 10% glycerol, 2.5 mM $MgCl_2$, and 2.5 mM dithiothreitol, and resuspended in a small volume of the same buffer supplemented with DNase I and lysozyme. Cells were lysed with a French press at 40,000 kPa and centrifuged for 20 min at 1,500 × *g* at 4°C, with the resulting crude cell extract used in the assays. The assay buffer contained 50 mM HEPES (pH 7.5), 2.5 mM $MgCl_2$, and either 5 mM benzyl viologen [reduced with Ti(III) citrate] or 0.5 mM 2,6-dichloroindophenol. Activity was measured at 578 nm in a 1.0-ml volume of buffer at 30°C. Fumarate reductase activity was measured by following the benzyl viologen absorbance decrease ($\varepsilon = 7.8\ mM^{-1}\ cm^{-1}$) after the addition of fumarate, and succinate dehydrogenase activity by the 2,6-dichloroindophenol absorbance decrease ($\varepsilon = 21\ mM^{-1}\ cm^{-1}$) after the addition of succinate (21). Protein concentrations were determined with the bicinchoninic acid method (36).

Metabolite analysis. Samples for organic acid analysis were filtered (0.2-μm pore diameter) and stored in 0.5 N HCl at −80°C. Samples were separated by high-pressure liquid chromatography (Aminex HPX-87H column, 300 × 7.8 mm; Bio-Rad Laboratories, Hercules, CA) with a mobile phase of 10.0 mM H_2SO_4 flowing at 0.6 ml/min, with detection at 215 nm. Peaks were identified and quantified based on standards of acetate, fumarate, succinate, and malate.

Nucleotide sequence accession numbers. The *frdCAB* open reading frames were given the NCBI accession numbers NP_952229, NP_952230, and NP_952231.

RESULTS AND DISCUSSION

Identification and analysis of the FrdCAB operon. The complete *G. sulfurreducens* genome (31) was searched with sequences of subunits from each of the different classes of fumarate reductases and succinate dehydrogenases (20). A single putative operon with three open reading frames was identified and designated *frdCAB* (Fig. 1A). There were no genes homologous to the periplasmic flavocytochrome fumarate reductases found in *Shewanella* species (30, 37). Comparison of the proteins encoded by the *G. sulfurreducens* operon to the heterotrimeric, or B-type, fumarate reductase from *W. succinogenes*, for which the structure has been solved (16), showed orthologs to the catalytic subunit, FrdA, with conserved flavin adenine dinucleotide and dicarboxylate binding residues; the Fe-S cluster subunit, FrdB, with three conserved cysteine-rich motifs; and the membrane anchor subunit, FrdC, with all five putative transmembrane helices (data not shown). Four conserved histidines, which have been shown to bind two *b*-type hemes in *W. succinogenes* (16), were also identified in the FrdC sequence. The succinate dehydrogenase of the gram-positive aerobe *Bacillus subtilis* is also a member of this B-type family of enzymes (20), and the *G. sulfurreducens* FrdCAB proteins are more similar to this enzyme (31% amino acid identity between A subunits) than to the fumarate reductases found in other *Proteobacteria*, such as *W. succinogenes* (23%) and *H. pylori* (20%).

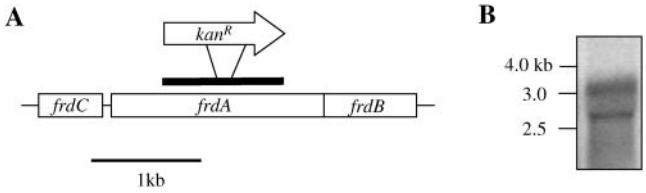

FIG. 1. Structure and mutagenesis of the fumarate reductase/succinate dehydrogenase operon of *G. sulfurreducens*. (A) The operon structure of the genes, GSU1176 (*frdC*), GSU1177 (*frdA*), and GSU1178 (*frdB*), showing the location (black bar) of the gene disruption with the kanamycin resistance cassette in the mutant strain. The homologous genes in *G. metallireducens* are organized and spaced identically, with 85%, 93%, and 91% amino acid sequence identity between the C, A, and B subunits, respectively. (B) Transcription of the operon in *G. sulfurreducens* grown with fumarate as electron acceptor, analyzed by Northern blot (10 μg RNA/lane) using a fragment of *frdA* as a probe. The expected size of a transcript of *frdCAB* is 3.3 kb and that of *frdAB* is 2.6 kb.

