Comparison of 16S rRNA, *nifD*, *recA*, *gyrB*, *rpoB* and *fusA* genes within the family *Geobacteraceae* fam. nov.

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The sequences of five conserved genes, in addition to the 16S rRNA gene, were investigated in 30 members of the *Geobacteraceae* fam. nov. All members of the *Geobacteraceae* examined contained *nifD*, suggesting that they are capable of nitrogen fixation, which may explain their ability to compete effectively in nitrogen-poor subsurface environments undergoing remediation for petroleum or metal contamination. The phylogenies predicted from *rpoB*, *gyrB*, *fusA*, *recA* and *nifD* were generally in agreement with the phylogeny predicted from 16S rRNA gene sequences. Furthermore, phylogenetic analysis of concatemers constructed from all five protein-coding genes corresponded closely with the 16S rRNA gene-based phylogeny. This study demonstrated that the *Geobacteraceae* is a phylogenetically coherent family within the δ -subclass of the *Proteobacteria* that is composed of three distinct phylogenetic clusters: *Geobacter*, *Desulfuromonas* and *Desulfuromusa*. The sequence data provided here will make it possible to discriminate better between physiologically distinct members of the *Geobacteraceae*, such as *Pelobacter propionicus* and *Geobacter* species, in *geobacteraceae*-dominated microbial communities and greatly expands the potential to identify *geobacteraceae* sequences in libraries of environmental genomic DNA.

INTRODUCTION

Investigations into the mechanisms for dissimilatory Fe(III) reduction in subsurface environments may be greatly simplified by the fact that micro-organisms in the *Geobacteraceae* fam. nov. are the predominant Fe(III)-reducing micro-organisms in a diversity of subsurface environments in which Fe(III) reduction is important (Anderson *et al.*, 2003; Holmes *et al.*, 2002; Roling *et al.*, 2001; Snoeyenbos-West *et al.*, 2000; Stein *et al.*, 2001). Although a wide diversity of bacteria and archaea are capable of dissimilatory Fe(III) reduction (Lovley, 2000a, b), most of these organisms do not appear to be important in Fe(III) reduction in typical subsurface environments, which are at circumneutral pH, freshwater or marine salinities and temperatures of about 10–20 °C. This finding, coupled with the fact that it is possible to recover *geobacteraceae* in culture, provides the rare potential opportunity to apply knowledge gained from pure culture physiology studies to the subsurface. Furthermore, the finding that *geobacteraceae* can account for about 40–90 % of the total microbial community under

Fe(III)-reducing conditions in some subsurface environments (Anderson *et al.*, 2003; Holmes *et al.*, 2002) suggests that subsurface Fe(III)-reducing communities may provide the type of low-diversity community that is most amenable to environmental genomics studies. This is also true for the surface of electrodes harvesting energy from aquatic sediments, in which *geobacteraceae* typically comprise about half of the microbial community (Bond *et al.*, 2002; Holmes *et al.*, 2004; Tender *et al.*, 2002).

However, more information than is available from 16S rRNA gene sequence analyses is required to characterize these *geobacteraceae*-dominated communities fully. Although 16S rRNA gene phylogeny correlates with the physiology of most species of the *Geobacteraceae* (Loneragan *et al.*, 1996), several organisms in this family have metabolisms that are notably different. Most *geobacteraceae* have the ability to oxidize acetate and other organic compounds to carbon dioxide with Fe(III) serving as the electron acceptor (Lovley, 2000a). However, *Pelobacter* species, which are phylogenetically intertwined among these acetate-oxidizing *geobacteraceae*, are primarily hydrogen-oxidizing Fe(III) reducers and are not able to oxidize organic acids completely (Loneragan *et al.*, 1996; Lovley, 2000a). Other unique physiological traits displayed by *Pelobacter* species include the ability to grow via the fermentation of unusual substrates, such as polyethylene glycol (Schink & Stieb, 1983) and acetylene (Schink, 1985),

The GenBank/EMBL/DDBJ accession numbers for the sequences reported in this paper are given in Fig. 1.

Genetic and synonymous distances for the genes analysed within the genera *Desulfuromonas*, *Desulfuromusa*, *Geobacter* and *Pelobacter* are available as supplementary material in IJSEM Online.

and syntrophic growth with methanogens (Schink, 1984; Schink & Pfennig, 1982; Schink & Stieb, 1983), a property not observed in non-*Pelobacter* species of the *Geobacteraceae*.

In order to define better the phylogeny of the *Geobacteraceae*, an intensive phylogenetic analysis of 30 strains of the *Geobacteraceae* was conducted. The nucleotide and amino acid sequences of the following genes were compared: *rpoB*, which encodes the β subunit of RNA polymerase; *recA*, which encodes the DNA repair protein RecA; *gyrB*, the structural gene for the DNA gyrase β subunit; *fusA*, which encodes the protein synthesis elongation factor-G (EF-G); *nifD*, which encodes the α subunit of the dinitrogenase protein; and the 16S rRNA gene. The results demonstrate that the *Geobacteraceae* is a phylogenetically and physiologically distinct family within the δ -subclass of *Proteobacteria*. This information significantly expands the number of genes that can be used for analysing the distribution of *geobacteraceae* in sedimentary environments and for identifying environmental genomic DNA that can be assigned to the *Geobacteraceae*.

