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Phylogenetic Analysis of Dissimilatory Fe(III)-Reducing Bacteria

DEBRA J. LONERGAN,[1,2] HARRY L. JENTER,[3] JOHN D. COATES,[3] ELIZABETH J. P. PHILLIPS,[3] THOMAS M. SCHMIDT,[4] AND DEREK R. LOVLEY[1]*

Department of Microbiology, University of Massachusetts, Amherst, Massachusetts 01003[1]; Department of Marine and Estuarine Environmental Sciences, University of Maryland, College Park, Maryland 20742[2]; Water Resources Division, U.S. Geological Survey, Reston, Virginia 22092[3]; and Department of Microbiology, Michigan State University, East Lansing, Michigan 48824[4]

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Evolutionary relationships among strictly anaerobic dissimilatory Fe(III)-reducing bacteria obtained from a diversity of sedimentary environments were examined by phylogenetic analysis of 16S rRNA gene sequences. Members of the genera *Geobacter*, *Desulfuromonas*, *Pelobacter*, and *Desulfuromusa* formed a monophyletic group within the delta subdivision of the class *Proteobacteria*. On the basis of their common ancestry and the shared ability to reduce Fe(III) and/or S^0, we propose that this group be considered a single family, *Geobacteraceae*. Bootstrap analysis, characteristic nucleotides, and higher-order secondary structures support the division of *Geobacteraceae* into two subgroups, designated the *Geobacter* and *Desulfuromonas* clusters. The genus *Desulfuromusa* and *Pelobacter acidigallici* make up a distinct branch within the *Desulfuromonas* cluster. Several members of the family *Geobacteraceae*, none of which reduce sulfate, were found to contain the target sequences of probes that have been previously used to define the distribution of sulfate-reducing bacteria and sulfate-reducing bacterium-like microorganisms. The recent isolations of Fe(III)-reducing microorganisms distributed throughout the domain *Bacteria* suggest that development of 16S rRNA probes that would specifically target all Fe(III) reducers may not be feasible. However, all of the evidence suggests that if a 16S rRNA sequence falls within the family *Geobacteraceae*, then the organism has the capacity for Fe(III) reduction. The suggestion, based on geological evidence, that Fe(III) reduction was the first globally significant process for oxidizing organic matter back to carbon dioxide is consistent with the finding that acetate-oxidizing Fe(III) reducers are phylogenetically diverse.

Dissimilatory Fe(III) reduction is being increasingly recognized as an environmentally significant process in both pristine and contaminated sedimentary environments (34). On the basis of studies with pure cultures and natural communities in sediments in which Fe(III) reduction was the predominant terminal electron-accepting process, it has been proposed that most of the microbially catalyzed Fe(III) reduction in anaerobic sedimentary environments is due to microorganisms which can completely oxidize acetate and other short-chain fatty acids to carbon dioxide, with Fe(III) serving as the sole electron acceptor (36, 37, 43).

Geobacter metallireducens was the first described acetate-oxidizing Fe(III) reducer (43). 16S rRNA-based phylogeny placed this organism in the delta subdivision of the class *Proteobacteria* (41). Of known organisms, *G. metallireducens* was most closely related to *Desulfuromonas acetoxidans*, a marine bacterium, which was isolated because of its ability to couple the oxidation of acetate to the reduction of S^0 (57). *D. acetoxidans* was subsequently found also to grow on acetate, with Fe(III) serving as the sole electron acceptor (59).

Since then, a number of other organisms that are closely related to *G. metallireducens* and *D. acetoxidans* have either been isolated from sediments as Fe(III) reducers or identified as Fe(III) reducers by screening of culture collections. Newly isolated organisms capable of conserving energy to support growth from acetate oxidation coupled to Fe(III) reduction include *G. sulfurreducens*, which was isolated from the submerged soil of a drainage ditch (11), and *D. palmitatis*, which was isolated from the marine sediments of San Diego Bay (14). *D. acetexigens* (26) and *Desulfuromusa kysingii* (33), which were isolated as acetate-oxidizing S^0 reducers, were also found to reduce Fe(III) (14, 25). *Pelobacter carbinolicus*, which is also closely related to *D. acetoxidans* (33, 64), does not grow as an acetate-oxidizing Fe(III) reducer but can grow with H_2 or ethanol as the electron donor and Fe(III) as the sole electron acceptor (45). A number of sulfate reducers which are also in the delta subdivision of the *Proteobacteria* (19) were found to reduce Fe(III) (16, 46). However, none of the sulfate reducers were found to be able to grow with Fe(III) as the sole electron acceptor.

In addition to the strict anaerobic, Fe(III)-reducing microorganisms in the delta subdivision of the *Proteobacteria*, several H_2-oxidizing isolates in the gamma subdivision of the class *Proteobacteria* have been described. These are *Shewanella putrefaciens* (44, 51), *S. alga* (formerly strain BrY) (10, 60), and *Pseudomonas* sp. strain Z-731 (6, 7). These organisms have the capacity to use a wide variety of electron acceptors, including oxygen. They can conserve energy to support growth from Fe(III) reduction, but their ability to use organic electron donors is extremely limited (39, 44, 53). Multicarbon electron donors such as lactate and pyruvate are only incompletely oxidized to acetate. Another facultative organism, "*Geospirillum barnesii*" (formerly strain SES-3), was recently found also to grow by oxidation of H_2 or incomplete oxidation of lactate to acetate, with Fe(III) serving as the electron acceptor (31). However, the phylogenetic placement of that organism was not reported.

