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Characterization of Citrate Synthase from *Geobacter sulfurreducens* and Evidence for a Family of Citrate Synthases Similar to Those of Eukaryotes throughout the *Geobacteraceae*

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Members of the family *Geobacteraceae* are commonly the predominant Fe(III)-reducing microorganisms in sedimentary environments, as well as on the surface of energy-harvesting electrodes, and are able to effectively couple the oxidation of acetate to the reduction of external electron acceptors. Citrate synthase activity of these organisms is of interest due to its key role in acetate metabolism. Prior sequencing of the genome of *Geobacter sulfurreducens* revealed a putative citrate synthase sequence related to the citrate synthases of eukaryotes. All citrate synthase activity in *G. sulfurreducens* could be resolved to a single 49-kDa protein via affinity chromatography. The enzyme was successfully expressed at high levels in *Escherichia coli* with similar properties as the native enzyme, and kinetic parameters were comparable to related citrate synthases (k_{cat} = 8.3 s^{-1}; K_m = 14.1 and 4.3 μM for acetyl coenzyme A and oxaloacetate, respectively). The enzyme was dimeric and was slightly inhibited by ATP (K_i = 1.9 mM for acetyl coenzyme A), which is a known inhibitor for many eukaryotic, dimeric citrate synthases. NADH, an allosteric inhibitor of prokaryotic hexameric citrate synthases, did not affect enzyme activity. Unlike most prokaryotic dimeric citrate synthases, the enzyme did not have any methylcitrate synthase activity. A unique feature of the enzyme, in contrast to citrate synthases from both eukaryotes and prokaryotes, was a lack of stimulation by K^+ ions. Similar citrate synthase sequences were detected in a diversity of other *Geobacteraceae* members. This first characterization of a eukaryotic-like citrate synthase from a prokaryote provides new insight into acetate metabolism in *Geobacteraceae* members and suggests a molecular target for tracking the presence and activity of these organisms in the environment.

Microorganisms that can transfer electrons to extracellular electron acceptors such as insoluble Fe(III) oxides (26, 30), humic substances (28), and electrodes (3) play an important role in the ecology and geochemistry of sedimentary environments. They produce geologically significant minerals such as magnetite (30), serve as models for metabolism on ancient Earth and in the deep subsurface (48, 54), contribute to the carbon cycle (6), are agents for bioremediation of environments contaminated with petroleum or toxic metals such as uranium (1, 26, 29), and show promise as a means of harvesting energy from waste organic matter (4). *Geobacter* species outcompete other Fe(III)-reducing microorganisms in Fe(III)-containing sedimentary environments (41, 42, 46), and a key factor in their competitiveness is their ability to completely oxidize acetate and other organic compounds to carbon dioxide in the subsurface.

Studies of model *Geobacteraceae* members, such as *Desulfuromonas acetoxidans* (14), *Geobacter metallireducens* (7), and *Geobacter sulfurreducens* (13), have demonstrated that enzymes necessary for a complete tricarboxylic acid (TCA) cycle are present in cell extracts, whereas enzymes indicative of a C_1-based pathway were not detected in any of these investigations. ^{14}C-labeling studies have also demonstrated an amino acid labeling pattern consistent with acetate oxidation via a TCA cycle and carbon assimilation via reductive carboxylation of acetate rather than assimilation of CO_2 (14). A CO_2-reducing (acetyl-coenzyme A [CoA]) pathway was recently proposed based on annotation of the *G. sulfurreducens* genome (33), but observations such as the lack of any known autotrophic member of the *Geobacteraceae*, the inability of *G. sulfurreducens* to grow with hydrogen without the addition of acetate as a carbon source (10), and the inability of organisms such as *D. acetoxidans* to grow via partial oxidation of substrates (such as butanol to butyrate) without the addition of a carbon source (44) suggest that a C_1 pathway is inactive under conditions used to study known members of the *Geobacteraceae* or that these genes are components of biosynthetic reactions unrelated to acetate oxidation.

Stereospecific citrate (s)-synthase is the only enzyme likely to be capable of catalyzing the Claisen condensation of acetyl-coenzyme A and oxaloacetate to produce citric acid in *Geobacteraceae*. This enzyme is of physiological and ecological interest, because it is commonly considered to exert significant control over flux into the TCA cycle. The genome sequence of *G. sulfurreducens* revealed only one putative citrate synthase gene. Surprisingly, the sequence was more closely related to eukaryotic citrate synthases than those described in prokaryotes (33).

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As part of an effort to model and understand the flux and regulation of key central metabolic processes in *Geobacter* species, citrate synthase was purified from *G. sulfurreducens*. These findings show that all detectable citrate synthase activity in *G. sulfurreducens* is found in a protein encoded by a eukaryotic-like citrate synthase gene and that this citrate synthase has a number of eukaryotic-like biochemical features. Furthermore, we report that a similar gene is found throughout the *Geobacteraceae*, providing the possibility that this unique *Geobacteraceae* sequence that is central to their metabolism can be used to track the presence and activity of these organisms.