METHODS

Source of organisms and culture conditions. *Desulfuromonas acetoxidans* DSM 624^T, *Desulfuromonas palmitatis* DSM 12391^T, 'Geothermobacter ehrlichii' SS015 (=ATCC BAA-635), *Desulfuromusa kysingii* DSM 7343^T, 'Geobacter bemidjensis' BEM, *Geobacter grbiciae* DSM 13689^T, 'Geobacter humireducens' JW-3, *Geobacter metallireducens* DSM 7210^T, *Geobacter sulfurreducens* DSM 12127^T, *Malonomonas rubra* DSM 5091^T, *Pelobacter carbinolicus* DSM 2380^T and *Pelobacter propionicus* DSM 2379^T were obtained from our laboratory culture collection. 'Geopsychrobacter electrodophilus' A2, 'Geopsychrobacter multivorans' A1 and *Desulfuromusa* sp. strain S1 were isolated from the surface of a current-harvesting electrode in a marine fuel cell (Holmes *et al.*, 2004). *Geobacter hydrogenophilus* DSM 13691^T and *Geobacter chapellei* DSM 13688^T were obtained from the laboratory of John Coates. 'Desulfuromonas michiganensis' BB1 was obtained from the laboratory of Frank Löffler (Sung *et al.*, 2003). *Desulfuromonas acetexigens* ATCC 51529^T, *Desulfuromonas chloroethenica* ATCC 700295^T and *Pelobacter acidigallici* ATCC 49970^T were obtained from the ATCC. *Desulfuromonas thiophila* DSM 8987^T, *Desulfuromusa bakii* DSM 7345^T, *Desulfuromusa succinoxidans* DSM 8270^T, *Geobacter bremensis* DSM 12179^T, *Geobacter pelophilus* DSM 12255^T, *Pelobacter acetylenicus* DSM 3246^T, *Pelobacter massiliensis* DSM 6233^T and *Pelobacter venetianus* DSM 2394^T were obtained from the DSMZ. *Trichlorobacter thiogenes* K1^T was obtained from the laboratory of James Tiedje (De Wever *et al.*, 2000).

Standard anaerobic techniques were used throughout (Balch *et al.*, 1979; Nottingham & Hungate, 1969). All media was sterilized by autoclaving and all incubations were at 30 °C, unless otherwise noted. 'Geobacter bemidjensis' BEM, 'Geobacter humireducens' JW-3, *Geobacter hydrogenophilus*, *Geobacter metallireducens* and *Geobacter sulfurreducens* were grown in freshwater medium (Lovley & Phillips, 1988) with 50 mM Fe(III) citrate as electron acceptor and 20 mM acetate as electron donor, as were *Geobacter bremensis* and *Geobacter pelophilus* with 1 mM ascorbic acid as reductant. *Geobacter grbiciae* was grown in freshwater medium (Lovley & Phillips, 1988) with either 5 mM anthraquinone 2,6-disulfonate (AQDS) or 100 mM Fe(III) oxide as electron acceptor and 10 mM acetate as electron donor. *Geobacter chapellei* was grown in freshwater medium (Lovley &