Although these pure culture models for dissimilatory Fe(III) reduction are available, the microorganisms actually responsible for Fe(III) reduction in sedimentary environments have

* Corresponding author. Mailing address: Department of Microbiology, University of Massachusetts, Amherst, MA 01003. Phone: (413) 545-9651. Fax: (413) 545-1578. Electronic mail address: dlovley@microbio.umass.edu.

not been determined. In fact, many of the known Fe(III) reducers have been found by screening of culture collections. Phylogenetic analysis of 16S rRNA sequences in environments and the use of probes targeted to specific regions of the 16S rRNA can be powerful tools for defining the structure of microbial communities (3, 17, 22, 29, 54, 56, 65, 69). With these techniques, many of the biases and much of the labor involved in the isolation and characterization of environmental isolates can be avoided. However, for the rRNA approach to be useful it is necessary to have a clear understanding of how conserved the physiological trait of interest is among closely related organisms.

To evaluate the potential of applying the rRNA approach to the study of dissimilatory Fe(III) reduction, the 16S rRNA sequences of strict anaerobic Fe(III) reducers were evaluated in detail. The results suggest that when all known organisms are considered, 16S rRNA sequence data show potential to determine if an organism has the capacity for dissimilatory Fe(III) reduction. However, unless the sequence is an exact match with a previously described Fe(III) reducer, it is not possible to predict reliably the electron donors that an organism with a putative Fe(III) reducer sequence is capable of utilizing. The analysis also indicates that some 16S rRNA probes previously designed to study the distribution of dissimilatory sulfate-reducing bacteria (SRB) and SRB-like microorganisms target Fe(III)-reducing microorganisms that do not reduce sulfate.

MATERIALS AND METHODS

Organisms and culturing techniques. Cultures of *P. acetylenicus* (DSM 3246), *P. acidigallici* (DSM 2377), *P. propionicus* (DSM 2379), and *P. venetianus* (DSM 2394) were purchased from the German Collection of Microorganisms (DSM), Braunschweig, Germany. Cultures of *D. bakii*, *D. kysingii*, and *D. succinoxidans* were kindly provided by K. Finster. "*G. barnesii*" was kindly provided by R. Oremland. *G. metallireducens*, "*Geothrix fermentans*," "*G. hydrogenophilus*," and "*G. chapelleii*" were from our culture collection.

Strict anaerobic culturing techniques were employed throughout as previously described (43). *D. bakii*, *D. kysingii*, and *D. succinoxidans* were grown in medium as previously described (25), with Fe(III)-nitrilotriacetic acid (NTA) (20 mM) (59) as the electron acceptor and acetate (10 mM) as the electron donor.

P. venetianus was grown either in the fermentative medium recommended by the DSM or in previously described Fe(III)-NTA medium (45) with ethanol (10 mM) as the electron donor. *P. propionicus* was grown in medium recommended by the DSM with Fe(III)-NTA (10 mM) and lactate (10 mM) as the electron donor. *P. acetylenicus* was grown with either ethanol (10 mM) or H_2 as the electron donor and Fe(III)-NTA (10 mM) in the fermentative medium recommended by the DSM. *P. acidigallici* was grown in the fermentative medium recommended by the DSM with gallic acid as the substrate. Where noted, S^0 was added to fermentative medium in the form of colloidal sulfur (ca. 3 mM) (8). The HCl-extractable Fe(II) concentration was determined as previously described (43).

16S rRNA gene (rDNA) sequencing. Nucleic acids were isolated from frozen cell pellets of "*G. chapelleii*," "*G. hydrogenophilus*," "*G. fermentans*," "*G. barnesii*," and *G. metallireducens* as previously described (5) and treated with RNase. Nearly (greater than 95%) complete 16S rDNAs were amplified from the DNAs and from a 1:10 dilution of a liquid culture of *P. venetianus* by using eubacterial primer 8F (5′-AGAGTTTGATCCTGGCTCAG-3′) and universal primer 1492R (5′-GGTTACCTTGTTACGACTT-3′) (22, 70). The partial 16S rDNA products were purified with a Wizard PCR Prep System (Promega Corp., Madison, Wis.) and resuspended in sterile water. Both strands of the purified PCR products were sequenced by automated dye dideoxy terminator sequencing at the Michigan State University Sequencing Facility by using a 373A DNA sequencing system (Applied Biosystems, Foster City, Calif.). Oligonucleotides complementary to conserved regions of the eubacterial 16S rRNA were chosen to prime the sequencing reactions.

Phylogenetic analysis. Sequence alignments were either performed manually or obtained from the Ribosomal Database Project (49). Phylogenetic trees were inferred from aligned sequence data by using the maximum-likelihood method (24, 55), the neighbor-joining method (62), and the distance matrix method with the least-squares algorithm (18). Evolutionary distances for the distance trees were computed by the method of Jukes and Cantor (30).