MATERIALS AND METHODS

Growth conditions and media. *G. sulfurreducens* strain PCA (ATCC 51573) was maintained in our laboratory under strict anaerobic conditions using a bicarbonate-CO_2-buffered medium as previously described (4), with 20 mM acetate as the electron donor and 40 mM fumarate as the electron acceptor. Where indicated, acetate was replaced with 20 mM sodium lactate or H_2 (added to the headspace to a final concentration of 50% H_2 at 2 atm and supplemented with 0.1 mM Na acetate as carbon source), or fumarate was replaced with 55 mM Fe(III) as ferric citrate. *G. metallireducens* strain GS-15 (ATCC 53774) was cultured using identical medium with ferric citrate as the acceptor and acetate or propionate (20 mM) as the electron donor.

Purification of citrate synthase. Ten grams of cells collected by centrifugation was washed with chilled 50 mM Tris-HCl (pH 7.5) buffer and resuspended in lysis buffer containing 50 mM Tris (pH 7.5), protease inhibitor cocktail (Roche), and 10% glycerol. Cells were frozen until further use. Cells were broken with two passes through a French pressure cell, and cell debris was removed by centrifugation (10,000 × *g*) at 4°C. Cytosolic proteins were separated from membranes with ultracentrifugation (100,000 × *g*, 1 h, 4°C).

Citrate synthase was purified from cytosolic fractions in three steps with fast protein liquid chromatography. Crude extract was applied to a Q Sepharose HP column (1.6 by 10 cm; Amersham Biosciences, Piscataway, NJ) equilibrated with 50 mM Tris-HCl (pH 8.0) and eluted with a linear gradient of KCl (0 to 1 M). Active fractions were pooled, concentrated, and desalted by ultrafiltration with a PM-10 membrane (Amicon, Billerica, MA). Samples were loaded onto a MonoQ column (0.5 by 5 cm; Amersham) using 50 mM Tris buffer, pH 8.0, and eluted with a stepwise gradient of KCl (0 to 0.5 M). The last step was an affinity column (1 by 10 cm) of MimeticRed 2 A6XL (Prometic BioScience, Ltd.). Citrate synthase was eluted with 1 mM oxaloacetate and 0.2 mM acetyl coenzyme A in 50 mM Tris-HCl (pH 8.0).

In-gel tryptic digests of purified proteins were performed according to Gharahdaghi et al. (16) with the addition of 0.01% *n*-octylglucopyranoside in the digest buffer. Digests were concentrated using ZipTip C18 pipette tips (Millipore) according to the recommended protocol, with the exception of 1% formic acid instead of trifluoroacetic acid. Matrix-assisted-laser-desorption–ionization time-of-flight mass spectrometry (MALDI-TOF MS) and PSD (post-source-decay) data were obtained on a Kratos Axima CFR (Kratos Analytical, Manchester, England). Samples were cocrystallized with alpha-cyano-4-hydroxy-cinnamic acid (10 mg/ml in 1% formic acid, 50% acetonitrile) via the dried drop method. External calibration in MS mode was accomplished by a three-point peptide mixture, which covered the mass range typical of tryptic digests (600 to 2,500 Da). PSD fragment ions were fitted to a generated curve, which was calibrated with PSD fragments from a synthetic peptide (P14R).

Searches of the *G. sulfurreducens* draft genome were performed using the Protein Prospector MSFit program (http://prospector.ucsf.edu). Monoisotopic peptide masses were searched using 150 ppm mass tolerances, and PSD fragments were searched using 0.2 Da as the parent tolerance and 0.5 Da as the fragment ion tolerance.

Expression of citrate synthase in *Escherichia coli*. *G. sulfurreducens* citrate synthase was amplified from genomic DNA with KOD DNA polymerase using conditions and reagents provided by the vendor (Novagen, Madison, WI) and was cloned into a pMCSG7 vector (49) using a modified ligation-independent cloning protocol (49). This process generated an expression clone producing a fusion protein with a N-terminal 6× His tag and a tobacco etch virus protease recognition site, ENLYFQ ↓ S. The fusion protein was overproduced in an *E. coli* BL21 derivative harboring a plasmid encoding three rare *E. coli* tRNAs (Arg [AGG/AGA] and Ile [ATA]). *E. coli* was grown and the His-tagged-containing enzyme was purified with standard procedures as described by the supplier (Stratagene, La Jolla, CA).

