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ORIGINAL ARTICLE

Subsurface clade of *Geobacteraceae* that predominates in a diversity of Fe(III)-reducing subsurface environments

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There are distinct differences in the physiology of *Geobacter* species available in pure culture. Therefore, to understand the ecology of *Geobacter* species in subsurface environments, it is important to know which species predominate. Clone libraries were assembled with 16S rRNA genes and transcripts amplified from three subsurface environments in which *Geobacter* species are known to be important members of the microbial community: (1) a uranium-contaminated aquifer located in Rifle, CO, USA undergoing *in situ* bioremediation; (2) an acetate-impacted aquifer that serves as an analog for the long-term acetate amendments proposed for *in situ* uranium bioremediation and (3) a petroleum-contaminated aquifer in which *Geobacter* species play a role in the oxidation of aromatic hydrocarbons coupled with the reduction of Fe(III). The majority of *Geobacteraceae* 16S rRNA sequences found in these environments clustered in a phylogenetically coherent subsurface clade, which also contains a number of *Geobacter* species isolated from subsurface environments. Concatamers constructed with 43 *Geobacter* genes amplified from these sites also clustered within this subsurface clade. 16S rRNA transcript and gene sequences in the sediments and groundwater at the Rifle site were highly similar, suggesting that sampling groundwater via monitoring wells can recover the most active *Geobacter* species. These results suggest that further study of *Geobacter* species in the subsurface clade is necessary to accurately model the behavior of *Geobacter* species during subsurface bioremediation of metal and organic contaminants.
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Introduction

Advances in genome-based analysis of microbial communities and modeling of microbial physiology may make it possible to predictively model the activity of microorganisms responsible for various bioremediation processes in subsurface environments (Lovley, 2003). If so, then it may be feasible to predict the likely outcome of potential bioremediation strategies prior to implementation, making bioremediation a less empirical practice. This approach may be applicable to the *in situ* bioremediation of uranium-contaminated groundwater where stimulation of microbial U(VI) reduction generally results in significant enrichments of microorganisms closely related to known U(VI)-reducing *Geobacter* species that are responsible for the bioremediation process (Holmes *et al.*, 2002, 2005; Anderson *et al.*, 2003; Petrie *et al.*, 2003; North *et al.*, 2004; Chang *et al.*, 2005; Vrionis *et al.*, 2005). This suggests that *in silico* models of *Geobacter* species (Mahadevan *et al.*, 2006) might be able to predict how *Geobacter* species in the subsurface will respond to native geochemical conditions as well as amendments introduced into the subsurface that could enhance the bioremediation process. Furthermore, these findings suggest that monitoring *in situ* gene expression of *Geobacter* in the groundwater may provide insights into the rates of metabolism and the metabolic state of the microorganisms responsible for the U(VI) bioremediation process (Lovley, 2003; Holmes *et al.*, 2005).

However, important questions about the ecology of the *in situ* uranium bioremediation process remain. For example, although it is clear from

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previous studies that *Geobacter* species are often the predominant organisms during *in situ* uranium bioremediation, there has been little attention paid to which *Geobacter* species are most prevalent. This is important because as more *Geobacter* species have been isolated, their physiology studied and their genomes sequenced, it has become apparent that there can be significant differences between *Geobacter* species that may have an important impact on their ecology.

Furthermore, it is necessary to know whether the *Geobacter* species that can be recovered from groundwater samples accurately represent the most metabolically active organisms in the subsurface. This is important, because it is necessary to monitor groundwater samples taken from preestablished wells to assess subsurface microbial communities associated with U(VI) bioremediation. Taking sediment core samples during the bioremediation process is not only expensive, but it can also disrupt groundwater flow patterns, as well as the composition of the microbial community. Therefore, it is important to know whether the microbial community that can be analyzed in the groundwater is representative of active microorganisms that might also be attached to the sediments.

To address these questions, we conducted detailed field experiments for two consecutive summers at the previously described (Anderson *et al.*, 2003; Holmes *et al.*, 2005; Vrionis *et al.*, 2005) uranium-contaminated site located in Rifle, CO. We also investigated two other sites: (1) an acetate-impacted aquifer in Plymouth, MA which serves as an analog for the long-term inputs of acetate that could be expected in subsurface environments during prolonged *in situ* bioremediation of uranium contamination; and (2) a petroleum-contaminated aquifer in which *Geobacter* species are considered to play an important role in the oxidation of aromatic hydrocarbon contaminants coupled to Fe(III) reduction (Anderson *et al.*, 1998; Rooney-Varga *et al.*, 1999; Holmes *et al.*, 2004b). The results suggest that a phylogenetically coherent subsurface clade of *Geobacter* species dominates the microbial communities associated with these diverse Fe(III)-reducing subsurface environments, as well as several previously investigated subsurface environments.

Materials and methods

Site and description of uranium-contaminated aquifer
Two small-scale *in situ* bioremediation experiments were conducted on the grounds of a former uranium ore processing facility in Rifle, CO during the months of August and September in 2004 and 2005. This site, designated the Old Rifle site, is part of the Uranium Mill Tailings Remedial Action program of the US Department of Energy. Both test plots were adjacent to a previously studied larger experimental plot at the site (Anderson *et al.*, 2003; Vrionis *et al.*, 2005). These smaller plots were about one quarter the size but with a similar design.

During the 2004 field experiment, a concentrated acetate/bromide solution (100:10 mM) mixed with native groundwater was injected into the subsurface to provide <10 mM acetate to the groundwater over the course of 28 days via an injection gallery composed of five injection wells. The injection gallery was fed from a manifold connected to a stainless steel tank containing the concentrated acetate/solution as previously described (Anderson *et al.*, 2003; Vrionis *et al.*, 2005). The first monitoring well (M16) was 6 ft from the injection gallery in line with the second injection well. A background-monitoring well was placed 6 ft upstream of the injection gallery. Sediment cores were collected as previously described (Vrionis *et al.*, 2005) 29 days after the initial acetate injection at a depth of 17 ft.

In 2005, a second mini gallery was constructed that was similar to that outlined above for the 2004 experiment. This gallery was 3.8 m to the southeast, and was perpendicular to groundwater flow. A critical difference in the two experiments was that in 2005, the flowmeters were replaced by a pump configuration for subsurface delivery of the concentrated acetate/bromide solution. Two variable speed console pumps (Cole Parmer, Vernon Hills, IL, USA) were utilized with the first pump being used to deliver solution to the three depths (4, 4.2 and 5.6 m) within each injection gallery, while the second pump was used to provide cross-well mixing in the subsurface to help maximize uniformity of delivery and minimize gaps in acetate delivery between the injection galleries. Pump flow-rate for the injection gallery was $\sim 3.5\ \mathrm{ml\ min^{-1}}$ while constant cross-well mixing was performed at a rate of $16\ \mathrm{ml\ min^{-1}}$. Sediment cores were collected on day 29 of acetate injection at a depth of 17 ft.

Site and description of calcium magnesium acetate-impacted aquifer
This site consisted of a highway runoff recharge pool located adjacent to State Route 25 (SR25) in Plymouth, MA. The pool was constructed to collect runoff generated by SR25, which opened in August 1987 (Church *et al.*, 1996). The Massachusetts Department of Environmental Protection enacted restrictions on this area requiring the use of nonchloride deicing agents along a 1900 m section of highway impacting nearby cranberry bogs. Since opening, the primary road-deicing agent used on this stretch of highway has been calcium magnesium acetate (CMA). The unconfined aquifer underlying the study site is part of the Wareham Outwash Plain consisting of fine to coarse-grained sand. The concentration of acetate in the groundwater varies between 0 and 5 mM (Ostendorf, 1997–2004).

Site and description of petroleum-contaminated aquifer

Aquifer sediments at the USGS Groundwater Toxics Site in Bemidji, MN have been contaminated with crude oil for over 18 years as a result of a break in an oil pipeline (Hult, 1984; Baedecker *et al.*, 1989; Cozzarelli *et al.*, 1990). Fe(III) reduction is an important terminal electron-accepting process in portions of the aquifer (Lovley *et al.*, 1989; Lovley, 1995; Anderson *et al.*, 1998; Rooney-Varga *et al.*, 1999; Holmes *et al.*, 2005; Nevin *et al.*, 2005). Sediments were collected from the Fe(III) reduction zone of the contaminant plume in 2004 with split spoon sample cores and transported immediately to the laboratory as previously described (Holmes *et al.*, 2005).

Analytical techniques

Acetate concentrations were determined with an HP series 1100 high-pressure liquid chromatograph (Hewlett Packard, Palo Alto, CA, USA) with a Fast Acid Analysis column (Bio-Rad Laboratories, Hercules, CA, USA) with an eluent of 8 mM H_2SO_4 and absorbance detection at 210 nm as previously described (Anderson *et al.*, 2003; Vrionis *et al.*, 2005).