Nucleotide sequence accession numbers. The GenBank and EMBL accession numbers for the sequences used in the phylogenetic analyses are as follows: *A. disciformis*, M94374; *B. bacteriovorus*, M59297; *C. pasteurianum*, M29390; *D. propionicus*, M34410; *D. autotrophicum*, M34409; *D. multivorans*, M34405; *D. tiedjei*, M26635; *D. bakii*, X79412; *D. kysingii*, X79414; *D. succinoxidans*, X79415; *D. curvatus*, M34413; *D. hydrogenophilus*, M34412; *D. postgatei*, M26630; *D. acetexigens*, U23140; *D. acetoxidans*, M26634; *D. palmitatis*, U28172; *Desulfuromonas* sp. strain 2, M80617; *D. pigra*, M34404; *D. variabilis*, M26632; *D. baarsii*, M34403; *D. desulfuricans*, M34113; *D. gigas*, M34400; *D. salexigens*, M34401; *D. vulgaris*, M34399; *G. sulfurreducens*, U13928; *H. foetida*, X77215; *M. xanthus*, M34114; *P. acetylenicus*, X70955; *P. acidigallici*, X77216; *P. carbinolicus*, U23141; *P. propionicus*, X70954; *S. putrefaciens*, X81623; *S. alga*, X81622; *W. succinogenes*, M88159. The 16S rRNA sequence of *Escherichia coli* was obtained from the Ribosomal Database Project (49).

The 16S rDNA sequences of "*G. chapelleii*," "*G. hydrogenophilus*," *P. venetianus*, "*G. barnesii*," and "*G. fermentans*" have been submitted to GenBank (accession numbers U41561, U28173, U41562, U41564, and U41563, respectively). Suggested revisions to the sequence of *G. metallireducens* (accession number L07834) have also been submitted to GenBank.

RESULTS AND DISCUSSION

Phylogeny of "*G. chapelleii*," "*G. hydrogenophilus*," and *P. venetianus*. Phylogenetic analysis of the nearly complete 16S rDNA sequences of "*G. chapelleii*," "*G. hydrogenophilus*," and *P. venetianus* placed these organisms in the delta subdivision of the *Proteobacteria*. Detailed inspection of the sequences indicated the presence of secondary structures and signature nucleotides characteristic of the delta subdivision of the *Proteobacteria* (71).

"*G. chapelleii*" was isolated from an acetate-oxidizing, Fe(III)-reducing enrichment obtained from deep subsurface sediments in South Carolina (40). The closest known relatives of "*G. chapelleii*" are *P. propionicus* (93.9% sequence identity; 1,387 nucleotides considered) (Fig. 1) and the molecular isolate *Desulfuromonas* sp. strain 2 (94.6% sequence identity; 540 nucleotides considered) (4). "*G. chapelleii*" shares several characteristics with previously isolated *Geobacter* species (11, 15, 41, 43). "*G. chapelleii*" was able to gain energy to support growth from the oxidation of acetate coupled to the reduction of Fe(III) (15). "*G. chapelleii*" was also able to reduce sulfur, but it was not determined whether this metabolism can support growth. The phylogenetic analysis and physiological characterization of "*G. chapelleii*" indicated that it should be placed in the genus *Geobacter.*

"*G. hydrogenophilus*" was enriched from the sediments of a hydrocarbon-contaminated aquifer in South Carolina (15). The closest known relative of "*G. hydrogenophilus*" is *G. metallireducens* (99.0% sequence identity; 1,385 nucleotides considered). "*G. hydrogenophilus*" was able to gain energy to support growth from the oxidation of acetate with Fe(III) as the sole electron acceptor (15), as were *G. metallireducens* (43), *G. sulfurreducens* (11), and "*G. chapelleii*" (15). S^0 is reduced by "*G. hydrogenophilus*," but as with *G. metallireducens*, S^0 reduction does not provide energy to support growth. Oxidation of H_2 coupled to reduction of Fe(III) provides energy to support the growth of "*G. hydrogenophilus*" (15) and *G. sulfurreducens* (11). On the basis of the results of a phylogenetic analysis and physiological characterization, "*G. hydrogenophilus*" has been placed in the genus *Geobacter.*

Previous studies (64) based on similarity coefficients for partial 16S rRNA sequences placed *P. venetianus* within the delta subdivision of the *Proteobacteria*, close to *P. carbinolicus* and *P. acetylenicus*. The phylogenetic analysis presented here, based on the nearly complete 16S rDNA sequence of *P. venetianus*, supports the original phylogenetic placement of *P. venetianus* within the delta subdivision of the *Proteobacteria*, with 97.8% sequence identity to *P. acetylenicus* (1,367 nucleotides considered) and 96.8% sequence identity to *P. carbinolicus* (1,363 nucleotides considered).

Phylogeny of acetate-oxidizing Fe(III) reducers. Previous

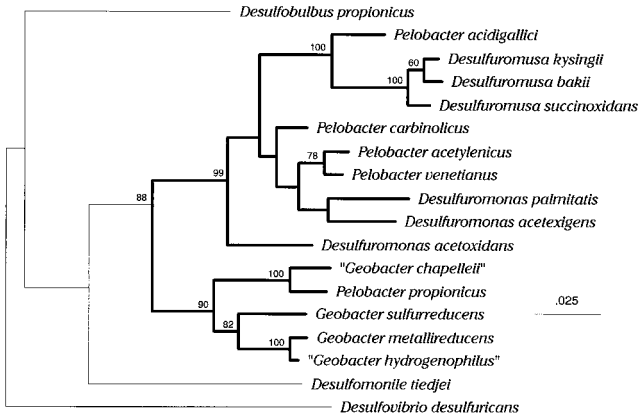

FIG. 1. Phylogenetic tree of the family *Geobacteraceae* (bold lines) inferred from 16S rRNA sequences by the maximum-likelihood method (55). Bootstrap values above 50% from 100 bootstrap analyses are given at branch nodes; 1,232 positions were considered. The bar on the right represents a 0.025% sequence difference.