Size determination. Samples from purification steps were electrophoresed on 12% sodium dodecyl sulfate (SDS)–polyacrylamide gels and visualized via Coomassie staining with Seeblueplus prestained proteins (Invitrogen). Size exclusion chromatography in Sephadex 75 (30 by 1 cm) and Superdex 200 (60 by 1.6 cm) columns was calibrated with high- and low-molecular-weight protein standards, respectively (Sigma), in 50 mM Tris-HCl buffer (pH 7.5) containing 150 mM KCl at the flow rate of 0.5 ml/min. Elution of active citrate synthase was verified via activity measurements of eluted fractions.

Assay of citrate synthase activity. Citrate synthase activity was assayed spectrophotometrically at 412 nm (47) using a UV2401-PC dual-beam spectrophotometer equipped with a temperature-controlled cuvette holder (Shimadzu, Columbia, MD). Unless otherwise indicated, the assay conditions were the following: 50 mM Tris-HCl buffer (pH 8.0), 0.1 mM acetyl coenzyme A, 0.2 mM oxaloacetate, 0.2 mM 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB). Reactions were initiated with the addition of citrate synthase or cell extract at 30°C. The enzyme reaction was monitored by the reaction of coenzyme A with DTNB (ε_{412} = 13,600 M^{-1} cm^{-1}). Controls containing only acetyl-CoA demonstrated no activity under these conditions. Methylcitrate synthase activity was performed using exactly the same conditions but substituting propionyl-CoA for acetyl-CoA. Oxaloacetate, 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), acetyl-coenzyme A, propionyl-CoA, and porcine citrate synthase were purchased from Sigma (St. Louis, MO), and all chemicals were used without further purification.

Accession numbers and primer sequences. The draft sequence for *Geobacter sulfurreducens* was obtained from The Institute for Genomic Research (www.tigr.org). The draft sequences for *G. metallireducens*, *Desulfuromonas acetoxidans*, and *Pelobacter carbinolicus* were obtained from the Joint Genome Institute (www.jgi.doe.gov). All *Geobacteraceae* genomic citrate synthase sequences were resequenced to correct errors in the draft sequences.

Comparisons with *Myxococcus xanthus* were performed using preliminary sequence data obtained from The Institute for Genomic Research (www.tigr.org). Comparisons with *Desulfotaelea psychrophila* and *Desulfobacterium autotrophicum* were performed using preliminary data made available at the REGX-project website (BLAST.mpi-bremen.de/BLAST/index.html).

Additional sequences were obtained from pure cultures of *Malonomonas rubra* (DSM 5091), *Desulfuromonas palmitatis* (DSM 12391), *Desulfuromonas acetexigens* (DSM 1397), and "*Geobacter bemidjiensis*" (recently isolated from petroleum-contaminated aquifer sediments [42] using primers developed for this purpose [192F, TTCCGYGGYAWGACMATTCC; and 1248R, TCCCARGTGAT GTTBGCCAWGC]). The identity of each organism was verified by also amplifying and sequencing the 16S rRNA of each DNA sample from which a citrate synthase was obtained.

Phylogenetic methods. Amino acid sequences were first aligned in CLUSTALX (53) and then checked and edited by eye in Se-Al version 2.0. Protein maximum-likelihood phylogenies were estimated by use of PMBML (54a), a modified version of PROML within the PHYLIP package, version 3.6a2 (12a), using a JTT + G model, global rearrangements, and 10 random addition replicates. The α parameter was estimated in PUZZLE. Confidence of branch points was estimated by 100 bootstrap replications and 100 puzzling quartets (TREE-PUZZLE), applying the settings described above.

Nucleotide sequence accession numbers. All *Geobacteraceae* citrate synthase sequences were submitted to GenBank (accession nos. AY490255 to AY490264).

RESULTS AND DISCUSSION

Citrate synthase activity in growing cultures. Citrate synthase activity was measured to determine activity levels in crude extracts as a function of time or medium conditions. Citrate synthase activity was detected at all times and was highest (21.5 U/mg protein) in mid-exponential phase for cultures grown with acetate as the electron donor and fumarate as the electron acceptor (Table 1). Cultures grown with hydrogen as the electron donor had the lowest specific activity. Activity in acetate-fumarate cultures decreased to 50% of maximal levels after cultures entered stationary phase, and thus mid-log acetate-fumarate cultures were used for all subsequent determinations.

TABLE 1. Specific activity of citrate synthase measured in the mid-log phase of growth of *G. sulfurreducens* cultured with various electron donors and acceptors

Electron donor	Electron acceptor	Sp act (U/mg protein)
Acetate	Fumarate	21.5
Lactate	Fumarate	8.6
Acetate	Ferric citrate	2.3
Hydrogen	Fumarate	0.3

Purification of citrate synthase. Several steps were needed to reach at least 30-fold purification of the citrate synthase activity (Table 2, Fig. 1). The mimetic red affinity column alone could not produce pure citrate synthase from crude extracts. Activity of citrate synthase decayed during purification, and agents such as 20% glycerol and/or 100 mM KCl, which have been used as stabilization agents for citrate synthase from other organisms (40, 45), did not improve the stability of the enzyme. When enzyme was needed for crucial kinetic, inhibitor, or size characterizations, it was purified as rapidly as possible (within 10 h).