Phillips, 1988) with 5 mM Fe(III) nitrilotriacetic acid as electron acceptor and 10 mM acetate as electron donor at 25 °C. *Desulfuromonas chloroethenica* was grown in ATCC medium 2063 with 40 mM fumarate as electron acceptor and 10 mM acetate as electron donor. *Desulfuromonas palmitatis* was grown in APW medium (Coates *et al.*, 1995) with 50 mM Fe(III) citrate as electron acceptor and 20 mM acetate as electron donor and 40 % of the recommended amount of MgCl₂·6H₂O and CaCl₂·2H₂O. *Desulfuromonas thiophila* was grown in DSM medium 647 with 20 g elemental sulfur l⁻¹ as electron acceptor and 5 mM pyruvate as electron donor. Medium 647 was steam sterilized at 100 °C for 1 h on three consecutive days. 'Desulfuromonas michiganensis' BB1 was grown in either DSM medium 298 or other anaerobic medium (Löffler *et al.*, 2000) with 40 mM fumarate as electron acceptor and 10 mM acetate as electron donor and 0.36 g NaS.9H₂O l⁻¹ as reductant at 25 °C. *Desulfuromonas acetoxidans*, *Desulfuromonas acetexigens*, *Desulfuromusa bakii*, *Desulfuromusa kysingii* and *Desulfuromusa succinoxidans* were grown in APW medium (Coates *et al.*, 1995) with 40 mM fumarate as electron acceptor, 20 mM acetate as electron donor and 40 % of the recommended amount of MgCl₂·6H₂O and CaCl₂·2H₂O and 0.36 g NaS.9H₂O l⁻¹ as reductant. 'Geothermobacter ehrlichii' SS015 was grown in marine medium (Grayson *et al.*, 1999) with 10 mM nitrate as electron acceptor and 10 mM malate as electron donor at 55 °C. *Desulfuromusa* sp. strain S1, 'Geopsychrobacter electrodophilus' A2 and 'Geopsychrobacter multivorans' A1 were grown at 20 °C in APW medium (Coates *et al.*, 1995) with Fe(III) oxide (60 mM) or colloidal S⁰ (10 mM) as electron acceptor and acetate (5 mM) as electron donor (Holmes *et al.*, 2004). *Malonomonas rubra* was grown in APW medium (Coates *et al.*, 1995) with 40 mM fumarate, 40 % of the recommended amount of MgCl₂·6H₂O and CaCl₂·2H₂O and 0.36 g NaS.9H₂O l⁻¹ as reductant. *Pelobacter acetylenicus* and *Pelobacter propionicus* were grown in DSM medium 298 with 1 g acetoin l⁻¹ and 0.9 g 2,3-butanediol l⁻¹, respectively. *Pelobacter acidigallici*, *Pelobacter carbinolicus*, *Pelobacter venetianus* and *Pelobacter massiliensis* were grown in DSM medium 293 with 0.68 g gallic acid l⁻¹, 0.9 g 2,3-butanediol l⁻¹, 1 g polyethylene glycol l⁻¹ and 0.5 g pyrogallol l⁻¹, respectively. *Trichlorobacter thiogenes* was grown in basal salts medium (De Wever *et al.*, 2000) with 10 mM fumarate as electron acceptor and 5 mM acetate as electron donor and 0.36 g NaS.9H₂O l⁻¹ as reductant at 25 °C for DNA sequence studies.

DNA extraction. Cells were collected by centrifugation and genomic DNA was extracted using the BIO 101 FastDNA Spin kit following the manufacturer's instructions. Cultures grown with poorly crystalline Fe(III) oxide as the electron acceptor were first treated with an equal volume of filter-sterilized oxalate solution (Phillips & Lovley, 1987) to remove Fe(III), which inhibits *Taq* polymerase.

Determination of *nifD*. In order to determine whether or not the various species of the *Geobacteraceae* carried the *nifD* gene, a genomic dot blot was carried out on DNA extracted from the 30 different strains. Genomic DNA was denatured at 90 °C for 10 min and 1–10 µg denatured DNA was hybridized to a Zeta-Probe GT charged membrane (Bio-Rad) according to the manufacturer's instructions. A digoxigenin-labelled probe was constructed using primers targeting the *nifD* gene of *Geobacter sulfurreducens* with the PCR DIG Probe Synthesis kit (Roche Diagnostics). The probe was denatured and allowed to hybridize to single-stranded genomic DNA immobilized on the membrane. Addition of NBT/BCIP alkaline phosphatase solution (Sigma) to the membrane allowed visualization of bound probe.

Amplification of targeted genes. The total volume of each PCR mixture was 100 µl. The amount of DNA template varied for each reaction, ranging from ~6 ng for more specific primers to ~25 ng for more degenerate primers. Every PCR contained 10 µl Qiagen 10 × buffer (15 mM MgCl₂), 8 µl of a 0.25 µM dNTP solution (Sigma), 60 pmol forward and reverse primers, 160 µg BSA ml⁻¹

(Sigma) and 3 U *Taq* polymerase (Qiagen). If Buffer Q (Qiagen) was added to the PCR mixture, the amounts ranged from 5 to 20 µl for each 100 µl reaction; additional MgCl₂ was also included at concentrations ranging from 1 to 4 mM (final reaction concentration). The following components (all from Sigma) were sometimes added to improve the PCR: 5 µl DMSO per 100 µl, 1–25–10% (w/v) formamide, 5 µl Ipegal with 5% (w/v) acetamide, 15–30 mM (NH₄)SO₄ (final reaction concentration) or 15–20% (w/v) glycerol. To ensure sterility, the PCR mixtures were exposed to UV radiation for 8 min prior to the addition of template and *Taq* polymerase. All PCR amplifications were performed in a DNA Engine thermal cycler (MJ Research, Inc.). PCR products were agarose gel-purified with the QIAquick gel extraction kit (Qiagen), ligated into the TOPO TA cloning vector and transformed into *Escherichia coli* cells with the TOPO TA cloning kit, version K2 (Invitrogen), according to the manufacturer's instructions. Plasmid inserts were then amplified with M13 forward and reverse primers (Invitrogen) and PCR products were prepared for sequencing using a QIAquick PCR purification kit (Qiagen).