Fe(III) reduction was monitored by measuring the formation of Fe(II) over time with a ferrozine assay in a split-beam dual-detector spectrophotometer (Spectronic Genosys2; Thermo Electron Corp., Mountain View, CA, USA) at an absorbance of 562 nm after a 1 h extraction with 0.5 N HCl as previously described (Lovley and Phillips, 1987; Lovley *et al.*, 1989). Sulfate and bromide concentrations were measured with a Dionex DX-100 ion chromatograph (Sunnyvale, CA, USA) (Lovley and Phillips, 1994). Uranium was measured by kinetic phosphorescence analysis as previously described (Finneran *et al.*, 2002; Anderson *et al.*, 2003).

Cell numbers were determined by counting acridine orange-stained cells with fluorescence microscopy on a Nikon Eclipse E600 microscope as previously described (Lovley and Phillips, 1988b).

Extraction of genomic DNA from environmental samples

Groundwater was collected for DNA extraction by filtering 1.5 l of groundwater through 0.2 μm pore size Sterivex-GP filters (Millipore Corp., Bedford, MA, USA). Sediment samples were collected in 15 ml conical tubes. Prior to DNA extraction, all samples were placed into whirl-pack bags, flash frozen in a dry ice/ethanol bath and shipped back to the laboratory where they were stored at −80 °C.

Genomic DNA was extracted from the cartridge filters and sediment with the FastDNA SPIN kit (Bio101 Inc., Carlsbad, CA, USA) according to the manufacturer's instructions.

Extraction of RNA from environmental samples

To obtain sufficient biomass from the groundwater for RNA extraction, it was necessary to concentrate 15 l of groundwater by impact filtration on 293 mm diameter Supor membrane disk filters (Pall Life Sciences, Ann Arbor, MI, USA). RNA was extracted from sediment collected in 15 ml conical tubes. All samples were placed into whirl-pack bags, flash frozen in a dry ice/ethanol bath and shipped back to the laboratory where they were stored at −80 °C.

RNA could only be extracted from half of a disk filter at a time. The frozen filter halves were first crushed into a fine powder, dispensed into eight different 2 ml screw-cap tubes and suspended in 800 ml of TPE buffer. RNA was extracted from this filter suspension with the acetone precipitation protocol as previously described (Holmes *et al.*, 2004b, 2005). To extract RNA from the frozen sediment cores, sediment was dispensed into 16 different 2 ml screw-cap tubes. A total of 1 ml TE-sucrose buffer (10 mM Tris-HCl, 1 mM EDTA, 6.7% sucrose, pH 8.0), 30 μl 10% sodium dodecyl sulfate, 10 μl lysozyme (50 mg ml^{-1}) and 3 μl Proteinase K (20 mg ml^{-1}) were added to each tube. Samples were then incubated at 37 °C for 10 min, and centrifuged at 13 200 r.p.m. for 15 min. The supernatant was allocated into 2 ml screw-cap tubes and a hot acidic phenol/chloroform extraction followed by isopropanol precipitation at −30 °C was done as previously described (Holmes *et al.*, 2005).

Degenerate primer design

Degenerate primers (Table 1) were designed from nucleotide and amino acid sequences extracted from the following *Geobacteraceae* genomes: *Geobacter sulfurreducens* (Methe *et al.*, 2003), *Geobacter metallireducens*, strain FRC-32, *Geobacter uraniumreducens, Desulfuromonas acetoxidans*, *Pelobacter carbinolicus*, *Pelobacter propionicus* and *Geobacter bemidjiensis*. Preliminary genome sequence data for *G. metallireducens,* strain FRC-32, *G. uraniumreducens*, *D. acetoxidans, P. carbinolicus*, *P. propionicus* and *G. bemidjiensis* were obtained from the DOE Joint Genome Institute website www.jgi.doe.gov. The primer sets were used to amplify 43 different gene fragments from genomic DNA extracted from the uranium-contaminated, CMA-impacted and petroleum-contaminated aquifers (Table 1).

PCR amplification parameters and clone library construction

16S rRNA was amplified from genomic DNA and cDNA with the following bacterial primer sets targeting different regions of 16S rRNA; 8F (Eden *et al.*, 1991) with 519R (Lane *et al.*, 1985) and 338F (Amann *et al.*, 1990) with 907R (Lane *et al.*, 1985). A DuraScript enhanced avian RT single-strand synthesis kit (Sigma-Aldrich, St Louis, MO, USA) was used to generate cDNA from 16S rRNA transcripts as

Table 1 Primers used to amplify 43 different *Geobacteraceae* gene fragments from the uranium-contaminated, CMA-impacted and petroleum-contaminated aquifers

Gene name	*Forward primer*	*Reverse primer*
Acetyl-CoA carboxylase	accC700F/GGACTGCTCCATCCAGCG	accC1200R/CKGGTTTCKGCATGGACGAT
Alanyl-tRNA synthetase	alaS130F/GGSATGAACCAGTTCAAGGA	alaS620R/CGRTTGAACTGCATGAAGAC
Acetylornithine aminotransferase	argD160F/GKGTKGCSGTGAACAACCTG	argD840R/GAAGGTKGAACCGTGGGTGC
Chorismate synthase	aroC320F/GACGTSCGTAACATCCTGGA	aroC830R/TSGGRATCGGCTTCATGGC
ATP synthase F1 (α-subunit)	atpA190F/AACCTTGARGARGATAACGTTGG	atpA985R/ATRACGTTGGTCGGRATRTAVGC
Cold-shock protein	csp30F/GTTCAATGACRSCAARGGTT	csp200R/TTKTTGACGTTGGCGGCCTG
Heavy-metal efflux pump (CzcA family)	cusA1150F/GTCASCTCCAACATCATGTCC	cusA1400R/CGATGAAGCCGACCCAGAC
Dihydrodipicolinate synthase	dapA160F/GGTGARKCHTCGACSCTGGATTA	dapA750R/GGTTSSWCTCGATGAACATGGC
Chaperone protein dnaK	dnaK270F/GCGSCTCATCGGCCGTAAGT	dnaK800R/AGGWGGAGAGCTCGCACTTG
Dinitrogenase reductase-activating glycohydrolase	draG100f/GCGCCGGTGGAATTCATGAC	draG740R/GCRCCGGTGGTGTCGGCATC
Enolase	eno200f/CGTCAACRACATCATTGCCGAG	eno1020R/AGSGWGCCGATCTGGTTCAG
Flagellar biosynthetic protein FlhB	flhB50F/GAAGARCTGGATGAGGCGA	flhB740R/CATRTGRTCGACRCCCTTGGC
Cell division protein FtsA	ftsA260F/CCACATCAAGGGRWTCAATTC	ftsA870R/ACCCGVGGTTCMAGGATTACVGMCA
Translation elongation factor-G	fusA710F/TGCSTTGATGGAGAAGTATCT	fusA1020R/CTRAWRAAGCAGAGCTGACC
UDP-N-acetylglucosamine pyrophosphorylase	glmU475f/TGCGCCGAGGCRTCSTTTCT	glmU940R/CCTGRTGATTTTCTTGGTTTC
Nitrogen regulatory protein P-II	glnK100F/CTGAACGAAATCGGCATCCA	glnK300R/CGATGCGICCIGTCTCTTGGC
Citrate synthase	gltA100F/TCARTSTATTGGCGGTGC	gltA850R/CGGAGTCTTSAIGCAGAACTC
Glycyl-tRNA synthetase (A-subunit)	glyQ300f/CAGGTSATCATGAAGCCGTC	glyQ700R/AIGCACTCCTTCTCGTACAT
DNA gyrase (A-subunit)	gyrA2030F/ATCATTGCCGTGAATCTGGA	gyrA2405R/GTGATGAGCATSAGGTCGTT
DNA gyrase (B–subunit)	gyrB840F/CAACAACATCAATACCCACGA	gyrB1520R/CACGTCSGCRTCGGTCAT
Imidazoleglycerol phosphate synthase (cyclase subunit)	hisF270F/GTGGACGATATCCGCAATCT	hisF580R/TCGACAATGGCGCGRGTCA
Histidyl-tRNA synthetase	hisS350F/GCTCAGGTTCTCACCATGCT	hisS740R/CCATGGCAAAKCCRATACC
Malate dehydrogenase	mdh260F/RCCTTCATCGCCTGGGAACT	mdh610R/CMRGGWACRCCGACRTAGTA
Gliding motility protein A	mglA150F/CACCGAGACCGARCGGAC	mglA420R/AGATCCCGCTTGTTGTACTG
Virulence factor mviN protein	mviN125F/CTSGCCAACGTSTGTTTCAC	mviN960R/TTCGTRTCCTTSAKAGCGT
Nitrogenase molybdenum-iron protein (α-chain)	nifD225F/ATCGGTGACGATATCAACGCC	nifD560R/TAGTTCATGGAACGGTAGCAGT
Nitrogenase iron protein	nifH100F/GCAAGARGGTSATGATCGTCGG	nifH630R/CGGGGWACGWARTGRATCATCTGG
NifU family protein	nifU60f/GCGGTGATGTGGAGCTGGT	nifU210R/CTGGACGGAWATCACTTCCT
Cytochrome *c* nitrite reductase (catalytic subunit NrfA)	nrfA350F/TGGCGCTKCTCTATAACGG	nrfA1100R/ATGGTCCGGTCCTGGATGGC
Phosphate transport system regulatory protein PhoU	phoU290f/ACCTGGAGCGGATCGGCGAC	phoU650R/TCGTGTGGCGGATGTCCTTC
Type IV pilus biogenesis protein PilB	pilB560F/GTSAAGCTCGTGAACCTGAT	pilB1025R/ATGTTTTCRGTRGTCTTGTTG
Twitching motility protein PilT	pilT360F/GCSGCCATGATCGATWCATCA	pilT860R/AGGGCGAARTCRTCSGGGTTG
Pyrroline-5-carboxylate reductase	proC75f/TSFATWGGIGGIGGIAATGGC	proC470R/CCCCACCAGGTCGAAGATT
Pyruvate ferredoxin/flavodoxin oxidoreductase	pf2970F/TGCTGGTKCTSGACACCGAG	pf3250R/ATGCAGTGSGCRTAGGCGATG
recA protein	recA202F/ATCTWCGGICCSGAGTCGTCG	recA670R/CCSTCGCCGTAGWAGATGTCGAA
DNA-directed RNA polymerase, β-subunit (Lee *et al*., 2000)	BF/GATGATATTGACCATTTAGG	BR/TTCAGGGGTTTCAATAGGAC
RNA polymerase σ-factor RpoD	rpoD60F/CAGGAGGGGAACATCGGTCTC	rpoD525R/CTCGATCTGACGAATCCGCTC
Ribosomal protein L14	rplN50F/TGGATGTKGCKGATAATTC	rplN310R/CCTSAGTTCTCTWGCGACCG
Ribosomal protein S3	rpsC30F/CWRTGGATTCAGGCTTGGTGT	rpsC490R/CCTTCDCKGTACCATTCAGT
Superoxide dismutase	sodA200F/TGGGARTTTAACGGCATGCG	sodA550R/TCGGCRGCCTTCCAGTCGAT
Inositol-1-monophosphatase	suhB200F/TATCGCTGGATCGTGGACC	suhB720R/TGGATMAGRCCGTTGGAAGC
Translation elongation factor-τ	tuf230F/TGARACCGAVAAGCGTCACT	tuf880R/ACGCTCGATGTCTTCACGCT
Excinuclease ABC (β-subunit)	uvrB320F/CACSGACACCTTCATCGAGA	uvrB850R/TGRGATTCGTCCACGAAGAG