Characteristics of the purified enzyme. Purified citrate synthase was found to correspond to a single ORF in the genome of *G. sulfurreducens* (identified with MALDI-TOF and PSD, 47% coverage). The predicted pI and molecular mass of the protein were 6.46 and 49.8 kDa, respectively. These values were consistent with the behavior of the protein during purification. As noted previously (33), alignment of the purified citrate synthase from *G. sulfurreducens* (Fig. 2) indicated that the citrate synthase sequence of *G. sulfurreducens* was most similar to those of eukaryotic organisms (2, 11, 21, 38). More specifically, all residues implicated in citrate and acetyl-CoA binding by citrate synthase as well as features common to eukaryotic rather than prokaryotic enzymes were present (Fig. 2). For example, based on crystal structures of related dimeric citrate synthases, arginine residues from each monomer have been shown to participate in citrate and acetyl-CoA binding, and these arginine residues appeared to be conserved in the *G. sulfurreducens* enzyme.

Contrary to the prediction of dimerization, initial gel filtration experiments indicated that the citrate synthase was monomeric under native conditions. To investigate this discrepancy, *G. sulfurreducens* citrate synthase was expressed in *E. coli* to obtain larger quantities of enzyme. The recombinant enzyme had comparable kinetic properties as the native enzyme (Table 3), and at protein concentrations of 0.3 mg ml^{-1} and above, *G. sulfurreducens* citrate synthase behaved as a homodimer, eluting at a molecular mass of 100 kDa. However, at concentrations of 0.16 and 0.08 mg ml^{-1}, the enzyme eluted

TABLE 2. Purification of the citrate synthase

Purification step	Total protein (mg)	Total act (U)	Sp act (U/mg protein)	Purification factor	Yield (%)
Crude extract	508.1	304.8	0.6	1.0	100
Q-Sepharose	20.2	72.7	3.6	6.0	24
MonoQ	5.3	52.5	9.9	16.5	17
Affinity	0.8	15.6	19.6	32.6	5

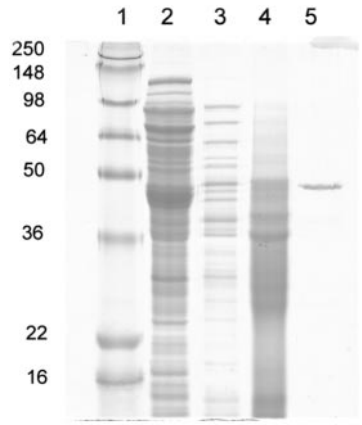

FIG. 1. SDS-PAGE profile of the purification of citrate synthase from *G. sulfurreducens*. Lane 1, molecular markers (numbers represent molecular mass in kilodaltons); lane 2, cell extract; lane 3, Q Sepharose column; lane 4, MonoQ column; lane 5, affinity column.

with apparent molecular masses of 78 kDa and 68 kDa, respectively, whereas below 0.05 mg ml^{-1} both the recombinant and wild-type enzymes eluted as monomers. This phenomenon, that a partially dissociated homodimeric protein elutes at different apparent sizes, has been observed with other enzyme systems (32, 39), including porcine citrate synthase (31). The fact that this enzyme dissociated at low protein concentrations also explained low specific activities obtained from purified fractions and samples where small amounts of biomass were lysed for activity measurements.

Dimeric citrate synthase with significant methylcitrate synthase activity has been found in bacteria such as *E. coli* and *Rhodothermus marinus* (13, 31), and the fact that this enzyme was dimeric raised the possibility that it was a methylcitrate synthase. However, no methylcitrate synthase activity was detected with the *G. sulfurreducens* crude extract or the purified enzyme using propionyl-CoA as substrate.

None of the compounds evaluated (oxaloacetate, acetyl-coenzyme A, acetate, glycerol, NAD^+, NADH, ATP, ADP, AMP, 2-oxoglutarate) aided in maintaining the dimeric form of the enzyme. Increasing concentrations of KCl or NaCl were also not effective in promoting dimerization or improving activity of the *Geobacter* citrate synthase. This was surprising, as KCl is well known to stimulate citrate synthase activity of both prokaryotic and eukaryotic enzymes (12, 40), and it is thought that KCl is essential to keep the proper configuration of these enzymes regardless of their origin (36).