Although the *G. sulfurreducens* gene products are similar to these well-studied heterotrimeric enzymes, they form a distinct phylogenetic group with proteins from diverse organisms, including *Cytophaga-Flavobacterium-Bacteroides*, green sulfur, high-GC gram-positive cyanobacteria and spirochete species (Fig. 2). This grouping is supported by phylogenetic analysis for each of the three subunits, with the membrane-bound FrdC subunit being the least conserved (data not shown).

Northern blot analysis was performed to determine whether the *frdCAB* cluster constituted an operon. When a fragment of *frdA* was used as a probe, two bands of 3.8 kb and 2.7 kb were detected in cells grown with either fumarate or Fe(III) as the electron acceptor and acetate as the sole carbon and energy source (Fig. 1B). Thus, expression of the *frdCAB* enzyme is not specific to conditions under which a terminal fumarate reductase is required. When a fragment of *frdC* was used as a probe, a single 3.8-kb band was detected (data not shown). The size of the larger band is consistent with cotranscription of all three genes, and the size of the smaller band is consistent with the

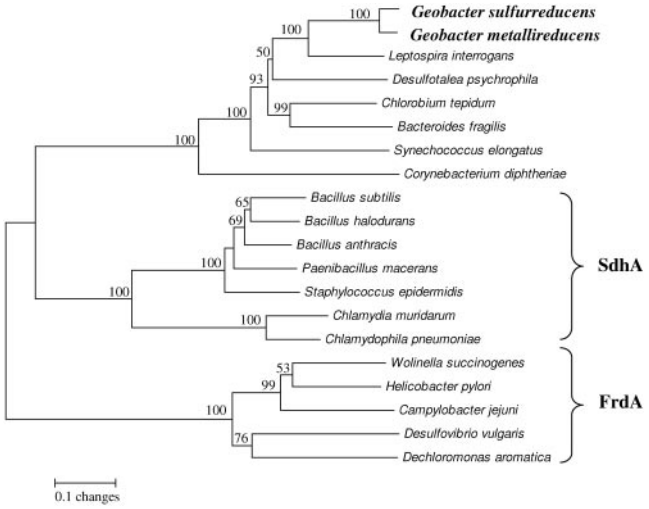

FIG. 2. Phylogenetic tree of representative catalytic subunits from putative B-type, heterotrimeric enzymes which were most similar to *G. sulfurreducens*. Sequences were aligned using Clustal X (38), and distances and branching order were determined using the neighbor-joining method (32). A total of 1,000 replicates were used for bootstrap analysis. The clade containing the *W. succinogenes* fumarate reductase is indicated by FrdA and the clade containing the *B. subtilis* succinate dehydrogenase is indicated by SdhA. *Geobacter* species subunits are shown in bold.

size of *frdAB*. Two transcript sizes have also been reported in *Paenibacillus macerans* and *E. coli* (33, 40).

Dual function of FrdCAB as the fumarate reductase and the succinate dehydrogenase. To determine the in vivo role of the enzyme encoded by this operon, the gene for the putative catalytic subunit, *frdA*, was mutated by insertion of a kanamycin resistance cassette, with concurrent deletion of 57% of the gene (Fig. 1A). The mutant strain was isolated using Fe(III) as the electron acceptor and hydrogen as the electron donor with acetate as the carbon source. The *frdA*-deficient strain did not grow with fumarate as the electron acceptor in solid or liquid medium with acetate, hydrogen, or both provided as the electron donor(s) (data not shown), and there was no detectable fumarate reductase activity in crude cell extracts of the mutant strain, compared to 123 ± 17.8 nmol min^{-1} mg $protein^{-1}$ in extracts of the wild type.

In addition, the *frdA*-deficient strain did not grow with Fe(III) as the electron acceptor when acetate was the electron donor (Fig. 3B), a growth condition under which no fumarate reductase activity should be required. This result supports the Northern blot analysis showing that the enzyme in expressed when Fe(III) is the terminal electron acceptor and suggests that the enzyme may also function as the succinate dehydrogenase required for acetate oxidation via the TCA cycle. This hypothesis was confirmed by determining the succinate dehydrogenase enzymatic activity in the mutant strain. There was no detectable succinate dehydrogenase activity in cell extracts of the mutant strain, compared to 55 ± 7.0 nmol min^{-1} mg $protein^{-1}$ in the wild type.