Primers and PCR conditions used to amplify the 16S rRNA gene, *gyrB*, *fusA*, *nifD*, *recA* and *rpoB*. 16S rRNA gene sequences for '*Geopsychrobacter multivorans*' A1, '*Geopsychrobacter*

electrodophilus' A2, '*Geothermobacter erlichii*' SS015 and *Pelobacter massiliensis* were amplified with primers 8 forward (Eden *et al.*, 1991) and 1492 reverse (Amann *et al.*, 1990; Achenbach & Woese, 1995) using the following PCR conditions: an initial denaturation step at 94 °C for 5 min, followed by 35 cycles of 94 °C (45 s), 50 °C (1 min) and 72 °C (1 min), with a final extension at 72 °C for 10 min.

The initial degenerate primers targeting the five protein-coding genes *gyrB*, *fusA*, *nifD*, *recA* and *rpoB* were designed using protein sequences from various bacterial and archaeal species available in the GenBank database. An alignment of amino acid sequences for each protein was done in CLUSTAL X (Thompson *et al.*, 1997) and the Genetic Computer Group (GCG) sequence editor (Wisconsin Package, version 10). This alignment was then examined and conserved regions of the proteins were targeted for primer design. More specific primer sets were designed based on alignments constructed from sequences from members of the *Geobacteraceae* obtained further along in the study.

Gene fragments were amplified by either the primer pairs and PCR conditions outlined in Table 1 or the following primers and conditions: *fusA*, FUSAF/FUSAR (Berchet *et al.*, 2000); *gyrB*, UP1E/

Table 1. Universal and specific primers used to amplify *gyrB*, *nifD*, *fusA*, *rpoB* and *recA* gene fragments

Locations refer to the *Escherichia coli* numbering system.

Primer pair*	Primer sequences (5'–3')	Location
<i>fusA</i> primers		
EF-GF/EF-GR ^a	ACIATGGATTGGATGGADCA/TTCIACCTTCATCATIGGTTTC	360–1420
EF-G30F/EF-G510R ^b	GTGCAGAMTCTICTTGAT/TAATGCCAGCTTTTCCTGGTC	30–510
<i>gyrB</i> primers		
GYR48F/GYR1010R ^b	GACGATGATCCGGTGGTAGC/CGATGATACCATCTTCGAGAC	48–1010
GYR130F/GYR1130R ^b	ATCTCCTGGGAGGAGAGCATC/CTGGAGCTGGAGATTCGCCG	130–1130
GYRBF/GYRBR ^a	CATCATATGGTNTGGGAGATWGTNGATAAT/CATTTTGTGCAAIKIGCTTTTTCIACGTT	100–1180
GYR838F/GYR1541R ^b	GGAAGGTGTACCGCCAGT/GAGGGTGGAACCCACTTGG	838–1541
<i>nifD</i> primers		
NIFDF/NIFDR ^a	GATTTTCADGADAADGATATT/CCAIGGIATICCDTATTTTC	405–963
NIFDF2/NIFDR2 ^b	CAGGAAAIGATATTGTCTACGG/TAGTCGTCCTIGTGGGCGAACTCGTA	180–987
<i>recA</i> primers		
RECAF/RECAR ^a	TGTGCITTTATWGATGCIGAGCATGC/CCCATGTCICCTTCKATTTTCIGCTTT	21–618
REC258F/REC858R ^c	AAGACGGACGACCTGCTCG/TGAGGAAAAGGCGGGAATTT	258–858
RECA11F/RECA479R ^b	CATCTGGCAAGACTACCCTG/GGATGTCCATGCGCACGAAG	11–479
RECA11F/RECA560R ^b	CATCTGGCAAGACTACCCTG/TGCGACTTTGTTATTAACGACCTT	11–560
RECA50F/RECA563R ^b	AGCCTTGGAATTGCCGAG/TCCGCCGTAGGAGAACCAG	50–563
REC300F/REC630R ^b	TGTTTGTGTATGGCCGAGCA/GGTTGCCGAACATGACACCGAT	300–630
REC300F/REC910R ^b	TGTTTGTGTATGGCCGAGCA/CCCGCCGTAGGAGAAACCAGGC	300–910
<i>rpoB</i> primers		
RPOBF/RPOBR ^a	CAGTTHATGGATCAG/GTHTGNCDTTGCATGTT	117–642
RPOBF2/RPOBR2 ^b	ACTCGTGAGCGTGCTGGTTTT/ATCAAAGCCCGGTGAGCAT	9–636
RPO216F/RPO666R ^b	CAGGAACGGGATGAGCGAT/CTCCGAGGTTACCCACAAGC	216–666
RPO175F/RPO800R ^b	ATGTTGACCCCATGAGTGC/GAGCGGATGAGTCTCCAGGA	175–800
RPO310F/RPO940R ^b	GAGCGSATGAGTCTSCAGGA/CITGTCATGTTSGAGCCCAT	310–940

*Amplification parameters: a, 92–94 °C for 5 min, 20 cycles of 92–94 °C for 1 min, 41–55 °C for 1.5–3 min (–0.5 °C per cycle) and 70–71 °C for 1.5–3 min and then 20 cycles of 92–94 °C for 1 min, 38–57 °C for 1–1.5 min and 71–72 °C for 1.5 min, followed by 71 °C for 10 min; b, 94 °C for 5 min, 35 cycles of 94 °C for 45–60 s, 47–60 °C for 1–1.5 min and 72 °C for 1–1.5 min; followed by 72 °C for 10 min; c, 94 °C for 5 min, 10 cycles of 92 °C for 45 s, 55 °C for 1.5 min (–0.2 °C per cycle) and 72 °C for 1.5 min, 20 cycles of 94 °C for 45 s, 58 °C for 1 min and 72 °C for 1 min, followed by 72 °C for 10 min.