Citrate synthase had a broad pH optimum with peak activity at pH 8, which decreased by only 30% at pH 6 and 10. The optimal temperature for citrate synthase activity was 50°C, higher than the optimal growth temperature for *G. sulfurreducens* of 30 to 35°C. Several metabolites were tested for their ability to inhibit or stimulate citrate synthase activity. NADH strongly inhibits prokaryotic hexameric citrate synthases (35, 43) but had no effect on the activity of the *Geobacter* citrate synthase at physiologically relevant concentrations (1 to 5 mM). 2-Oxoglutarate, also a specific inhibitor of some hexameric citrate synthases (56), did not inhibit the *Geobacter* enzyme. ATP inhibits many dimeric citrate synthases (17, 40, 55),

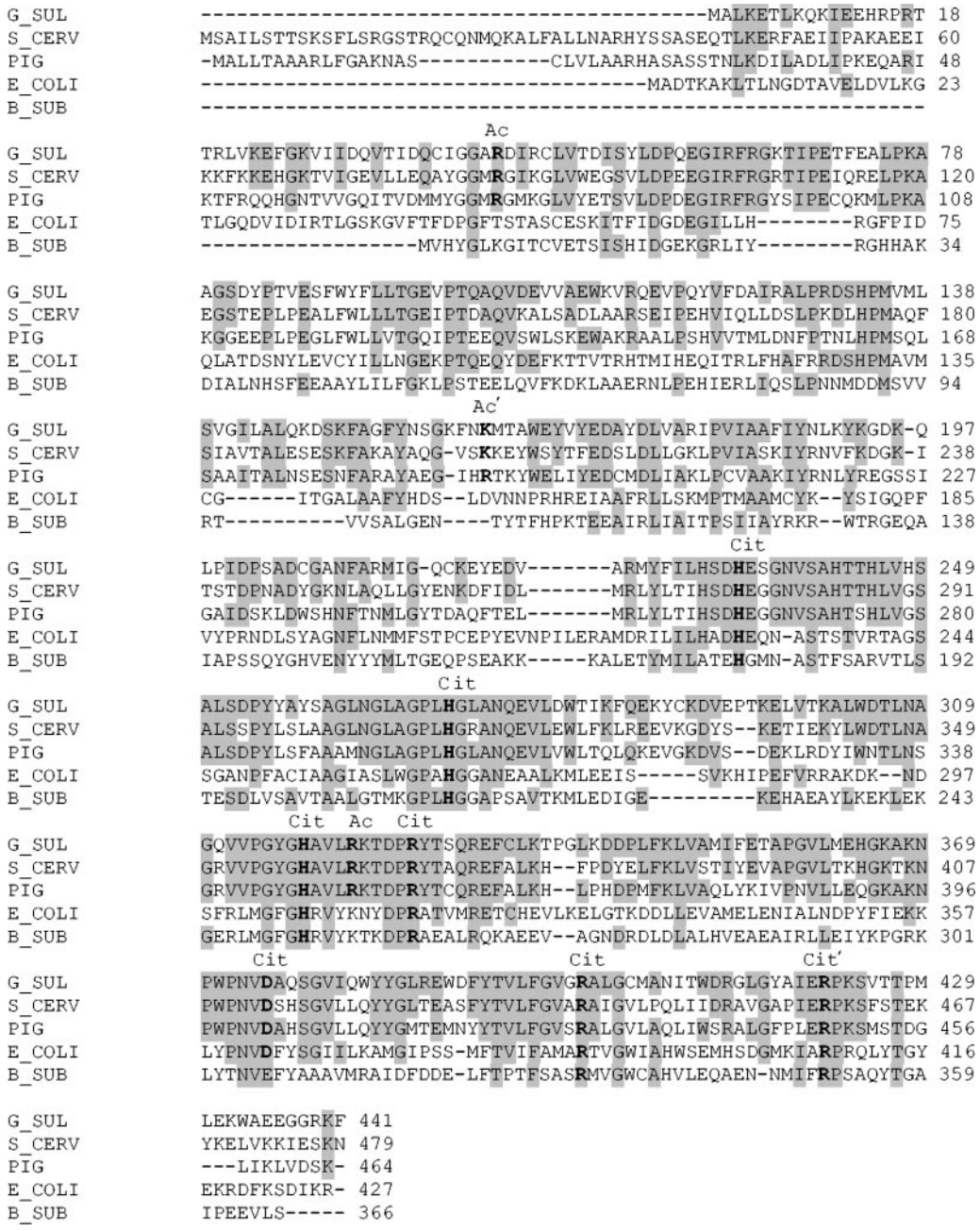

FIG. 2. Alignment of amino acid sequences with the citrate synthase purified from *G. sulfurreducens.* Residues implicated in citrate binding are marked "(Cit)". Residues implicated in acetyl-CoA binding are marked "(Ac)". A prime (′) denotes a residue contributed by a second monomer in the dimeric enzyme. Amino acids identical to those of *Geobacter* spp. are highlighted in green. Alignment was made by ClustalW. G_SUL, *Geobacter sulfurreducens*; S_CERV, *Saccharomyces cerevisiae* (gi:6324328); PIG, pig (gi:116470); E_COLI, *Escherichia coli* (gi:16128695); B_SUB, *Bacillus subtilis* (gi:729145).

and ATP exhibited a weak competitive inhibition of acetyl-CoA, presumably due to the similar adenosine-3-phosphate moiety with a K_i of 1.9 mM. Inhibition of oxaloacetate binding by high levels of ATP was noncompetitive. No other TCA cycle metabolites affected citrate synthase activity.