studies placed *G. metallireducens*, *G. sulfurreducens*, *D. acetoxidans*, *D. palmitatis*, and *D. acetexigens* within the delta subdivision of the *Proteobacteria* (11, 14, 15, 27, 41). The phylogenetic relationships inferred from 16S rRNA sequences by the maximum-likelihood, neighbor-joining, and distance methods were consistent with the previous studies and indicated that "*G. chapelleii*," "*G. hydrogenophilus*," and the previously known acetate-oxidizing Fe(III) reducers evolved from a common origin within the delta subdivision (Fig. 1), close to the sulfate reducers. The genera *Geobacter* and *Desulfuromonas* are phylogenetically intermixed with members of the genus *Pelobacter*, as had been indicated by earlier phylogenetic analyses (23, 64). This assemblage of bacteria appears to have branched evolutionarily to form two closely related subgroups (Fig. 1). The genus *Geobacter*, *P. propionicus*, and the molecular isolate *Desulfuromonas* sp. strain 2 (Fig. 2) make up one branch and will be referred to here as the *Geobacter* cluster. The other branch contains the remainder of the genera *Desulfuromonas* and *Pelobacter* and the genus *Desulfuromusa* and will be referred to as the *Desulfuromonas* cluster. Detailed inspection of 16S rRNA sequences revealed the presence of nucleotides characteristic of either the *Geobacter* or the *Desulfuromonas* cluster at positions 122, 200, 217, 239, 286, 453, 454, 681, 690, 822, 859, 878, 888, 1117, 1122, 1151, 1168, 1254, and 1283 (*E. coli* numbering). Higher-order secondary structures starting at positions 1024 and 1443 (*E. coli* numbering) distinguishing the two clusters were also discovered (Fig. 3).

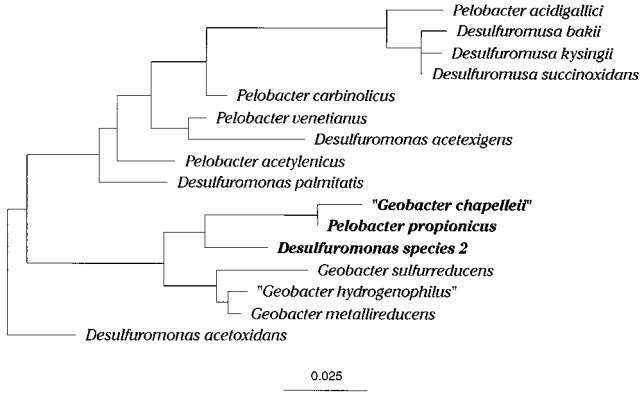

FIG. 2. Phylogenetic tree inferred from 16S rRNA sequences showing the placement of the molecular isolate *Desulfuromonas* sp. strain 2 within the *Geobacter* cluster. The maximum-likelihood method (55) was used to infer the phylogenetic tree from 545 positions. The bar represents a 0.025% sequence difference.

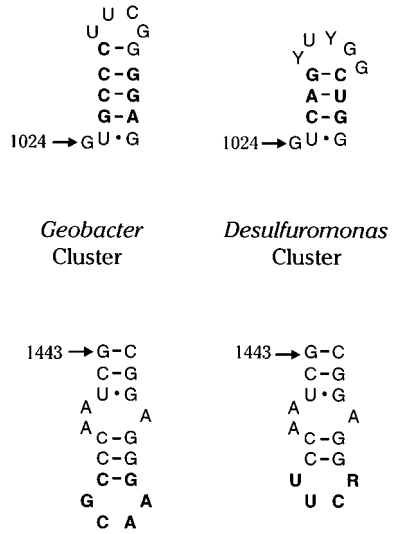

FIG. 3. Differences in higher-order structural detail between the *Geobacter* and *Desulfuromonas* clusters. Position numbers are based on the corresponding positions in the *E. coli* 16S rRNA.

The phylogenetic analyses and previous studies (33) indicated that the genus *Desulfuromusa* and *P. acidigallici* share the origin within the delta subdivision common to the *Geobacter* and *Desulfuromonas* clusters (Fig. 1). The genus *Desulfuromusa* and *P. acidigallici* appear to have evolved into a separate subgroup branching from the *Desulfuromonas* cluster. The genus *Desulfuromusa* and *P. acidigallici* contain nucleotides at positions 122, 286, 454, 681, 888, 1117, 1122, 1151, and 1168 (*E. coli* numbering) and secondary structures (Fig. 3) characteristic of the *Desulfuromonas* cluster. Bootstrap analysis and deletions present in the 16S rRNA sequences of the genus *Desulfuromusa* and *P. acidigallici* at positions 200 to 207, 212 to 217, 459 to 463, and 469 to 475 (*E. coli* numbering) not present in the *Desulfuromonas* cluster support the phylogenetic separation of the genus *Desulfuromusa* and *P. acidigallici* from the genus *Desulfuromonas*, *P. acetylenicus*, *P. carbinolicus*, and *P. venetianus* within the *Desulfuromonas* cluster. The three different methods of comparative 16S rRNA analysis used in this study consistently inferred the branching pattern that resulted in the apparent evolution of the *Geobacter* and *Desulfuromonas* clusters as described above.