APRU (Yamamoto *et al.*, 2000); *nifD*, NIFD883F/NIFD1337R (Ueda *et al.*, 1995); *recA*, FGPR-476/FGPR-477 (Maréchal *et al.*, 2000); *rpoB*, BF/BR (Lee *et al.*, 2000).

Phylogenetic analysis of sequences. 16S rRNA, *gyrB*, *fusA*, *nifD*, *recA* and *rpoB* gene sequences were compared to the GenBank nucleotide and protein databases using BLASTN and BLASTX

algorithms (Altschul *et al.*, 1990). Nucleotide and amino acid sequences for each gene were initially aligned in CLUSTAL X (Thompson *et al.*, 1997) and imported into the GCG sequence editor, where alignments were checked and hypervariable regions were masked.

Similarity matrices for each gene were generated using CLUSTAL W and CLUSTAL X (Thompson *et al.*, 1994, 1997). Translation products were compared using the PAM120 amino acid substitution matrix (Dayhoff, 1978) and the PAM 1 matrix was used to determine similarity values for nucleotide sequences. Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 2.1 (Kumar *et al.*, 2001). Genetic distances were calculated by the Kimura two-parameter (Kimura, 1980) and Tamura–Nei models (Tamura & Nei, 1993). The method of Nei & Gojobori (1986) was applied to the various sequences to obtain synonymous distances (multiple substitutions adjusted by the Jukes–Cantor formula).

All distance matrices constructed with 16S rRNA gene sequences considered 1323 nucleotides. The *gyrB* matrices used 883 nucleotides and 265 amino acids; 590 nucleotides and 182 amino acids were used in the *fusA* matrices; 412 nucleotides and 170 amino acids were used in the *recA* matrices; 440 nucleotides and 150 amino acids were used in the *nifD* matrices; and 540 nucleotides and 176 amino acid sequences were considered in the *rpoB* matrices.

Construction of phylogenetic trees. Aligned sequences were imported into PAUP 4.0b 4a (Swofford, 1998), where phylogenetic trees were inferred. Distances and branching order were determined and compared using character-based (maximum-parsimony and maximum-likelihood) and distance-based algorithms [HKY85 (Hasegawa *et al.*, 1985) and Jukes–Cantor (Jukes & Cantor, 1969)]. *Chlorobium tepidum* TLS^T and *Nostoc* sp. PCC7120 were the outgroups used for phylogenetic comparisons of 16S rRNA, *gyrB*, *fusA*, *nifD*, *recA* and *rpoB* gene fragments, and bootstrap analyses were performed using 'Fast' stepwise-addition, with 1000 replicates. Nucleotide-based phylogenetic trees were generated by a heuristic search with maximum-likelihood settings, while amino acid-based trees were generated with maximum-parsimony settings. Phylogenetic trees constructed with amino acid and nucleotide sequences yielded similar results.

Preliminary sequence data from *Geobacter sulfurreducens*, *Geobacter metallireducens*, *Desulfuromonas acetoxidans* and *Pelobacter carbinolicus* were obtained from The Institute for Genomic Research (TIGR) website (<http://www.tigr.org>) and the DOE Joint Genome Institute (JGI) website (<http://www.jgi.doe.gov>).

RESULTS AND DISCUSSION

Comparison of 16S rRNA gene, *fusA*, *rpoB*, *recA*, *nifD* and *gyrB* phylogenetic tree topologies

There were slight differences between phylogenetic structures for the *Geobacteraceae* predicted by analysis of the 16S rRNA, *fusA*, *gyrB*, *nifD*, *recA* and *rpoB* genes. The topologies of phylogenetic trees constructed from 16S rRNA, *rpoB*, *gyrB*, *fusA* and *recA* genes were in close agreement. According

to analysis of these genes, the family *Geobacteraceae* can be further divided into three distinct monophyletic clusters: *Geobacter*, *Desulfuromonas* and *Desulfuromusa* (Fig. 1a–c, e, f). Phylogenies based on *nifD* suggested that the *Desulfuromonas* and *Desulfuromusa* species were phylogenetically intertwined, while *Geobacter* species formed a distinct cluster (Fig. 1d).