previously described (Holmes *et al.*, 2004b). Previously described thermal cycler parameters were used to amplify 16S rRNA from the environment (Holmes *et al.*, 2004b). The following parameters were used to amplify genes used for construction of concatamated alignments: an initial denaturation step at 95 °C for 5 min; 20 cycles of 95 °C for 1 min, 60–50 °C for 1:30 min (−0.5 °C per cycle) and 72 °C for 1:30 min; 20 cycles of 95 °C for 1 min, 55–48 °C for 1:30 min and 72 °C for 1:30 min; followed by a final extension step at 72 °C for 10 min. To ensure sterility, the PCR mixtures were exposed to UV radiation for 8 min prior to addition of DNA or cDNA template and Taq polymerase.

For clone library construction, PCR products were purified with the Gel Extraction Kit (Qiagen, Valencia, CA, USA), and clone libraries were constructed with a TOPO TA cloning kit, version M (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. A total of 100 plasmid inserts from each clone library were then sequenced with the M13F primer at the University of Massachusetts Sequencing Facility.

Quantification of Geobacteraceae *abundance by MPN-PCR analysis*

Optimal amplification conditions for primers designed for most-probable-number (MPN)-PCR of *Geobacteraceae nifD* genes was determined in a gradient thermal cycler (MJ Research Inc., Waltham, MA, USA). Geo_nifD225f/560r (Holmes *et al.*, 2004b) was used to amplify *Geobacter nifD* genes.

Five-tube MPN-PCR analyses were performed as previously described (Holmes *et al.*, 2002). Serial 10-fold dilutions of DNA template were made, and *Geobacteraceae nifD* gene fragments were amplified by PCR. PCR products were visualized on an ethidium bromide-stained agarose gel. The highest dilution that yielded product was noted, and a standard five-tube MPN chart was consulted to estimate the number of target genes in each sample.

Quantification of Geobacteraceae *abundance by MPN culture analysis*

Groundwater (0.9 ml) was aliquoted into sterilized 2 ml falcon tubes containing 0.1 ml dimethyl sulfoxide. Samples were immediately flash frozen in a dry ice/ethanol bath, and shipped back to the laboratory for analysis. Anoxic pressure tubes containing modified nutrient broth medium (Lovley *et al.*, 1999; Holmes *et al.*, 2004a) with poorly crystalline Fe(III) oxide (100 mM) (Lovley and Phillips, 1988a, b) provided as the electron acceptor and acetate (5 mM) provided as the electron donor were inoculated with these groundwater samples. Each culture was serially diluted to 10^{-8} in triplicate. After ~1 month of growth at 18 °C in the dark, the highest dilution with growth was noted, and a standard three-tube MPN chart was consulted. 16S rRNA gene clone library analysis of the highest positive dilutions of MPN culture tubes was also conducted (100 colonies were analyzed from each MPN enrichment culture).

Phylogenetic analysis

16S rRNA and functional gene sequences were compared to GenBank nucleotide and protein databases using the blastn and blastx algorithms (Altschul *et al.*, 1990). Nucleotide sequences were initially aligned in Clustal X (Thompson *et al.*, 1997) and imported into the Genetic Computer Group sequence editor (Wisconsin Package version 10; Madison, WI, USA). These alignments were then imported into Clustal W (Thompson *et al.*, 1994), Mview (Brown *et al.*, 1998) and ALIGN (Pearson, 1990) where identity matrices were generated.

Aligned sequences were imported into PAUP 4.0b10 (Swofford, 1998) where phylogenetic trees were inferred. Distances and branching order were determined and compared using maximum parsimony and distance-based algorithms (HKY85 and Jukes-Cantor). Bootstrap values were obtained from 100 replicates.

Nucleotide sequence accession numbers

The nucleotide sequences of cloned 16S rRNA and functional genes have been deposited in the GenBank database under accession numbers EF414512-EF414961 and EF668010-EF669472.

Results and discussion

Identification of a subsurface clade of pure culture Geobacter *species*

Analysis of the 16S rRNA gene sequences of the *Geobacter* species available in pure culture revealed that most isolates recovered from subsurface environments fall into two distinct phylogenetic clades, designated here as 'subsurface clade 1', and 'subsurface clade 2' (Figure 1). Subsurface clade 1 includes *Geobacter bremensis*, *Geobacter bemidjiensis*, strain FRC-32, *Geobacter humireducens*, strain Ply4, *G. uraniumreducens*, strain M21 and strain M18. Subsurface clade 2 includes: *Geobacter chapellei*, *P. propionicus*, *Geobacter psychrophilus* and strain Ply1. These subsurface isolates were recovered from a diversity of environments and geographic locations (Table 2).