Fe(III) and S^0 reduction by *Pelobacter* and *Desulfuromusa* species. Since the genus *Pelobacter* is phylogenetically intertwined with the Fe(III)- and/or S^0-reducing members of the genera *Geobacter*, *Desulfuromonas*, and *Desulfuromusa*, the possibility of Fe(III) and/or S^0 reduction was evaluated for *P. acidigallici*, *P. acetylenicus*, *P. propionicus*, and *P. venetianus.* *P. carbinolicus* had previously been shown to obtain energy for growth from the reduction of Fe(III) or S^0 with ethanol or H_2 serving as the electron donor (45). *P. venetianus* grew with Fe(III)-NTA as the electron acceptor and ethanol as the electron donor (Fig. 4) and also reduced Fe(III)-NTA with H_2 or formate. *P. propionicus* reduced Fe(III)-NTA in medium with lactate. *P. acetylenicus* reduced Fe(III)-NTA with ethanol or H_2 as the electron donor but was never successfully transferred

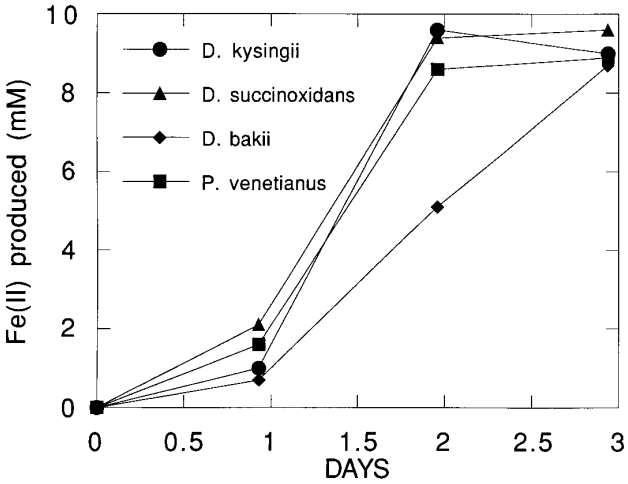

FIG. 4. Fe(II) production over time by *P. venetianus*, *D. bakii*, *D. kysingii*, and *D. succinoxidans* with either acetate (*Desulfuromusa* species) or ethanol (*P. venetianus*) as the electron donor and Fe(III) as the electron acceptor. The data from one representative culture of duplicate treatments are shown.

with Fe(III)-NTA as the electron acceptor. However, in our study its growth was not robust on any medium, suggesting that it has a special growth requirement not met under the current culturing conditions. Electron donors for *P. acidigallici*, such as gallic acid and phloroglucinol, chemically reduce Fe(III); therefore, Fe(III) reduction by *P. acidigallici* was not investigated. However, *P. acidigallici* was evaluated for S^0, which it reduced, as did *P. venetianus*, *P. propionicus*, and *P. acetylenicus* when it was added to their fermentative medium.

Because of their close phylogenetic relationship with the acetate-oxidizing Fe(III) reducers, members of the genus *Desulfuromusa* were tested for the ability to use Fe(III) as an electron acceptor. Of the three known *Desulfuromusa* species (25), only *D. kysingii* was found to reduce Fe(III)-citrate. However, *D. bakii*, and *D. succinoxidans*, and *D. kysingii* all could be grown in medium with acetate as the electron donor and Fe(III)-NTA as the electron acceptor with significant Fe(III) reduction over time (Fig. 4).

The family *Geobacteraceae*. The finding that all members of the genera *Geobacter*, *Desulfuromonas*, *Pelobacter*, and *Desulfuromusa* can use Fe(III) and/or S^0 as terminal electron acceptors links them physiologically, as well as phylogenetically, and suggests that their ancestor was a dissimilatory Fe(III)- and S^0-reducing bacterium. However, the phylogenetic differences between the genera are sufficiently great that they should not be reclassified into a single genus (20) but should be grouped into a single family, *Geobacteraceae*. The designation of this assemblage as a family would recognize the monophyletic origin and the unifying physiological characteristics of the genera *Geobacter*, *Desulfuromonas*, *Desulfuromusa*, and *Pelobacter.*

Members of the genus *Pelobacter* are distributed throughout the family *Geobacteraceae*, indicating the need for taxonomic revision. Until recently, the apparent lack of physiological similarities between *Pelobacter* species and the remainder of the *Geobacteraceae* hindered revision (23, 64). Subsequent studies with *P. carbinolicus* (45) and the results presented here provide the missing physiological similarities and further demonstrate the potential of 16S rRNA sequence analysis to predict physiology (45, 59). The results of the phylogenetic analysis suggest the need to reassign *P. acetylenicus*, *P. carbinolicus*, and *P. venetianus* to the genus *Desulfuromonas* and *P. propionicus* to the genus *Geobacter*. Further characterization of *P. acidigallici* and the *Desulfuromusa* species may indicate the need to reassign *P. acidigallici* to the genus *Desulfuromusa.*