It was previously hypothesized that succinate dehydrogenase activity might not be necessary when fumarate served as the electron acceptor for *G. sulfurreducens*, because exogenous fumarate could serve as the substrate for malate and oxaloacetate synthesis (8). In accordance with this hypothesis, adding fumarate to acetate-Fe(III) medium permitted the *frdA*-deficient strain to grow with acetate as the electron donor and Fe(III) as the electron acceptor despite the lack of succinate dehydrogenase activity (Fig. 3B). Thus, unlike previously studied organisms, *G. sulfurreducens* uses a single enzyme as both the terminal fumarate reductase in anaerobic respiration and the succinate dehydrogenase in acetate oxidation via the TCA cycle. Although previously described fumarate reductases and succinate dehydrogenases typically catalyze both fumarate reduction and succinate oxidation in vitro, these enzymes have been found to catalyze the reaction in just one direction in vivo (4, 9, 14, 17).

Energetic cost of the succinate dehydrogenase reaction. Both wild-type and mutant strains growing in Fe(III) medium with excess acetate as the electron donor grew faster when supplemented with fumarate (8.3 ± 0.4 and 7.6 ± 0.4 h generation time, respectively) compared to wild type growing without fumarate supplementation (9.5 ± 0.1 h generation time) (Fig. 3). Furthermore, the peak cell density was more than

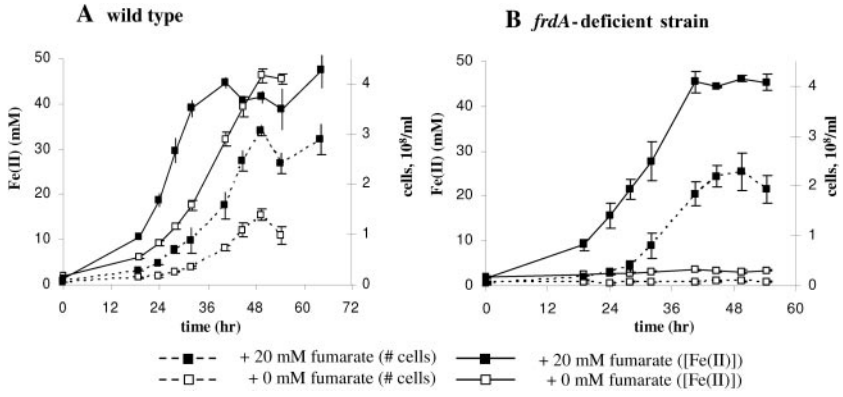

FIG. 3. Growth of wild-type and *frdA*-deficient *G. sulfurreducens* strains with excess acetate as the electron donor and Fe(III) as the electron acceptor. (A) Cell growth and Fe(III) reduction of the wild type with 20 mM fumarate supplementation and with no fumarate supplementation. (B) Cell growth and Fe(III) reduction of the *frdA*-deficient strain with 20 mM fumarate supplementation and with no fumarate supplementation. Experiments were run in parallel with inocula of ca. 6×10^6 late-log-phase cells adapted to the appropriate medium. Data are means ± standard deviations of triplicate cultures.

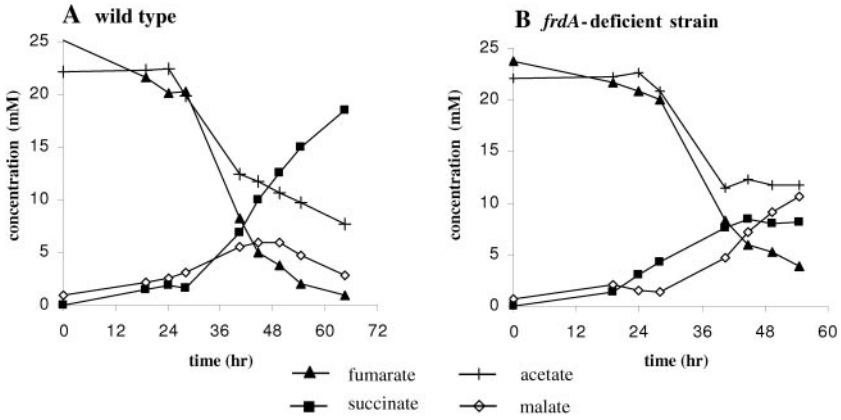

FIG. 4. Metabolism of organic acids in wild-type and *frdA*-deficient *G. sulfurreducens* strains grown with excess acetate as the electron donor and Fe(III) as the electron acceptor. Data are means of analyses by high-pressure liquid chromatography of the fumarate supplemented cultures shown in Fig. 3. (A) Metabolism of acetate, fumarate, succinate, and malate in the wild-type strain. (B) Metabolism of acetate, fumarate, succinate, and malate in the *frdA*-deficient mutant strain. Samples were taken from the triplicate cultures at the same time as those from Fig. 3.