Predominant uncultured Geobacter *species from multiple subsurface environments cluster within subsurface clade 1*

Analysis of 16S rRNA gene and transcript sequences in three subsurface environments in which Fe(III) reduction is an important process suggested that most of the uncultured *Geobacter* species in these environments were phylogenetically aligned within

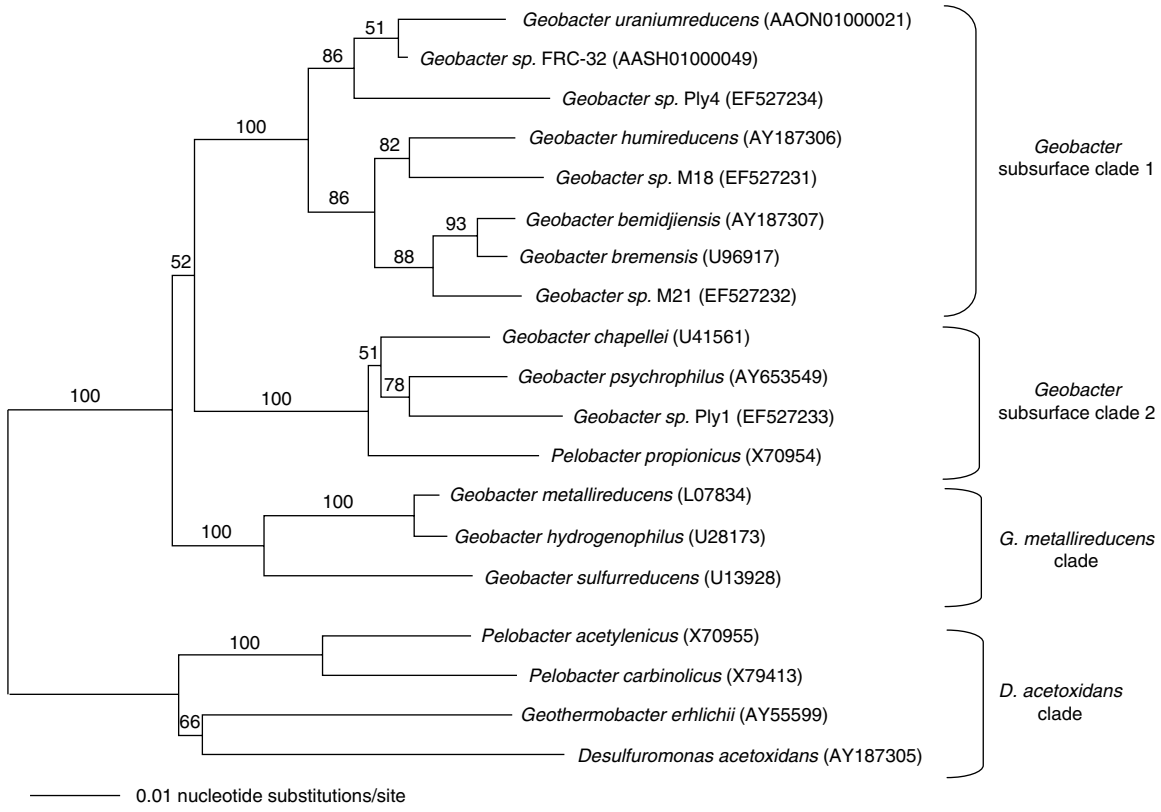

Figure 1 Phylogenetic tree comparing 16S rRNA gene sequences from *Geobacteraceae* species that are most similar to uncultivated *Geobacteraceae* that are detected in Fe(III)-reducing subsurface environments. These organisms form four phylogenetic clades; *Geobacter* subsurface clade 1, *Geobacter* subsurface clade 2, *G. metallireducens* clade, and *D. acetoxidans* clade. Branching lengths and bootstrap values were determined by maximum parsimony analysis with 100 replicates. *D. acetoxidans* and *G. erhlichii* were used as outgroups for construction of the tree.

Table 2 Description of environments from which subsurface isolates were recovered

	Subsurface environment	*Geographic location*	*Reference*
Subsurface clade 1			
Geobacter bremensis	Sediments obtained from a freshwater ditch	Bremen, Germany	(Straub and Buchholz-Cleven, 2001)
Geobacter bemidjiensis	Fe(III)-reducing sediments from a petroleum-contaminated aquifer	Bemidji, MN	(Nevin *et al.*, 2005)
Geobacter humireducens	Fe(III)-reducing freshwater wetland sediments	Hale County, AL	(Coates *et al.*, 1998)
Geobacter uraniumreducens	Aquifer contaminated with uranium that was amended with acetate to promote dissimilatory metal reduction	Rifle, CO	This study
Geobacter sp. M21			
Geobacter sp. M18			
Geobacter sp. FRC-32	Aquifer co-contaminated with uranium and nitrate that was amended with either glucose or ethanol to promote metal reduction	Oakridge, TN	Kostka, JE, personal communication (www.jgi.doe.gov)
Geobacter sp. Ply4	Aquifer that has been exposed to calcium magnesium acetate (CMA) as a road-deicing agent for more than 15 years	Plymouth, MA	This study
Subsurface clade 2			
Geobacter chapellei	Deep pristine aquifer sediments	Atlantic Coastal Plain, SC	(Coates *et al.*, 2001)
Pelobacter propionicus	Anoxic muds collected from a freshwater creek	Hanover, FRG	(Schink, 1984)
Geobacter psychrophilus	Aquifer that has been exposed to calcium magnesium acetate (CMA) as a road-deicing agent for more than 15 years	Plymouth, MA	(Nevin *et al.*, 2005)
Geobacter sp. Ply1			This study

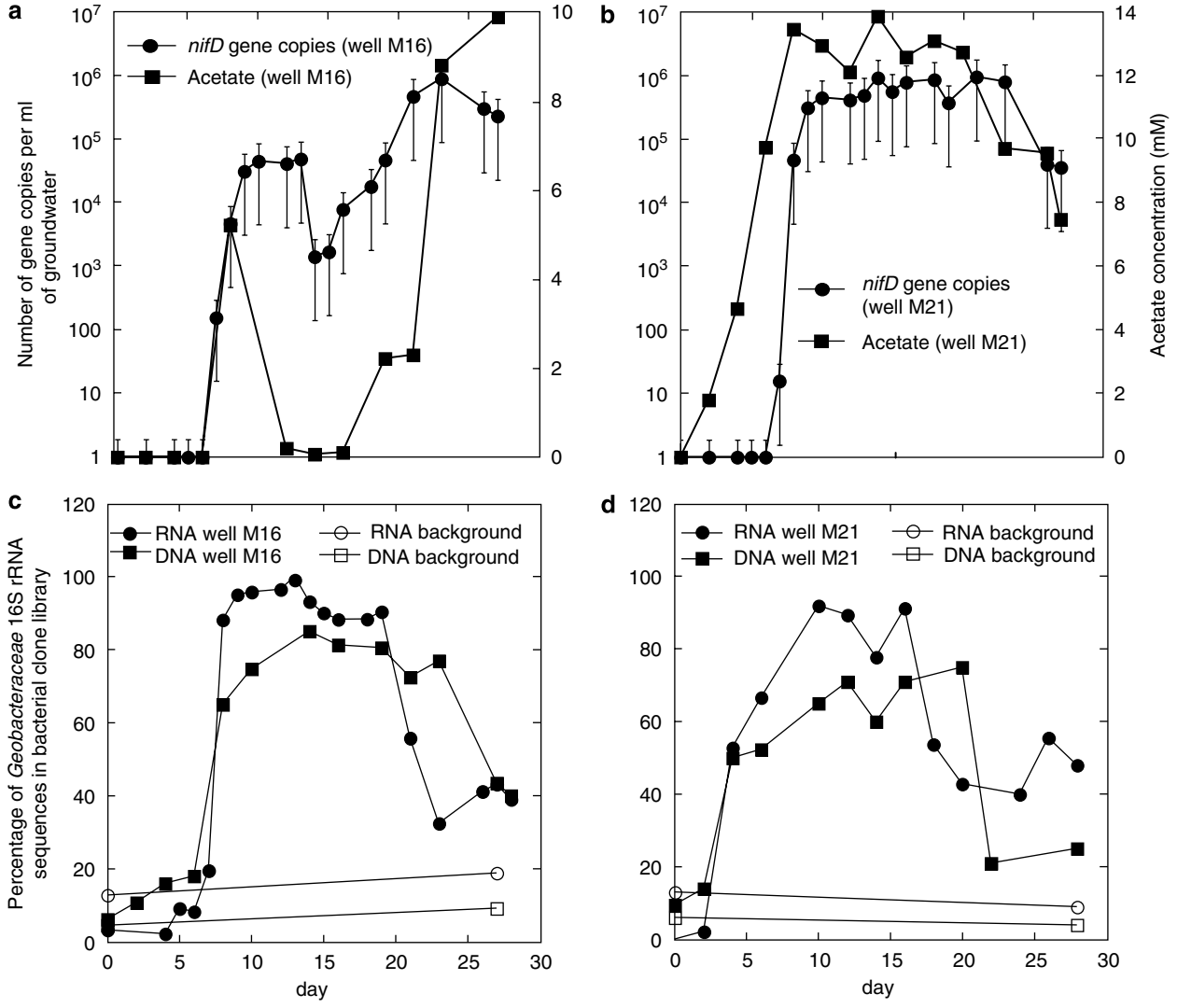

Figure 2 Results from field studies at the Rifle, CO study site. MPN-PCR results with primers targeting *Geobacteraceae*-specific *nifD* compared to groundwater acetate concentrations (**a** and **b**), as well as percentages of *Geobacteraceae* 16S rRNA transcript and 16S rRNA gene sequences in groundwater bacterial clone libraries (**c** and **d**) during the 2004 and 2005 field experiments.