The phylogeny of the family *Geobacteraceae* corresponds to the ability to reduce Fe(III) and/or S^0; however, the family *Geobacteraceae* encompasses a diversity of other metabolisms. The genera *Geobacter* (11, 15, 43), *Desulfuromonas* (14, 28), and *Desulfuromusa* (33) oxidize acetate with the reduction of Fe(III). "*G. hydrogenophilus*" (15), *G. sulfurreducens* (11), *P. carbinolicus* (45), *P. acetylenicus*, *P. propionicus*, and *P. venetianus* (38) oxidize H_2 with the reduction of Fe(III) and/or S^0. *G. metallireducens* oxidizes aromatic compounds (15, 42), *D. palmitatis* oxidizes long-chain fatty acids (14), and *Desulfuromusa* species utilize dicarboxylic and amino acids (33). *Pelobacter* species are capable of fermentative metabolism, as are some *Desulfuromusa* (25, 63) and *Desulfuromonas* (57) species. Thus, a molecular isolate whose 16S rRNA-based phylogeny places it among the *Geobacteraceae* is likely to be a dissimilatory Fe(III) and S^0 reducer, but little else can be determined about its metabolic potential. For example, the closest relatives of the molecular isolate *Desulfuromonas* sp. strain 2 are the dissimilatory Fe(III) reducers "*G. chapelleii*" and *P. propionicus*, suggesting that *Desulfuromonas* sp. strain 2 is a dissimilatory Fe(III) reducer. However, solely on the basis of the phylogenetic analysis, it is impossible to know whether *Desulfuromonas* sp. strain 2 is capable of oxidizing acetate, as is "*G. chapelleii*," or fermenting butanediol and lactate, as is *P. propionicus.*

Molecular isolates whose phylogeny places them within the family *Geobacteraceae* may tentatively be considered Fe(III) reducers. The closest known relative of the *Geobacteraceae* is the sulfate reducer *Desulfomonile tiedjei*, which does not reduce Fe(III) (46). On the basis of the ability of *Desulfobulbus propionicus*, another close relative of the *Geobacteraceae*, and several other sulfate reducers to reduce Fe(III) (16, 35, 46), had *D. tiedjei* been a molecular isolate, it would have been considered an Fe(III)-reducing sulfate reducer. Thus, if the phylogenetic analysis places the 16S rRNA sequence of a molecular isolate slightly outside of the family *Geobacteraceae*, it is pure speculation as to whether the organism is an Fe(III) reducer.

Evaluation of SRB rRNA probe target sites. As the closest known phylogenetic relatives of the *Geobacteraceae* are sulfate reducers (41), the 16S rRNA sequences of the *Geobacteraceae* were compared to the target sequences of SRB and SRB-like 16S rRNA probes (Fig. 5). SRB probe 385 was originally designed to hybridize specifically with most species of the delta *Proteobacteria* (1). It has since been determined that several

	SRB Probe 385	Desulfovibrio Probe 687	Population Type 1 Probe
Target	CCTGACGCAGCGACGCCG	AGGAGTGAAATCCGTA	CAGACTTGAATACGGGAGA
G.metall	CCTGACGCAGC**A**ACGCCG	AGGAGTGAAATCCGTA	**G**AGACTTGA**G**TACGGGAGA
G.hydrog	CCTGACGCAGC**A**ACGCCG	AGGAGTGAAATCCGTA	**G**AA**A**CTTGA**G**TACGGGAGA
G.sulfur	CCTGACGCAGC**A**ACGCCG	AGGAGTGAAATCCGTA	**G**AGACTTGA**G**TACGGGAGA
G.chapel	CCTGACGCASC**A**ACSCCG	AGGAGTGAAATCCGTA	CAGACTTGAATACGGGAGA
P.propio	CCTGACGCAGC**A**ACGCCG	AGGAGTGAAATCCGTA	CAGACTTGAATACGGGAGA
Dsm.sp.2	----------	AGGAGTGAAATCCGTA	CAGACTTGAATACGGGAGA
Dsm.acetox	CCTGACGCAGC**A**ACGCCG	AGG**G**GTGAAATCCGTA	CA**A**ACTTGA**G**TACGGGAGA
Dsm.acetex	CCTGACGCAGC**A**ACGCCG	AGG**G**GTGAAATCCGTA	CA**T**ACTTGA**G**TACGGGAGA
Dsm.palmit	CCTGACGCASNMACSCCG	AGG**G**GTGAAATCCGTA	C**C**G**G**CTTGA**G**TACGGGAGA
P.acetyl	CCTGACGCAGC**A**ACGCCG	AGG**G**GTGAAATCCGTA	CAGACTTGA**G**TACGGGAGA
P.carb	CCTGACGCAGC**A**ACGCCG	AGG**G**GTGAAATCCGTA	CAGACTTGA**G**TACGGGAGA
P.venet	CCTGACGCAGC**A**ACGCCG	AGG**G**GTGAAATCCGTA	CAGACTTGA**G**TACGGGAGA
P.acidig	CCTGACGCAGC**A**ACGCCG	AGGAGTGAAATCCGTA	CAG**T**CTTGA**G**TA**T**GGGAGA
Dmsa.bakii	CCTGACGCAGC**AA**TACCG	AGGAGTGAAATCCGTA	**TA**G**T**CTTGA**G**TA**T**GGGAGA
Dsma.kysin	CCTGACGCAGC**AA**TACCG	AGGAGTGAAATCCGTA	CAG**G**CTTGA**G**TA**T**GGGAGA
Dmsa.succi	CCTGACGCAGC**AA**TACCG	AGGAGTGAAATCCGTA	C-GACTTGA**G**TA**T**GGGAGA

FIG. 5. Comparison of SRB probe 385, *Desulfovibrio* probe 687, and population type 1 probe target sites with the aligned 16S rRNA sequences of the *Geobacteraceae*. Mismatches with the probe target sequences are in boldface.