Phylogenetic comparisons of all of the genes indicated that *Pelobacter* species, with the exception of *Pelobacter propionicus*, are scattered throughout the *Desulfuromonas* and *Desulfuromusa* clusters. *Pelobacter propionicus* consistently fell within the *Geobacter* cluster when all six genes were examined. *Trichlorobacter thiogenes* also clustered with *Geobacter* species for all of the genes. 'Geopsychrobacter electrodophilus' A2 and 'Geopsychrobacter multivorans' A1 consistently branched with the *Desulfuromusa* clade; however, the phylogenetic placement of 'Geothermobacter ehrlichii' SS015 was more variable. 'Geothermobacter ehrlichii' SS015 clustered with *Desulfuromonas* species for the 16S rRNA gene and *fusA*, while it branched with *Desulfuromusa* species for *recA* and *rpoB* genes. The phylogenies predicted for *Malonomonas rubra* were most similar to *Desulfuromusa* species.

Phylogenetic structure of *fusA*, *gyrB*, *nifD*, *recA* and *rpoB* genes within the *Geobacteraceae*

The genetic distances between the *fusA*, *gyrB*, *nifD*, *recA*, *rpoB* and 16S rRNA gene sequences within the same genus were within 0.56 of the Kimura two-parameter (Kimura, 1980) distances and within 0.96 of the synonymous distances (Nei & Gojobori, 1986). According to both phylogenetic models, *fusA* and *rpoB* sequences from species within the genus *Geobacter* showed the greatest variation, whereas *nifD*, *recA* and *gyrB* gene sequences within the genus *Pelobacter* were most divergent. Overall, sequences from *Desulfuromusa* varied the least, and the *nifD* gene seemed to have the fewest nucleotide substitutions across all four genera. A table showing the genetic and synonymous distances for the 16S rRNA, *fusA*, *gyrB*, *nifD*, *recA* and *rpoB* genes within the genera *Desulfuromonas*, *Desulfuromusa*, *Geobacter* and *Pelobacter* is available as supplementary material in IJSEM Online.

In addition, there was a significant correlation between the genetic distances calculated by the model of Tamura & Nei (1993) for *gyrB*, *rpoB* and *fusA* gene fragments and the corresponding 16S rRNA genes within the *Geobacteraceae*; *r* values were 0.81, 0.74 and 0.75 ($P < 0.005$) for the *gyrB*, *rpoB* and *fusA* genes. The evolutionary distances calculated for the *nifD* and *recA* gene fragments did not correspond as closely with 16S rRNA gene distances; *r* values were 0.62 and

Fig. 1. Phylogenetic trees predicted by maximum-likelihood analysis comparing sequences of the 16S rRNA gene (a), *fusA* (b), *gyrB* (c), *nifD* (d), *recA* (e) and *rpoB* (f) from species of the *Geobacteraceae*. *Chlorobium tepidum* and *Nostoc* sp. PCC 7120 were used as outgroups and 1000 replicates were used for maximum-likelihood (first bootstrap value) and maximum-parsimony (second value) bootstrap analyses. Taxa are represented by type strains unless the strain name is indicated.

0.64 ($P < 0.005$) for the *nifD* and *recA* gene fragments. However, the distances from concatemers constructed with all five protein-coding genes were correlated with 16S rRNA gene distances ($r = 0.79$; $P < 0.005$) (Fig. 2).

Intragenomic comparisons within the *Geobacteraceae*

In order to determine the genetic variation and gene redundancy of 16S rRNA, *fusA*, *gyrB*, *nifD*, *recA* and *rpoB* genes within the *Geobacteraceae*, the genomes of *Geobacter sulfurreducens* and *Geobacter metallireducens* were examined. Preliminary analysis of the *Geobacter sulfurreducens* genome indicated that two copies of the 16S rRNA gene, three copies of the *fusA* gene and one copy each of the *recA*, *nifD*, *rpoB* and *gyrB* genes are present in *Geobacter sulfurreducens*. Two copies of the *fusA* gene and a single copy of each of the 16S rRNA, *gyrB*, *nifD*, *recA* and *rpoB* genes were detected in the genome of *Geobacter metallireducens*.

Further intragenomic comparisons of multiple copy genes within these genomes from *geobacteraceae* indicated that the *fusA* gene is relatively divergent within the *Geobacter sulfurreducens* and *Geobacter metallireducens* genomes. In the *Geobacter sulfurreducens* genome, the genetic distances between the three *fusA* genes were within 0.63 to 0.76 of the Kimura two-parameter distances, within 0.59 to 0.81 of the synonymous distances and the *p*-distance values (based on amino acid sequences) ranged from 0.52 to 0.64. The Kimura two-parameter distance for the two *fusA* genes in *Geobacter metallireducens* was 0.63, while the synonymous and *p*-distance values were 0.80 and 0.54, respectively.

While these protein-coding genes were relatively divergent within the *Geobacteraceae*, the 16S rRNA genes within the *Geobacter sulfurreducens* genome were highly conserved, and very few nucleotide differences between intraspecies 16S

rRNA genes were detected; the Kimura two-parameter distance values were 0.00.