Geobacter subsurface clade 1. The most detailed information was available on *Geobacter* species detected at the uranium-contaminated aquifer located in Rifle, CO in which metal reduction was stimulated by the addition of acetate in field studies conducted in 2004 and 2005 (Figure 2). As expected from previous studies at this site (Anderson *et al.*, 2003; Vrionis *et al.*, 2005), the addition of acetate stimulated the growth of *Geobacter* (Figures 2a and b).

In the 2004 field experiment, the numbers of *Geobacter*, as estimated from the number of *Geobacter nifD* gene copies, increased by more than four orders of magnitude as acetate concentrations increased (Figure 2a). When acetate additions were stopped temporarily, acetate concentrations declined and the number of *Geobacter* species temporarily decreased and then increased again when acetate additions resumed. *Geobacter* numbers also tracked with acetate concentrations in the 2005 experiment (Figure 2b). An increase in the number of *Geobacter* species, comparable to that estimated from *Geobacter nifD* MPN-PCR analysis was observed in MPN culture estimates of acetate-oxidizing Fe(III)-reducing microorganisms (Table 3). Clone library analysis of the highest positive dilutions of MPN culture tubes from acetate-amended groundwater demonstrated that the 16S rRNA gene sequences were most closely related to pure cultures of *Geobacter* in subsurface clade 1 (Table 3). Direct counts of total cells in groundwater collected on day 19 from both years indicated that total cell numbers in 2004 and 2005 were 1.03×10^6 to 3.60×10^6 cells per ml and 2.94×10^6 to 8.44×10^6 cells per ml, respectively, which when coupled with the MPN-culturing techniques, suggested that the majority of cells present in the groundwater on day 19 were *Geobacter* species.

Table 3 MPN estimates of acetate-oxidizing Fe(III)-reducing microorganisms and phylogenetic affiliation of microorganisms in the highest positive dilutions

Groundwater sample	*Estimated cell number (cells ml^{-1} of groundwater)*	*Most similar organisms*	*Sequence similarity (%)*	*Proportion of sequences detected in enrichment (%)*	*Accession numbers*
Rifle 2004 (well M16) day 0	73 to 150	*Geobacter bemidjiensis*	95	66.67	EU120748
		Pseudomonas sp. LCY11	99	21.9	EU120750
		Pseudomonas stutzeri	98	11.43	EU120751
Rifle 2004 (well M16) day 19	1.5×10^6 to 4.6×10^6	*Geobacter bemidjiensis*	95	25.0	EU120744
		Geobacter bremensis	96	75.0	EU120745
Rifle 2005 (well M21) day 0	15 to 62	*Geobacter bremensis*	96	25.0	EU120742
		Geobacter bemidjiensis	96	41.67	EU120743
		Ideonella sp. 0-0013	95	8.33	EU120749
		Pseudomonas sp. LCY11	99	16.67	EU120752
		Dechloromonas denitrificans	97	8.33	EU120741
Rifle 2005 (well M21) day 19	3.60×10^5 to 2.40×10^6	*Geobacter bemidjiensis*	97	70.83	EU120746
		Geobacter bremensis	97	20.83	EU120747
		Pseudomonas synxantha	97	4.17	EU120753
		Pseudomonas fluorescens	99	4.17	EU120754

Serial dilutions were made in medium containing acetate (10 mM) provided as the electron donor, and poorly crystalline Fe(III)-oxide (100 mM) provided as the electron acceptor. A standard three-tube MPN chart was consulted to estimate cell numbers (95% confidence interval).

Direct analysis of the phylogenetic composition of the microbial community without culturing also demonstrated an increased importance of *Geobacter* species following the addition of acetate (Figures 2c and d). The proportion of the community comprised of *Geobacter* species increased substantially during the most active phase of the growth of *Geobacter* species. During the later phases of both field experiments, when Fe(III) oxides in the sediments were likely to have been depleted, the proportion and abundance of *Geobacter* in the groundwater declined. In both years, libraries generated from 16S rRNA transcripts generally indicated that *Geobacteraceae* accounted for a greater proportion of the microbial community than did libraries constructed from 16S rRNA genes, especially in the early phases of bioremediation. This may reflect the fact that during this period *Geobacteraceae* were the most active members of the microbial community and that other minor members of the community, although present and thus detectable with 16S rRNA gene sequence analysis, were not as metabolically active.

Further analysis of the clone libraries demonstrated that sequences that fell within *Geobacter* subsurface clade 1 predominated throughout the 2004 and 2005 field studies (Figure 3). In general, sequences in *Geobacter* subsurface clade 1 accounted for a higher proportion of the libraries constructed from 16S rRNA transcripts than from 16S rRNA genes, suggesting that when metabolically active organisms were considered, this subsurface clade had increased importance. Sequences that fell within *Geobacter* subsurface clade 2 were the next most abundant. Representatives of the *G. metallireducens* and *D. acetoxidans* clades were detected in most samples as well (Figure 3).

Geobacter species also comprised a high proportion of the previously described (Holmes *et al.*, 2005) acetate-impacted aquifer in Plymouth, MA which serves as an analog for the purposeful long-term acetate additions to the subsurface proposed for *in situ* uranium bioremediation. Groundwater collected in June of 2004 and 2005 had acetate concentrations of 1 mM and 600 μM, respectively. In the two successive years *Geobacteraceae* comprised 78% and 68% of the sequences in 16S rRNA transcript libraries and 55% and 54% of the sequences in 16S rRNA gene libraries. Sequences that fell within subsurface clade 1 accounted for the highest proportion of *Geobacter* sequences in both years, both in libraries constructed from 16S rRNA gene sequences as well as 16S rRNA transcript libraries (Figure 4). As noted in the uranium-contaminated sediments from the Rifle site, sequences that fell within subsurface clade 2 were second in relative abundance.

In sediments collected in 2004 from the previously described (Lovley *et al.*, 1989; Lovley, 1995; Anderson *et al.*, 1998; Rooney-Varga *et al.*, 1999; Holmes *et al.*, 2004b, 2005; Nevin *et al.*, 2005) petroleum-contaminated aquifer in which *Geobacter*

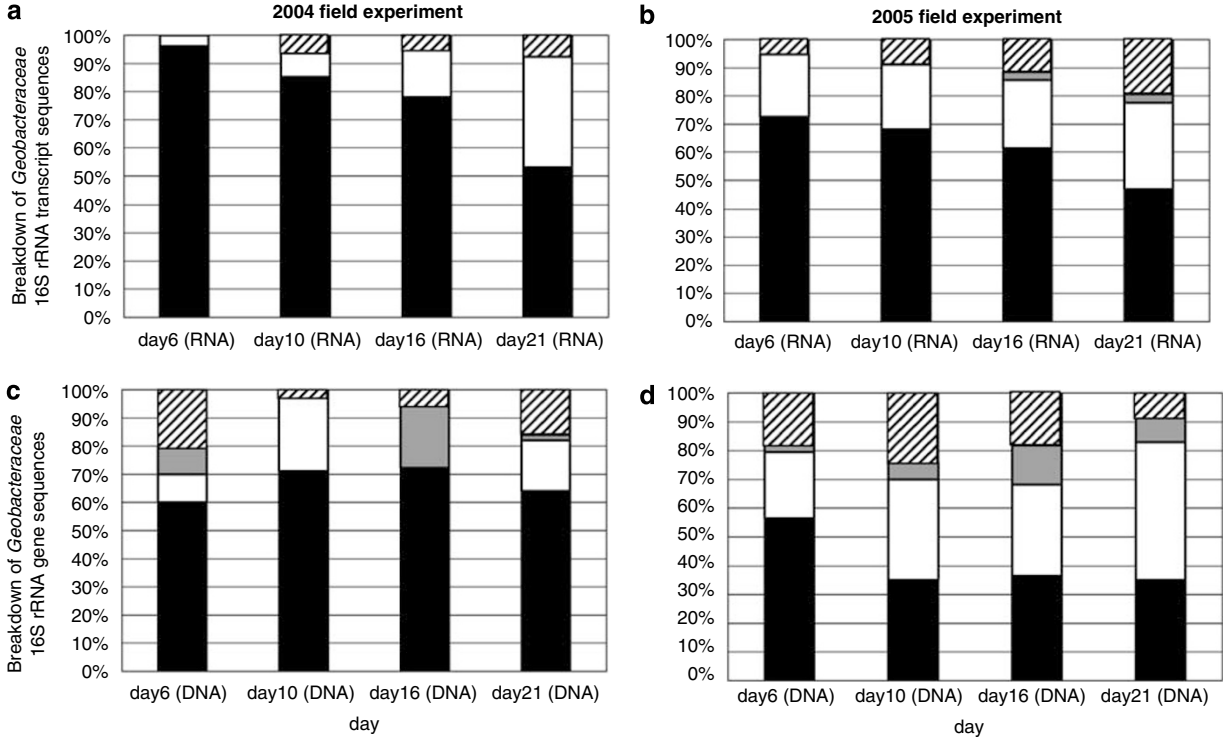

Figure 3 *Geobacteraceae* 16S rRNA transcript and gene sequences detected in clone libraries assembled from RNA and DNA extracted from groundwater collected from the uranium-contaminated aquifer on 6, 10, 16 and 21 days after initial acetate injections. (**a**) RNA extracted from groundwater collected during the 2004 field experiment, (**b**) RNA extracted from groundwater collected during the 2005 field experiment, (**c**) DNA extracted from groundwater collected during the 2004 field experiment and (**d**) DNA extracted from groundwater collected during the 2005 field experiment. ■ subsurface clade 1, □ subsurface clade 2, ▩ *G. metallireducens* clade, ▨ *D. acetoxidans* clade.