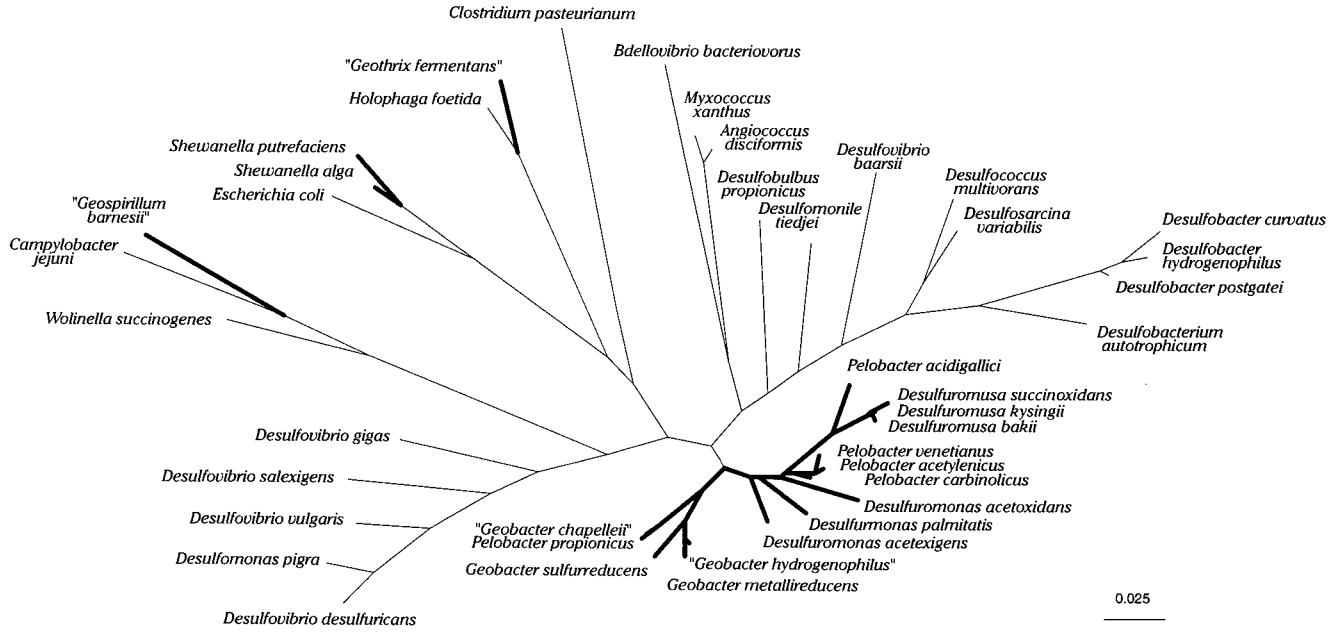

FIG. 6. Phylogenetic tree inferred from 16S rRNA sequences showing the lineages of Fe(III)-reducing bacteria (bold lines). The phylogenetic tree was inferred from 1,007 positions by using the maximum-likelihood method (55). The sequence of *Clostridium pasteurianum* was included as an outgroup. The bar represents a 0.025% sequence difference.

bacteria outside of the delta *Proteobacteria* share this target sequence (4). Originally, the target site of SRB probe 385 in the 16S rRNA sequence of *G. metallireducens* was believed to contain three mismatches and one deletion relative to the probe's actual target sequence. However, the unambiguously determined sequences of the remaining members of the genus *Geobacter*, as well as the sequences of the genera *Desulfuromonas* and *Pelobacter*, contained a single mismatch to the target site of SRB probe 385. The 16S rDNA of *G. metallireducens* was resequenced in this study and was also found to contain only one mismatch to the SRB probe target sequence. A single mismatch can provide specificity for in situ hybridization with rRNA-based probes (2).

The population type 1 SRB-like probe was designed from the 16S rRNA sequence of *Desulfuromonas* sp. strain 2, a molecular isolate obtained from a bioreactor by selective amplification of extracted DNA by using SRB probe 385 and universal primer 907 (4). The target sequence of the population type 1 probe was found in "*G. chapelleii*" and *P. propionicus*; thus, this probe hybridizes with 16S rRNAs from organisms that do not reduce sulfate. The probe had one mismatch to its target site in *P. acetylenicus*, *P. carbinolicus*, and *P. venetianus* and two or more mismatches to the target sites in the rest of the members of the genera *Desulfuromonas*, *Geobacter*, *Pelobacter*, and *Desulfuromusa.*

Another SRB probe is *Desulfovibrio* probe 687 (21). The 16S rRNA sequences of the *Geobacter* cluster, the genus *Desulfuromusa*, and *P. acidigallici* contain the target sequence of this probe. The 16S rRNAs of organisms in the *Desulfuromonas* cluster contain one mismatch to the target sequence of probe 687. These results demonstrate that investigators using this probe to track *Desulfovibrio* species should consider that organisms that do not reduce sulfate may also have been identified (58).

There are several reasons why the *Desulfovibrio* probe 687 target site in the *Geobacter* cluster, the genus *Desulfuromusa*, and *P. acidigallici* was not discovered when the SRB probes were designed in 1992. Previous phylogenetic studies had indicated that the genus *Pelobacter* is closely related to *D. acetoxidans* (23, 64), the only member of this family whose 16S rRNA had been sequenced when the probes were designed. The 16S rRNA sequence of *D. acetoxidans* does not contain the exact probe target site, and the 16S rRNA sequences of *P. acidigallici* and *P. propionicus* had not been obtained at that time. The first 16S rRNA sequence of a member of the family *Geobacteraceae* which contained the target site of *Desulfovibrio* probe 687, that of *G. metallireducens*, was not determined until 1993. The discovery of a probe target site in closely related but physiologically distinct nontarget bacteria indicates the need for caution in the design and application of 16S rRNA probes.