The intragenomic heterogeneity observed between multiple copies of the *fusA* gene in the *Geobacter sulfurreducens* and *Geobacter metallireducens* genomes is likely to influence the phylogenies generated with this gene. Therefore, *fusA* should not be considered definitive in the taxonomic classification of the *Geobacteraceae*. Although the 16S rRNA gene is also present in multiple copies, this gene appears to have a low nucleotide substitution rate within the genome of *geobacteraceae*. Therefore, intragenomic heterogeneity should not have a significant impact on the phylogeny predicted by the 16S rRNA gene.

Taxonomic classification of the *Geobacteraceae*

The *Geobacteraceae* form a phylogenetic clade within the δ -subclass of the *Proteobacteria* that can be divided into three distinct subclades: *Desulfuromonas*, *Desulfuromusa* and *Geobacter*. For the most part, conserved genes within the *Desulfuromusa* subclade have the fewest nucleotide substitutions. A possible explanation for the low phylogenetic diversity within this genus is that *Desulfuromusa* species evolved most recently within the family *Geobacteraceae*. Evolutionary distances within the *Geobacter* and *Desulfuromonas* clusters, on the other hand, are relatively large. It is likely that these organisms evolved relatively early and share a common ancestor within the δ -subclass of the *Proteobacteria*.

Six genera officially comprise these three taxonomic subclades: *Geobacter*, *Desulfuromonas*, *Desulfuromusa*, *Pelobacter*, *Malonomonas* and *Trichlorobacter*. Analysis of all six genes and concatenated alignments indicates that *Pelobacter* species other than *Pelobacter propionicus* are not phylogenetically coherent and are interspersed among *Desulfuromonas* and *Desulfuromusa* species. However, unique physiological characteristics such as their ability to ferment a number of unusual substrates (Schink, 1984, 1985, 1992; Schink & Pfennig, 1982; Schink & Stieb, 1983) and to grow syntrophically with methanogens (Schink, 1984; Schink & Pfennig, 1982; Schink & Stieb, 1983) suggest that these *Pelobacter* species are distinct from *Desulfuromonas* and *Desulfuromusa* species.

As previously noted (Lonergan *et al.*, 1996), phylogenetic analysis suggests that *Pelobacter propionicus* should also be placed in the genus *Geobacter*. However, *Pelobacter propionicus* is unique in that, unlike previously described *Geobacter* species (Lovley, 2000a) and *Trichlorobacter thiogenes* (Nevin *et al.*, 2003), *Pelobacter propionicus* cannot utilize acetate as an electron donor (Schink, 1992) or completely oxidize organic acids. Furthermore, although *Pelobacter propionicus* and other *Pelobacter* species can reduce Fe(III) (Lonergan *et al.*, 1996; Lovley *et al.*, 1995), unlike *Geobacter* species (Lovley, 2000a) and *Trichlorobacter thiogenes* (Nevin *et al.*, 2003), *Pelobacter propionicus* and other *Pelobacter* species do not contain the *c*-type cytochromes that are involved in

Fig. 2. Comparison between the Tamura–Nei genetic distances estimated from 16S rRNA genes and *fusA*–*gyrB*–*nifD*–*recA*–*rpoB* concatemers.

electron transfer in *Geobacter* species (Leang *et al.*, 2003; Lloyd *et al.*, 2003). Thus, due to its distinct physiological differences from *Geobacter* species, a separate genus designation for *Pelobacter propionicus* may be warranted.

The phylogenies generated from all six genes evaluated, as well as comparisons of concatenated alignments, demonstrate clearly that *Trichlorobacter thiogenes* falls within the phylogenetically coherent *Geobacter* cluster of the *Geobacteraceae*.

Malonomonas rubra, which is the only current representative of this genus, has recently been found to use Fe(III) as a terminal electron acceptor (Nevin *et al.*, 2003), contains *c*-type cytochromes (Dehning & Schink, 1989) and, like all of the described *Desulfuromusa* species (Liesack & Finster, 1994), is able to ferment both fumarate and malate (Dehning & Schink, 1989). These similarities with closely related *Desulfuromonas* and *Desulfuromusa* species suggests that the taxonomic classification of *Malonomonas rubra* may need to be re-evaluated.

Application to community analysis and environmental genomics

The data presented here will permit more refined analysis of the composition of subsurface microbial communities in which *geobacteraceae* predominate. This may aid in a better understanding of the physiological characteristics of the *geobacteraceae* community. For example, *Pelobacter propionicus* falls within the *Geobacter* clade; however, this organism has a significantly different physiology from the *Geobacter* species. Analysing the distribution of the genes whose phylogenies are described here, in addition to the 16S rRNA gene, should make it easier to use molecular analysis to differentiate between *Pelobacter* and *Geobacter* species.