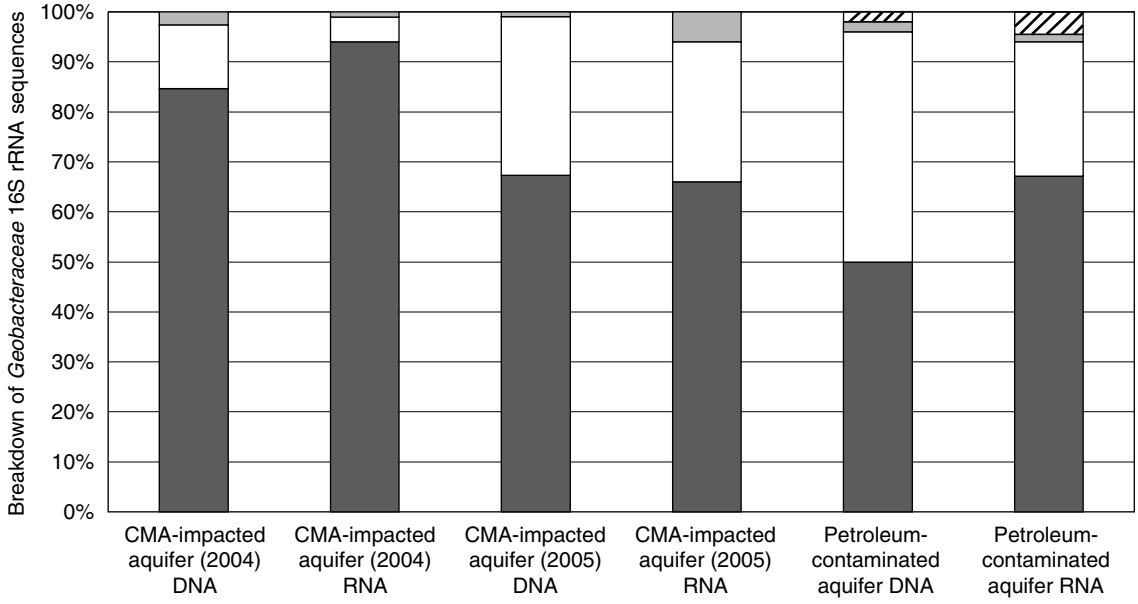

Figure 4 *Geobacteraceae* 16S rRNA gene and transcript sequences detected in clone libraries assembled from nucleic acids extracted from groundwater collected from the calcium magnesium acetate (CMA)-impacted aquifer located in Plymouth, MA in June of 2004 and 2005, and sediments collected from the petroleum-contaminated aquifer located in Bemidji, MN in 2004. ■ subsurface clade 1, □ subsurface clade 2, ▩ *G. metallireducens* clade, ▨ *D. acetoxidans* clade.

species are thought to play an important role in the oxidation of aromatic hydrocarbon contaminants coupled to the reduction of Fe(III), *Geobacteraceae* accounted for 35% and 41% of the sequences recovered from 16S rRNA gene and 16S rRNA transcript libraries, respectively. In these libraries,

sequences that fell within subsurface clade 1 predominated, with subsurface clade 2 sequences of secondary importance (Figure 4).

In a previous study conducted at a uranium-contaminated aquifer in Oak Ridge, TN in which dissimilatory metal reduction was stimulated with the addition of either ethanol or glucose, there was also an enrichment of *Geobacter* species associated with increased metal reduction in sediments collected from this site (North *et al*., 2004). Analysis of the four 16S rRNA gene sequences that were reported for that community demonstrated that they all fell within *Geobacter* subsurface clade 1.

Geobacteraceae 16S rRNA sequences were also predominant in clone libraries constructed from samples collected from Cretaceous shale and sandstone rock formations ~200 m below ground surface in Cerro Negro, New Mexico (Kovacik *et al*., 2006). Phylogenetic analysis of these *Geobacteraceae* sequences indicated that organisms that clustered within *Geobacter* subsurface clade 1 were significant members of the microbial community. In the Cubero sandstone clone libraries (112 clones analyzed) 50% of the reported *Geobacteraceae* sequences were in subsurface clade 1, and in the Clay Mesa shale clone libraries (96 clones analyzed) 24% of the reported *Geobacteraceae* sequences were in this clade.

Geobacter species were enriched in the Fe(III) reduction zone of a landfill leachate-contaminated aquifer in which Fe(III) reduction was considered to be involved in contaminant degradation (Roling *et al*., 2000, 2001; Lin *et al*., 2005; Mouser *et al*., 2005). Analysis of the 48 *Geobacter* sequences reported for this site indicated that in contrast to all of the other sites reported in this study sequences in *Geobacter* subsurface clade 2 were predominant.

Phylogeny based on concatamated alignments

To more intensively evaluate the phylogeny of the *Geobacter* species found in the three aquifers that were the focus of this study, portions of 43 genes, known to be conserved cross the genomes of pure cultures of *Geobacteraceae*, were amplified from genomic DNA extracted from either sediments collected from the petroleum-contaminated site in 2004, groundwater collected from the CMA-impacted site in 2005 or groundwater collected from the uranium-contaminated aquifer 10 days after initial acetate injections during the *in situ* uranium bioremediation experiment conducted in 2005.

Concatamated alignments of these genes from each environment were compared to mini genomes constructed from the genes available in pure culture genomes (Figure 5). Similar to 16S rRNA gene and transcript sequences, the concatamers constructed from all three subsurface samples clustered within *Geobacter* subsurface clade 1. Phylogenetic comparisons indicated that the similarity between the environmental concatamers from the uranium-contaminated, acetate-contaminated and petroleum-contaminated aquifers and the subsurface clade 1 isolate, *G. bemidjiensis* was 70%, 68.2% and 65.5%, respectively. These environmental gene assemblies were ~70% identical to each other.

Further analysis of the individual gene sequences that were used to construct these concatamated