The steady-state kinetics (Table 3) of both the native and recombinant citrate synthases (30°C, pH 8, in the absence of KCl) were generally comparable to those of other dimeric and hexameric citrate synthases (15, 23, 40, 51). Levels of acetyl-

TABLE 3. Steady-state kinetics of the condensation of oxaloacetate and acetyl-coenzyme A

Substrate	Native enzyme			Recombinant enzyme		
	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1} s^{-1})	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1} s^{-1})
Acetyl-CoA	14.1	8.3	5.89×10^5	12.3	8.8	7.15×10^5
Oxaloacetate	4.3		1.93×10^6	8.1		1.09×10^6

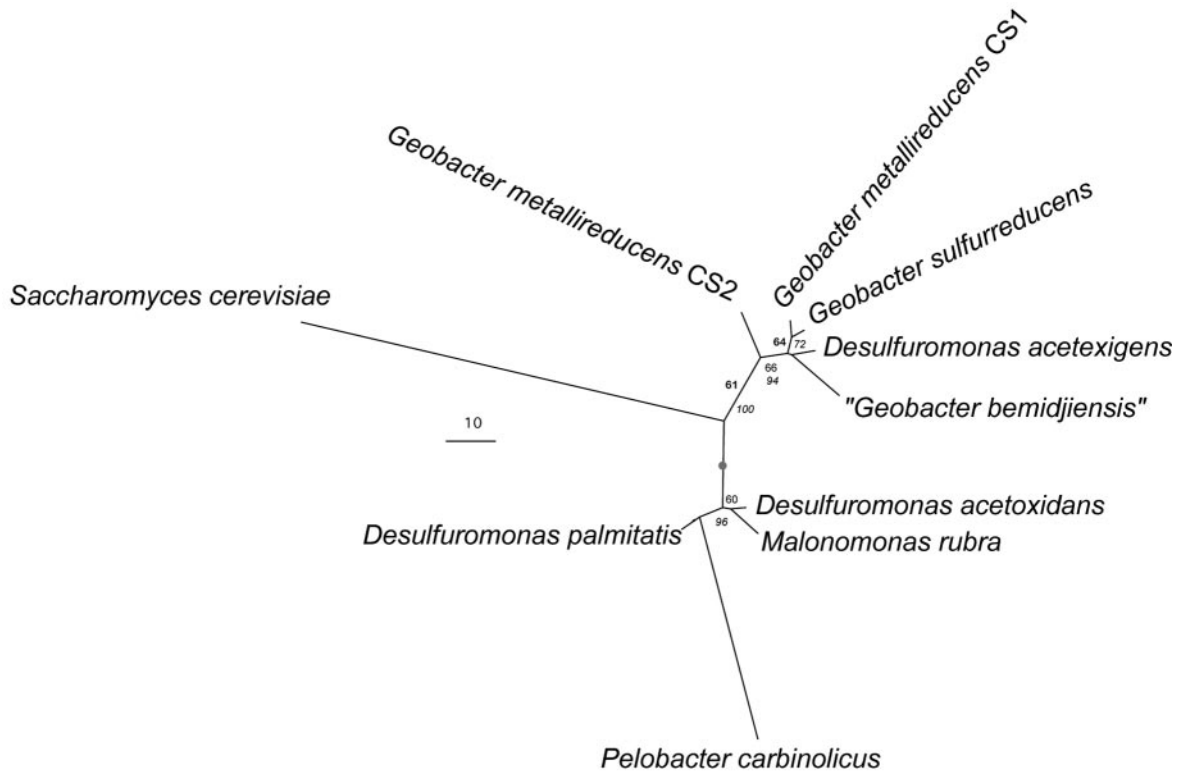

FIG. 3. Maximum likelihood tree of partial *Geobacteraceae* family citrate synthase sequences. The *Saccharomyces cerevisiae* sequence was included as a representative of eukaryotic citrate synthase sequences (see Fig. 1). The tree is based on a 258-amino-acid alignment. Values indicate number of times the node was recovered in 100 bootstrap replicates or PUZZLE support (italics). A grey circle indicates that both the bootstrap value and the PUZZLE support were above 70%.