1.6-fold higher in the fumarate-supplemented strains than in unsupplemented wild type (Fig. 3). In the case of the wild type, these increases in growth rate and cell yield could be due to the simultaneous exploitation of two terminal electron acceptors, fumarate and Fe(III), allowing the oxidation of more acetate, leading to the generation of more ATP. Examination of the organic acid content of the growth medium from the fumarate-supplemented wild-type cultures showed that the wild-type strain did exploit both electron acceptors and continued to oxidize acetate and convert fumarate to succinate after the depletion of Fe(III) (by 40 h) (Fig. 4A). Malate accumulated when fumarate was in excess, which is consistent with the activity of the reversible fumarase in *G. sulfurreducens* (8) and the lack of a glyoxylate shunt in this species (31).

However, the mutant strain cannot generate ATP via fumarate reduction, so the use of two electron acceptors does not account for the increases in growth rate and cell yield (Fig. 3B). Examination of the organic acid content of the growth medium from the fumarate supplemented *frdA*-deficient strain confirmed that acetate oxidation and succinate production ceased when Fe(III) was depleted (by 40 h) (Fig. 4B). This is consistent with a strict dependence of the succinate production on the TCA cycle in this strain (Fig. 5B).

The increases in growth rate and cell yield during bypass of the succinate dehydrogenase (Fig. 3A) indicate that there is an energetic cost for this reaction. This could be due to the unfavorable coupling of the oxidation of succinate (+30 mV) with the reduction of menaquinone (−80 mV), the only membrane-bound electron carrier in *Geobacter* species (3, 25). In *B. subtilis*, the succinate dehydrogenase is orthologous to FrdCAB from *G. sulfurreducens*, and the membrane-bound electron carrier is also menaquinone (9). Dissipation of the membrane potential has been proposed to drive succinate oxidation in *B. subtilis* (34, 35). In *G. sulfurreducens*, a succinate dehydrogenase that dissipates the membrane potential could explain the increases in cell yield and growth rate seen when the succinate dehydrogenase is bypassed (Fig. 3A). This also provides insight into the decreases in growth rate and cell yield previously observed in wild-type *G. sulfurreducens* growing with Fe(III) citrate as the electron acceptor compared to fu-

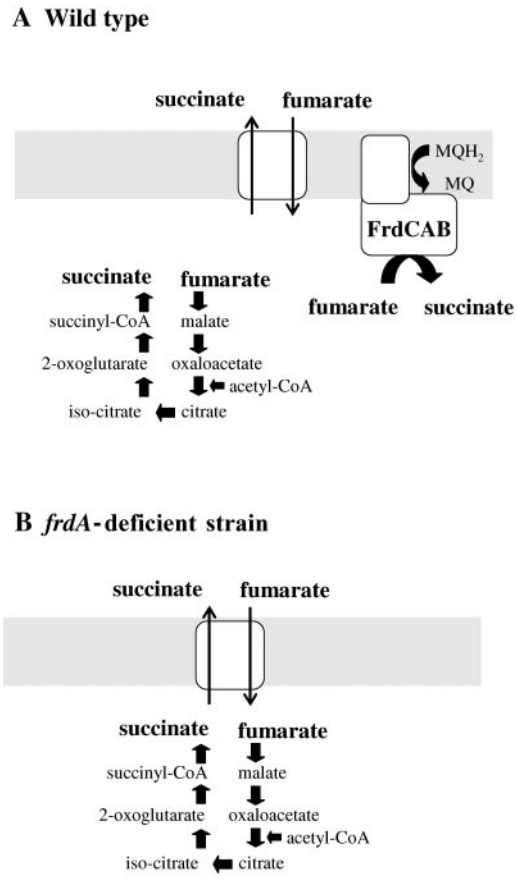

FIG. 5. A model for succinate production during growth with fumarate supplementation in the wild-type and *frdA*-deficient *G. sulfurreducens* strains. (A) In the wild-type strain, succinate can be produced by both the terminal fumarate reductase, FrdCAB, and by the TCA cycle reactions. Succinate can be exchanged with fumarate from external media, obviating the need for a succinate dehydrogenase. (B) In the *frdA*-deficient mutant strain, neither a terminal fumarate reductase nor a succinate dehydrogenase is present, so succinate is produced only by the TCA cycle reactions and must be exchanged with external fumarate to allow continued acetate oxidation.