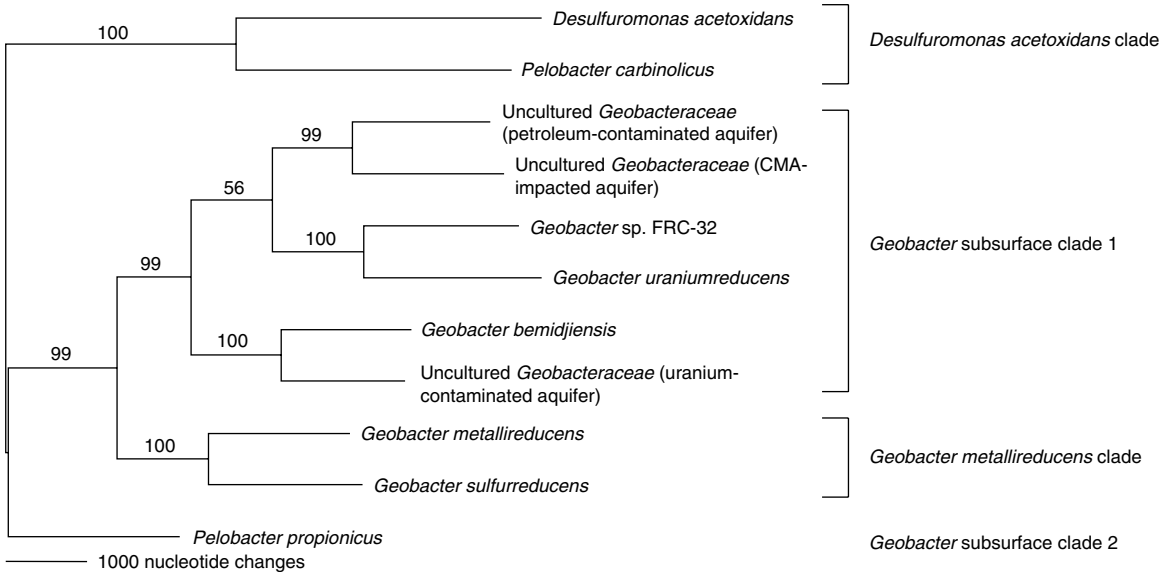

Figure 5 Phylogenetic tree comparing concatamers constructed with 43 conserved *Geobacteraceae* genes detected in the uranium-contaminated, calcium magnesium acetate (CMA)-impacted and petroleum-contaminated aquifers to concatamers constructed with genes from *Geobacteraceae* species available in culture. Branching lengths and bootstrap values were determined by maximum parsimony analysis with 100 replicates. *D. acetoxidans* and *P. carbinolicus* were used as outgroups for construction of the tree. Genomic DNA was extracted from sediments collected in the Fe(III) reduction zone of the petroleum-contaminated aquifer, groundwater collected from the CMA-impacted site in June, 2005 and groundwater collected from the uranium-contaminated aquifer 10 days after initial acetate injections during the *in situ* uranium bioremediation experiment conducted in 2005.

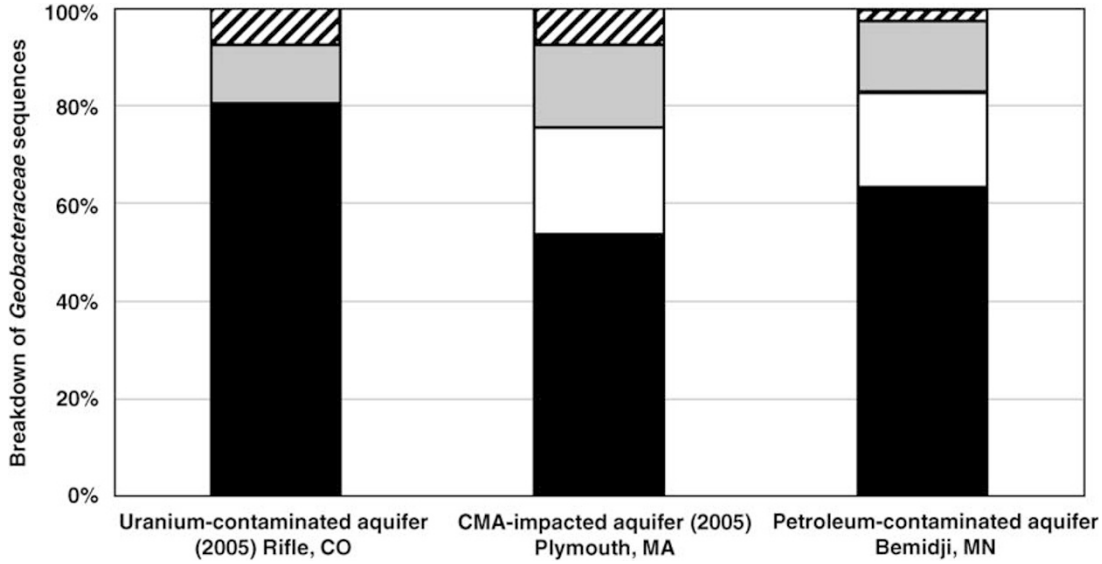

Figure 6 Percentage of sequences from the 43 conserved genes amplified from the petroleum-contaminated, calcium magnesium acetate (CMA)-impacted and uranium-contaminated aquifers that were most similar to sequences obtained from available genomes that cluster within: ■ subsurface clade 1 (*G. bemidjiensis*, *G. uraniumreducens* and strain FRC-32), □ subsurface clade 2 (*P. propionicus*), ▩ *G. metallireducens* clade (*G. metallireducens* and *G. sulfurreducens*) or ▨ *D. acetoxidans* clade (*D. acetoxidans* and *P. carbinolicus*). Genomic DNA samples were the same as in Figure 5.

alignments indicated that the majority of sequences from all three subsurface environments were most similar to organisms from the *Geobacter* subsurface clade 1 (Figure 6). For example, 80.5%, 63.4% and 53.7% of the genes amplified from the uranium-contaminated, CMA-impacted and petroleum-contaminated aquifers, respectively, were most similar to sequences that cluster within subsurface clade 1.

Phylogenetic comparison of Geobacteraceae *in sediments and groundwater*

For many subsurface sites, it is only technically or economically feasible to sample groundwater and not sediments. Therefore, potential differences between the composition of *Geobacteraceae* in the groundwater and sediments at the Rifle study site at the end of the field experiments were evaluated. In groundwater collected on day 28 in the 2004 and 2005 field experiments, *Geobacteraceae* accounted for between 25% and 48% of the bacterial 16S rRNA gene and transcript sequences (Figure 7a). *Geobacteraceae* accounted for between 21% and 57% of the 16S rRNA gene and transcript sequences detected in sediment cores extracted at the end of the 2004 and 2005 field experiments (day 29). Libraries generated from 16S rRNA transcripts indicated that *Geobacteraceae* accounted for a higher proportion of the microbial community than did libraries constructed from 16S rRNA genes. The proportion of *Geobacteraceae* 16S rRNA gene sequences detected in cores of background sediments not exposed to the acetate amendments was significantly lower; 5.8% and 10%, in 2004 and 2005, respectively. There was not enough biomass in the background samples to obtain high-quality RNA for clone library analysis.

Further analysis of these *Geobacteraceae* sequences indicated that the *Geobacter* communities found in the groundwater and sediment during the uranium bioremediation process were similar. In both years examined, the predominant 16S rRNA transcripts detected in the groundwater and sediment clustered within *Geobacter* subsurface clade 1 (Figure 7b). Phylogenetic comparisons of the predominant *Geobacteraceae* sequences detected in the groundwater and sediment samples indicated that these sequences were 97%–100% identical to each other (Figure 8).

Implications

These results demonstrate that *Geobacter* species in subsurface clade 1 are often the predominant *Geobacter* species in a diversity of subsurface environments in which Fe(III) reduction is an important process. To date, studies on the physiology of *Geobacter* species have focused on *G. sulfurreducens* (Caccavo *et al.*, 1994; Lovley *et al.*, 2004; Lovley, 2006) primarily because the complete genome sequence (Methe *et al.*, 2003) and a genetic system (Coppi *et al.*, 2001) are available for this organism. There have also been limited physiological studies on *G. metallireducens* (Lovley *et al.*, 1993, 2004; Lovley, 2006). However, neither of these *Geobacter* species clusters within subsurface clade 1. Preliminary analysis of the genomes (www.jgi.doe.gov) of *G. uraniumreducens*, *G. bemidjiensis* and strain FRC-32, which are subsurface clade 1 isolates, suggests that there may be significant physiological differences between *Geobacter* species in the subsurface clade and *G. sulfurreducens* and *G. metallireducens*.