CoA have been reported to range from about 50 to 500 μM in bacteria (9). Because of the unfavorable thermodynamics of the malate dehydrogenase reaction, the oxaloacetate/malate ratio in *Geobacter* spp. is predicted to be approximately 2×10^{-5}, which at an internal malate concentration on the order of 5 mM would predict an oxaloacetate concentration no higher than 0.1 μM (13, 34). Using these estimates to approximate magnitudes in *G. sulfurreducens*, citrate synthase activity should be more strongly influenced by oxaloacetate than acetyl-CoA availability.

Citrate synthases in other *Geobacteraceae* genomes. The *Geobacteraceae* family has consistently been shown to form a coherent phylogenetic cluster, separate from other *∂-proteobacteria*, based not only on analysis of 16S rDNA (24) but also on other conserved genes (22). Four draft genomes (*G. metallireducens*, *G. sulfurreducens*, *Desulfuromonas acetoxidans*, and *Pelobacter carbinolicus*) were analyzed for the presence of citrate synthase sequences. Putative sequences were resequenced to correct for possible errors in the draft sequences. All of the citrate synthase sequences detected had high similarity (BLAST e value, $\sim e^{-127}$) to eukaryotic citrate synthase sequences but only weak similarity to all known prokaryotic sequences (BLAST e value, $\sim e^{-27}$). No other putative citrate synthase sequences could be found in the genomes of these organisms.

Primers targeting conserved regions of *Geobacteraceae* citrate synthases were designed from the available sequences and then used to determine whether other branches of the *Geobacteraceae*, such as those originally isolated for their fermentative (*Malonomonas*), sulfur-reducing (*D. acetexigens*), or Fe(III)-reducing (*D. palmitatis*) abilities, also contained eukaryotic-like citrate synthases. Citrate synthase sequences were recovered from each organism. The phylogeny of the partial citrate synthase sequences within the *Geobacteraceae* family reflected previously derived topologies (Fig. 3), with the exception of *D. acetexigens*, which in all other analyses has been shown to cluster with *Desulfuromonas* isolates (19). *D. acetexigens* was originally isolated from a freshwater habitat (37), similar to where *Geobacter* species are typically found, raising the possibility that the citrate synthase of *D. acetexigens* reflects a transfer event associated with its adaptation to freshwater environments.

Visual inspection of aligned residues from eukaryotic and prokaryotic citrate synthase sequences also verified the relationship between nuclear-encoded eukaryotic mitochondrial citrate synthase sequences and the new set of *Geobacteraceae* sequences, based on insertions, deletions, and variable regions found only in eukaryotic and *Geobacteraceae* sequences. A search of recently available draft prokaryotic genomes, including those of closely related bacteria such as the aerobic *∂*-proteobacterium *Myxococcus xanthus* and the anaerobic sulfate-reducing *∂*-proteobacteria *Desulfotaelea psychrophila* and *Desulfobacterium autotrophicum*, failed to identify any prokary-

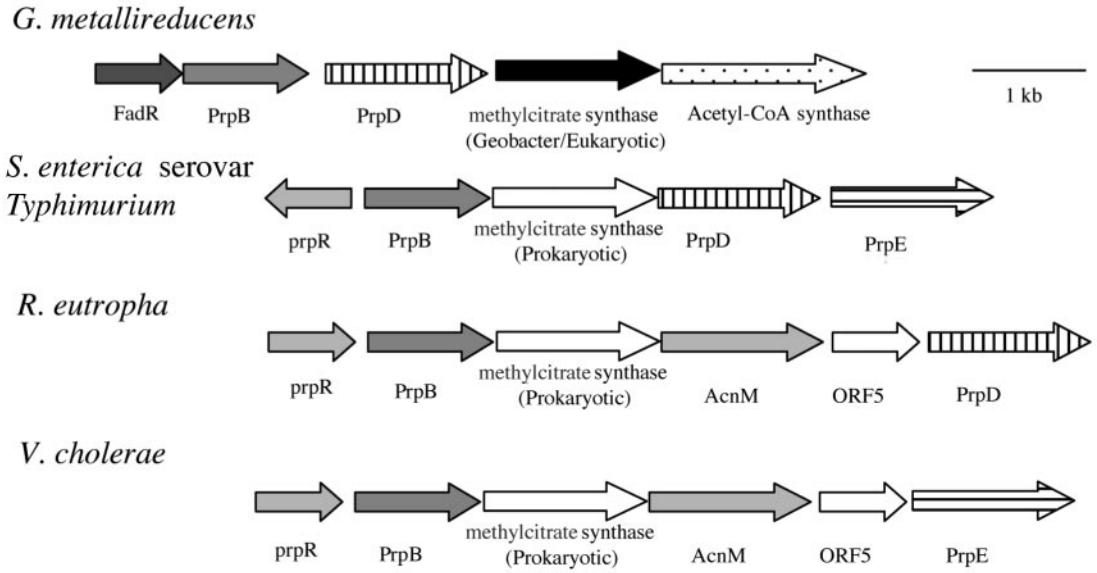

FIG. 4. Genome regions showing methylcitrate synthase pathway enzymes in selected organisms. Assignments in *G. metallireducens* genome are putative. While many ORFs in this region were highly similar to prokaryotic representatives (e.g., *prpB* and *prpD*), the likely methylcitrate synthase was most similar to the citrate synthase of *Geobacter* spp. and eukaryotes.

otic database entries which were similar to the *Geobacter* sequence.