Previous studies have suggested that microbial community analysis with a low-copy-number gene may be more accurate than analysis with high-copy-number genes, such as the 16S rRNA gene (Dahllöf *et al.*, 2000; Peixoto *et al.*, 2002). Preliminary analysis of the *Geobacter sulfurreducens* and *Geobacter metallireducens* genomes indicated that several of the conserved genes used in this study occur as single-copy or low-copy-number genes in the *Geobacteraceae*. For example, there is only one copy of the *nifD*, *recA* and *gyrB* genes in both of the genomes analysed from the *Geobacteraceae*.

Significant information about the physiology of uncultivated *geobacteraceae* in subsurface environments might also be derived from sequencing genomic DNA extracted from environments of interest (Lovley, 2002). Analysis of the genes reported here will make it possible to design specific PCR primers or probes that can be used to screen for fragments of *geobacteraceae* genomic DNA that contain these genes. This will significantly expand the amount of *geobacteraceae* genomic DNA that can be identified in genomic DNA libraries beyond what would be possible with screening only for 16S rRNA genes.

Significance of *nifD*

Previous physiological studies have demonstrated that *Geobacter metallireducens* and *Geobacter sulfurreducens* are capable of nitrogen fixation (Bazylnski *et al.*, 2000; Coppi *et al.*, 2001). The finding that *nifD* was present in all 30 species of the *Geobacteraceae* examined suggests that nitrogen fixation is a highly conserved physiological trait within this family. The ability to fix nitrogen might help *geobacteraceae* to compete successfully in subsurface environments, particularly those undergoing bioremediation of organic compounds or metals. For example, *geobacteraceae* were the predominant Fe(III)-reducing micro-organisms in subsurface environments contaminated with petroleum (Rooney-Varga *et al.*, 1999) and landfill leachate (Roling *et al.*, 2001). It is likely that the influx of organic carbon into these otherwise nutrient-poor subsurface environments resulted in a limitation of fixed nitrogen relative to the availability of carbon substrates. In a similar manner, *geobacteraceae* became the predominant dissimilatory metal-reducing micro-organisms when acetate, but no fixed nitrogen, was added to uranium-contaminated subsurface sediments to promote the reductive precipitation of uranium (Anderson *et al.*, 2003; Holmes *et al.*, 2002). The results suggest that the ability to adapt to such nitrogen-limited environments is typically found in *geobacteraceae*. In contrast, the genomes of other well-studied metal-reducing micro-organisms, such as *Shewanella oneidensis* and *Desulfovibrio vulgaris*, do not appear to contain *nifD*, suggesting that they do not have the adaptive feature of nitrogen fixation.

In summary, these results suggest that, in addition to the 16S rRNA gene, a number of other gene sequences may be useful for analysing the distribution of sequences from *geobacteraceae* in subsurface environments. Such studies may further aid in understanding why micro-organisms in this family are such successful competitors over other Fe(III)-reducing micro-organisms in many subsurface environments.

Description of *Geobacteraceae* fam. nov.

Geobacteraceae (Ge.o.bac'te.ra.ce.ae. N.L. masc. n. *Geobacter* the type genus of the family; -aceae ending to denote a family; N.L. neut. pl. n. *Geobacteraceae* the *Geobacter* family).

The family *Geobacteraceae* is located within the domain *Bacteria* and the δ -subclass of the *Proteobacteria*. This family branches phylogenetically between the orders '*Desulfovibrionales*' and '*Desulfobacterales*'. The family *Geobacteraceae* comprises five genera: *Geobacter*, *Desulfuromonas*, *Desulfuromusa*, *Pelobacter* and *Malonomonas*. The type genus is *Geobacter*.

Micro-organisms within this family have mostly been isolated from anoxic sedimentary environments. All species of the *Geobacteraceae* are able to reduce Fe(III) and some are able to utilize S^0 and/or the humics analogue AQDS as terminal electron acceptors. With the exception of

Pelobacter and *Malonomonas*, all genera of the *Geobacteraceae* contain species that can oxidize acetate and other multi-carbon organic substrates to carbon dioxide. This family includes species with rod-like morphology, and members of all of the genera within this family, except *Pelobacter*, contain abundant *c*-type cytochromes. In addition, cells of members of the *Geobacteraceae* are non-motile or are motile by means of flagella.

ACKNOWLEDGEMENTS

We would like to thank J. Coates, F. Löffler and J. Tiedje for providing us with cultures of *Geobacter hydrogenophilus*, *Geobacter chapellei*, *Desulfuromonas michiganensis* and *Trichlorobacter thiogenes*. This research was supported by the Office of Science (BER), US Department of Energy, with funds from the Natural and Accelerated Bioremediation Research (NABIR) program (grant DE-FG02-97ER62475) and the Genomes to Life Program (cooperative agreement no. DE-FC02-02ER63446). Preliminary sequence data from *Desulfovibrio desulfuricans* and *Geobacter sulfurreducens* were obtained from The Institute for Genomic Research (TIGR) website. Preliminary sequence data from *Geobacter metallireducens* and *Desulfuromonas acetoxidans* were obtained from the DOE Joint Genome Institute (JGI) website.

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