The use of 16S rRNA-directed probes for studying the distribution of dissimilatory Fe(III) reducers is further complicated by the fact that the ability of bacteria to use Fe(III) as a terminal electron acceptor is not limited to members of the delta subdivision of the *Proteobacteria*. The H_2-oxidizing dissimilatory Fe(III) reducers *Shewanella alga*, *Shewanella putrefaciens*, and *Pseudomonas* sp. strain Z-731, as well as the recently described species *Ferrimonas balearica* (61), are located within the gamma subdivision of *Proteobacteria* (6, 48, 60). The selenate-reducing microorganism "*Geospirillum barnesii*" (formerly strain SES-3) has been shown to obtain energy for growth from the reduction of Fe(III) with H_2 as the electron donor (31). Phylogenetic analysis of the nearly complete 16S rRNA sequence of "*G. barnesii*" placed it within the epsilon subdivision of the *Proteobacteria* (Fig. 6). "*Geothrix fermentens*" (13), an Fe(III)-reducing microorganism recently isolated from a petroleum-contaminated aquifer, is closely related to the gram-negative acetogen *Holophaga foetida* (32), which represents a novel line of descent in the domain *Bacteria* (Fig. 6). Another novel Fe(III) reducer, "*Geovibrio ferrireducens*" (12), isolated from a drainage ditch, is not closely related to any previously described bacteria. The phylogenetic placement of these new isolates suggests that the capacity for Fe(III) reduction is likely to be spread throughout the domain *Bacte-*

ria. This wide phylogenetic diversity of dissimilatory Fe(III) reduction indicates that so many different 16S rRNA-directed probes would be required just to cover the known Fe(III) reducers that it may not be feasible to use 16S rRNA probes to determine the structure of an Fe(III)-reducing microbial community.

Correspondence between phylogenetic and geological data. Geological studies of magnetite accumulations in the pre-Cambrian banded iron formations and other geochemical considerations have led to the suggestion that oxidation of organic matter coupled to the reduction of Fe(III) was the first globally significant biological process for completely oxidizing organic carbon back to carbon dioxide (67, 68). The discovery that *G. metallireducens* could completely oxidize multicarbon organic compounds to carbon dioxide with the reduction of Fe(III) to magnetite provided a microbial model for this process (47). Although the metabolism of organisms such as *S. putrefaciens* (44, 51), *S. alga* (10, 60), *F. balearica* (61), "*G. barnesii*" (31), and *Bacillus infernus* (9) is undoubtedly important in many environments, the capacity to oxidize multicarbon organic compounds (primarily acetate) completely to carbon dioxide is necessary to account for the oxidation of organic matter to carbon dioxide coupled to Fe(III) reduction in the banded iron formations (34).

The finding that the acetate-oxidizing Fe(III) reducers "*G. fermentens*" (this study) and "*G. ferrireducens*" (12) are not closely related to members of the family *Geobacteraceae*, or to each other, is important because previous findings had suggested that all acetate-oxidizing Fe(III) reducers are clustered in a tight phylogenetic group (11, 14, 59). However, if acetate oxidation coupled to Fe(III) reduction had been an early form of respiration, then it would be expected that this capacity would be widespread among the *Bacteria*. This is because there should have been strong selective pressure to retain this metabolism in other organisms in addition to those which evolved from the common *Geobacteraceae* ancestor because Fe(III) reduction continues to be an important process for organic matter oxidation in a wide variety of contemporary sedimentary environments (37).

The now apparent wide phylogenetic dispersion of acetate-oxidizing Fe(III) reducers does not prove that Fe(III) reduction was an early form of respiration, as a similar phylogenetic distribution might also be observed if this metabolism had independently evolved multiple times. However, the phylogenetic placement of the acetate-oxidizing Fe(III)-reducing bacteria is no longer inconsistent with their having an important role in the Archaean carbon cycle.

In summary, members of the *Geobacter*, *Desulfuromonas*, *Pelobacter*, and *Desulfuromusa* branch of the delta subdivision of the *Proteobacteria*, the *Geobacteraceae*, are united phylogenetically by a common origin and physiologically by the ability to use Fe(III) and/or S^0 as a terminal electron acceptor. The *Geobacteraceae* are the Fe(III)-reducing bacteria most readily isolated from a diversity of marine, estuarine, and freshwater sedimentary environments (15). However, the microorganisms actually responsible for Fe(III) reduction in sedimentary environments have not been determined. The existence of additional Fe(III) reducers in the gamma and epsilon subdivisions and the sulfate reducers of the delta subdivision of the *Proteobacteria*, as well as in novel lines of descent within the domain *Bacteria*, suggests that using 16S rRNA probes to determine the structure of microbial communities in Fe(III)-reducing environments may require an unwieldy battery of probes. Alternatively, 16S rRNA sequence analysis of molecular isolates obtained from Fe(III)-reducing environments by cloning (3, 56, 66, 69) or denaturing gradient gel electrophoresis (50, 52) has the potential to be diagnostic for the identification of dissimilatory Fe(III)-reducing bacteria, particularly if the phylogeny of Fe(III) reducers located outside of the delta subdivision correlates with physiology, as is the case for the family *Geobacteraceae.*

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