Putative methylcitrate synthase in *G. metallireducens*. Duplicate copies of citrate synthase were identified in *G. metallireducens*, with deduced amino acid sequences 95 and 86% identical to that of *G. sulfurreducens* citrate synthase. The paralog with lesser identity was flanked by genes highly similar to genes found in propionate-utilizing proteobacteria which encode propionate dehydratase (*prpD*), methylisocitrate lyase (*prpB*), an acetyl-CoA synthase-like enzyme, and a DNA-binding protein related to FadR–acyl-CoA-binding proteins (Fig. 4). In organisms with *prpBCD*-like genes, propionate metabolism proceeds via a pathway in which propionyl-CoA and oxaloacetate are condensed to form methylcitrate. This suggests that the cluster in *G. metallireducens*, which is able to oxidize propionate, is associated with propionate metabolism. To date, only two methylcitrate synthase enzyme families have been described, one which appears to have evolved from dimeric prokaryotic citrate synthases and the other which likely evolved from the eukaryotic form (5, 15). However, the citrate synthase-like enzyme found in the center of this *G. metallireducens* operon was most like the citrate synthases found in other *Geobacteraceae* spp. (>90% similarity) and was unlike methylcitrate synthases described in eukaryotes (~60% similarity) or prokaryotes (~25% similarity).

When the acetyl-CoA-dependent citrate synthase and propionyl-CoA-dependent methylcitrate synthase activity was monitored in cell extracts of *G. metallireducens* grown with acetate or propionate as the electron donor, citrate synthase activity was present (3.5 U/mg protein) irrespective of the type of electron donor, while methylcitrate synthase activity increased 10-fold (0.03 to 0.32 U/mg protein) when this organism was cultured with propionate. These results suggested that variants of the *Geobacter* citrate synthase have recently evolved with an altered substrate preference to act in concert with known bacterial methylcitrate pathway enzymes.

Ecological implications. The predominance of members of the *Geobacteraceae* in a diversity of environments in which organic matter is oxidized with the reduction of metals (1, 41, 42, 46) or electrodes (3, 18, 52) has been attributed, at least in part, to their ability to oxidize acetate, the key extracellular intermediate in the anaerobic degradation of organic matter (27). Other studied dissimilatory metal-reducing microorganisms, such as *Shewanella* and *Desulfovibrio* species, lack the ability to oxidize acetate and thus play a negligible role in metal reduction in many environments (25). Most organisms closely related to the *Geobacteraceae*, including many sulfate-reducing bacteria, cannot completely oxidize organic donors, and many do not even possess a complete citric acid cycle. For example, the recently sequenced ∂-proteobacterium *Desulfovibrio desulfuricans* appears to lack citrate synthase in its genome. This suggests that citrate synthase was an important requirement in evolution of *Geobacteraceae* to couple the oxidation of acetate with the reduction of Fe(III).

The citric acid cycle is known to be a dynamic pathway, containing numerous examples of nonhomologous gene displacement and refinement (20), but a biochemical reason for *Geobacteraceae* utilizing a eukaryotic form of citrate synthase rather than the common prokaryotic form is not obvious from studies with the purified enzyme. Most mechanisms described for the regulation and functioning of a complete citric acid cycle are derived from studies of aerobic or facultative aerobic organisms. Thus, investigation of this new form of citrate synthase is warranted to further evaluate how this enzyme contributes to metabolism, and possibly competitiveness, of *Geobacteraceae* members.

This described citrate synthase has characteristics that may make it useful for assessing the activity of *Geobacteraceae* in

sedimentary environments with molecular techniques. The gene sequence is conserved within the *Geobacteraceae* but is unlikely to be found in other prokaryotes living in the same environments. Further, given the key role of citrate synthase in central metabolism, measurements of expression of the *Geobacteraceae* citrate synthase could provide an indication of the metabolic state of *Geobacteraceae* in environments of interest. Recent studies have demonstrated that it is possible to quantitatively assess levels of mRNA for key *Geobacteraceae* genes in subsurface sediments and on the surface of electrodes, and that levels of mRNA transcripts for key respiratory genes in *Geobacteraceae* may be related to rates of metal reduction (8). However, environmental factors other than the rate of metal reduction may influence the expression of respiratory genes (8), whereas it might be expected that expression of citrate synthase would be more closely related to rates of central metabolism. Investigations into this possibility are under way.

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