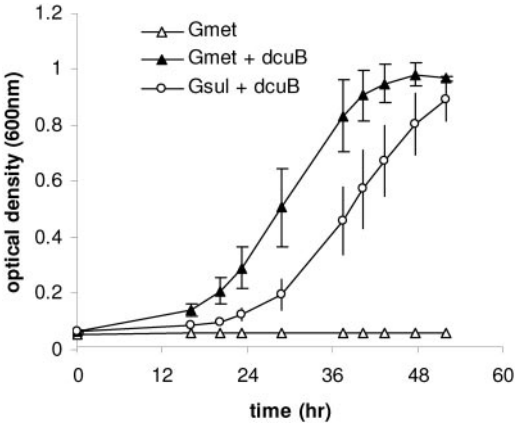

FIG. 6. Growth of *G. metallireducens* and *G. sulfurreducens* with excess acetate as the electron donor and fumarate as the sole electron acceptor. Shown are wild-type *G. metallireducens* and strains of *G. metallireducens* and *G. sulfurreducens* in which *dcuB*, the putative fumarate transporter from *G. sulfurreducens*, is being constitutively expressed in *trans*. Inocula were 2.5% late-log-phase cells adapted to the appropriate medium, with the strains carrying pRG*dcuB* grown in the presence of 275 μM spectinomycin. Data are means ± standard deviations of triplicate cultures.

marate as the electron acceptor (7). This decrease is unexpected, because the midpoint potential of the fumarate/succinate redox couple (+30 mV) is lower than that of the Fe(III)/Fe(II) citrate couple (+370 mV). Because the succinate dehydrogenase is required if exogenous fumarate is not present, the lower cell yield during growth with Fe(III) serving as the electron acceptor may be due in part to the cost of succinate oxidation. The proton translocation stoichiometry of other parts of the electron transport chain to Fe(III) is currently under investigation.

Engineering *G. metallireducens* to grow on fumarate. Unlike *G. sulfurreducens*, the closely related species *G. metallireducens* cannot grow with fumarate as the sole electron acceptor (Fig. 6). However, analysis of the draft genome of *G. metallireducens* identified a single putative operon (NCBI accession numbers ZP_00300178, ZP_00300178, and ZP_00300180) with ca. 90% identity to *frdCAB* of *G. sulfurreducens* (Fig. 1A). Since fumarate is reduced in the cytoplasm in *G. sulfurreducens* (8), the genome was also searched for genes homologous to known fumarate transporters. While the *G. sulfurreducens* genome contained an open reading frame (NCBI accession number NP_953796) whose product has 43% amino acid identity to a fumarate transporter protein, DcuB, found in *W. succinogenes* (NCBI accession number CAA10331) (39), no open reading frame with similarity to known dicarboxylate transporters (11) was found in *G. metallireducens*. To determine if lack of fumarate transport was the cause of the inability of *G. metallireducens* to grow with fumarate as the terminal electron acceptor, a copy of the *G. sulfurreducens dcuB* gene was constitutively expressed in *G. metallireducens* in *trans*. The *G. metallireducens* strain expressing *dcuB* was able to grow with fumarate as the sole electron acceptor with a generation time similar to that of the *G. sulfurreducens* strain expressing *dcuB*, 8.5 versus 8.4 h, with a somewhat shorter lag time and a slightly higher maximum optical density (Fig. 6). Thus, the primary role of this FrdCAB in *G. metallireducens* is likely to serve as the succinate dehydrogenase of the TCA cycle, but the presence of fumarate in the cell allows fumarate reduction as well, possibly by a mechanism similar to that shown in Fig. 5A. The conversion of *G. metallireducens* to a fumarate-respiring microorganism represents the first genetic engineering of this strain and the first engineering of a *Geobacter* species to expand its respiratory capabilities.

Implications. In summary, the results show that the FrdCAB enzyme of *G. sulfurreducens* acts as both the terminal fumarate reductase and the succinate dehydrogenase of the TCA cycle in vivo, with an apparent energetic cost when catalyzing succinate oxidation. It is similar to the fumarate reductase of *W. succinogenes* and the succinate dehydrogenase of *B. subtilis*, but the apparent role of the enzyme in *G. metallireducens* and the low availability of exogenous fumarate in the sedimentary environments in which these species predominate (23) indicate its primary function in both *Geobacter* species is likely to serve as a succinate dehydrogenase.

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