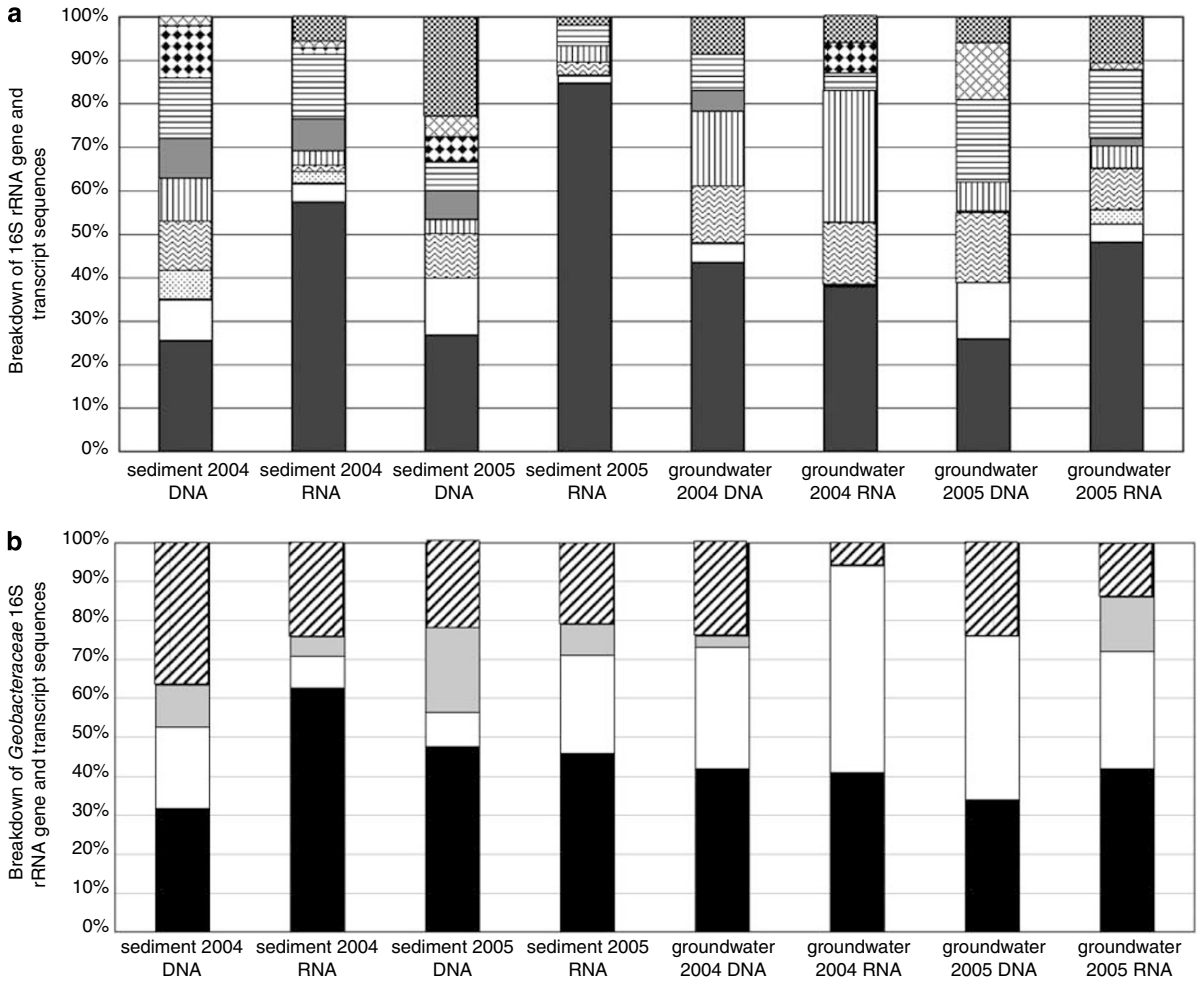

Figure 7 Bacterial (**a**) and *Geobacteraceae* (**b**) 16S rRNA gene and transcript sequences detected in clone libraries assembled from RNA and DNA extracted from groundwater collected at the end of the 2004 and 2005 field experiments (day 28), and sediment samples collected at the end of the 2004 and 2005 field experiments (day 29). (**a**) ■ *Geobacteraceae*, □ other *δ-proteobacteria*, ▨ *α-proteobacteria*, ▨ *β-proteobacteria*, ▥ *γ-proteobacteria*, ■ *acidobacteria*, ▤ *firmicutes*, ▣ *bacteroidetes*, ▨ *planctomycetes*, ▩ other. (**b**) ■ *Geobacter* subsurface clade 1, □ *Geobacter* subsurface clade 2, ■ *G. metallireducens* clade, ▨ *D. acetoxidans* clade.

The results also suggest that the most physiologically active *Geobacter* species in the subsurface may be monitored in groundwater samples. This is an important finding because, although it is relatively simple to sample groundwater from preestablished monitoring wells during field studies, sampling from sediments is often problematic. Sediment core sampling is generally expensive, labor intensive and technically difficult. In addition, sediment coring typically disrupts groundwater flow and geochemistry, at least temporarily, complicating the interpretation of changes in groundwater chemistry and microbial composition. Furthermore, the biomass in sediments is frequently too low to extract high-quality RNA.

Although previous studies have suggested that there may be significant differences in the microbial communities associated with sediments and groundwater in the subsurface (Pedersen and Ekendahl, 1990; Holm *et al.*, 1992; Lehman *et al.*, 2001, 2004; Reardon *et al.*, 2004), it is not too surprising that major differences were not observed in the *Geobacter*-dominated samples from the Rifle study site. This is because recent studies have noted that although *Geobacter* species must directly contact Fe(III) oxides to reduce them (Nevin and Lovley, 2000), *Geobacter* species are also highly motile during growth on Fe(III) oxides (Childers *et al.*, 2002). It is hypothesized that motility is required because Fe(III) oxides are heterogeneously dispersed in subsurface sediments and once Fe(III) oxides are depleted in one microsite, *Geobacter* species must locate another source of Fe(III) oxide (Childers *et al.*, 2002; Lovley *et al.*, 2004). The ability of *Geobacter* species to transfer electrons to Fe(III) oxides over distances of multiple cell lengths via conductive pili (Reguera *et al.*, 2005) is likely to aid in this planktonic behavior.

In summary, the recent recovery of a number of pure cultures from subsurface environments that fall within the *Geobacter* subsurface clade 1 makes it possible to further evaluate the physiology of these

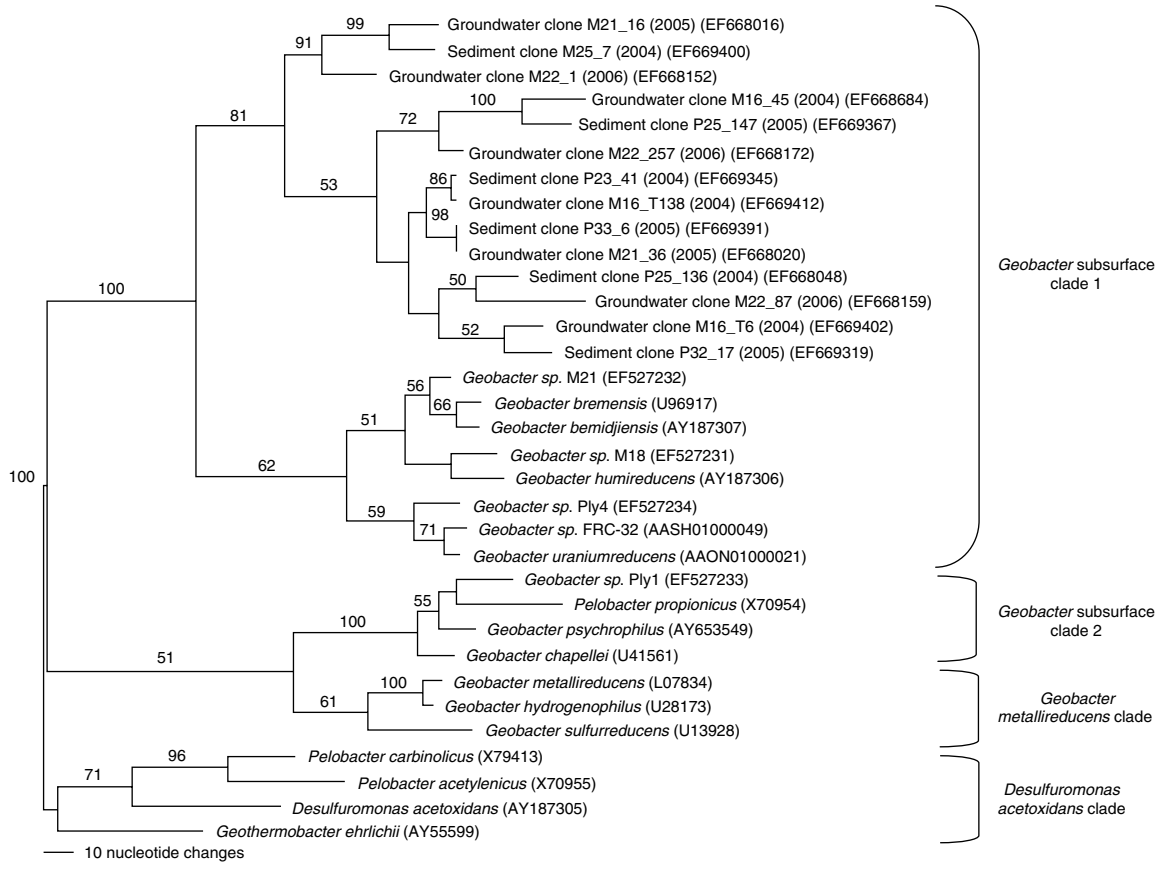

Figure 8 Phylogenetic tree comparing 16S rRNA gene sequences from known *Geobacter* isolates to the predominant *Geobacteraceae* sequences detected in the groundwater and sediment samples. Branching lengths and bootstrap values were determined by maximum parsimony analysis with 100 replicates. *D. acetoxidans* and *G. erhlichii* were used as outgroups for construction of the tree.

organisms. Such studies seem warranted given the predominance of this clade of *Geobacter* species in a number of subsurface environments of concern.

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