Change in Bacterial Community Structure during In Situ Biostimulation of Subsurface Sediment Cocontaminated with Uranium and Nitrate

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Previous studies have demonstrated that metal-reducing microorganisms can effectively promote the precipitation and removal of uranium from contaminated groundwater. Microbial communities were stimulated in the acidic subsurface by pH neutralization and addition of an electron donor to wells. In single-well push-pull tests at a number of treated sites, nitrate, Fe(III), and uranium were extensively reduced and electron donors (glucose, ethanol) were consumed. Examination of sediment chemistry in cores sampled immediately adjacent to treated wells 3.5 months after treatment revealed that sediment pH increased substantially (by 1 to 2 pH units) while nitrate was largely depleted. A large diversity of 16S rRNA gene sequences were retrieved from subsurface sediments, including species from the α, β, δ, and γ subdivisions of the class *Proteobacteria*, as well as low- and high-G+C gram-positive species. Following in situ biostimulation of microbial communities within contaminated sediments, sequences related to previously cultured metal-reducing δ-*Proteobacteria* increased from 5% to nearly 40% of the clone libraries. Quantitative PCR revealed that *Geobacter*-type 16S rRNA gene sequences increased in biostimulated sediments by 1 to 2 orders of magnitude at two of the four sites tested. Evidence from the quantitative PCR analysis corroborated information obtained from 16S rRNA gene clone libraries, indicating that members of the δ-*Proteobacteria* subdivision, including *Anaeromyxobacter dehalogenans*-related and *Geobacter*-related sequences, are important metal-reducing organisms in acidic subsurface sediments. This study provides the first cultivation-independent analysis of the change in metal-reducing microbial communities in subsurface sediments during an in situ bioremediation experiment.

Uranium [U(VI)] is the most common radionuclide contaminant found within the U.S. nuclear weapon complex managed by the U.S. Department of Energy. Nitrate is often a cocontaminant with U(VI) in subsurface sedimentary environments because of the use of nitric acid in the processing of U(VI) waste (39, 45). U(VI) can be microbiologically immobilized from groundwater by its reduction from UO_2^{2+} to insoluble U(IV) oxide (28, 31, 33). One of the more promising strategies for the in situ remediation of U(VI) waste involves the "biostimulation" of U(VI) immobilization (39). Biostimulation is defined as addition of nutrients (carbon and other nutrient sources) that serve to increase the number or activity of indigenous microorganisms available for bioremediation activity. Biostimulation can lead to creation of a permeable treatment zone in contaminated aquifers that removes radionuclides from the aqueous phase before they enter sensitive water supplies.

Nitrate serves as a competing and energetically more favorable electron acceptor for metal-reducing bacteria in nitric acid-contaminated sediments (9). Previous studies of microbial U(VI) reduction in sediments cocontaminated with nitrate have indicated that no net U(VI) reduction occurs until nitrate is reduced. Once nitrate is depleted, U(VI) and Fe(III) are reduced concurrently (12, 13, 49). Thus, denitrification intimately affects the fate of U(VI) in anoxic subsurface environments. Subsurface denitrification is believed to be mediated by a group of facultative anaerobes that are phylogenetically and metabolically diverse (35). Many Fe(III)-reducing bacteria (FeRB) and sulfate-reducing bacteria (SRB), such as members of the *Geobacteraceae* and *Shewanellae* families, possess the ability to reduce nitrate (9, 31, 43), but few have been observed to carry out complete denitrification.

Optimization of U(VI) bioremediation strategies requires an extensive understanding of metal-reducing microorganisms and the environmental parameters controlling their activity. Most previous work on U(VI)-reducing bacteria has been conducted with pure cultures or enrichments in the laboratory, where it is difficult to reconstruct field conditions. Few studies of known metal reducers capable of U(VI) utilization have been carried out by cultivation-independent techniques with subsurface sediments, and structure-function relationships have not been examined extensively. Thus far, for the FeRB, members of the *Geobacteraceae* family within the δ-*Proteobacteria* subdivision were most often detected in abundance in subsurface sediment slurries at neutral pH upon stimulation of concurrent U(VI) and Fe(III) reduction by addition of acetate as an electron donor (18).

Several FeRB and SRB have the ability to reduce U(VI), and some conserve energy for growth from this reaction (6, 32, 57). Not only do these organisms have the ability to enzymatically reduce U(VI), but the products of microbial sulfate and Fe(III) reduction, hydrogen sulfide and Fe(II), can chemically reduce U(VI) (26, 34, 37). In the terrestrial subsurface, oxidized iron is often the most abundant electron acceptor available under anoxic conditions, thus providing a metabolic advantage for FeRB (28). Previous culture studies conducted in our laboratory suggested that upon pH neutralization, mem-

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bers of the δ-*Proteobacteria* subdivision (*Geobacter* spp., *Anaeromyxobacter* spp.) will be important metal reducers in initially acidic subsurface sediments cocontaminated with U(VI) and nitrate (42). Iron(III)-reducing consortia, enriched from the U.S. Department of Energy Natural and Accelerated Bioremediation Research Field Research Center (FRC), where our study was conducted, utilized Fe(III) minerals as the sole electron acceptor (24) and rapidly reduced U(VI) in culture. A most-probable-number (MPN) serial dilution assay for SRB and FeRB revealed that FeRB are far more abundant than SRB in subsurface FRC sediments (L. Petrie, unpublished results). Therefore, the FeRB were the focus of this study, as they represent indigenous microorganisms likely to catalyze the immobilization of U(VI) contamination in situ. Further evidence from preliminary field experiments at the FRC site suggested that subsurface microbial activity was carbon substrate limited (20). Thus, we hypothesized that (i) biostimulation, through the addition of a carbon substrate and pH neutralization, would catalyze the reduction of nitrate, Fe(III), and U(VI) in contaminated FRC sediments and (ii) metal-reducing members of the δ-*Proteobacteria* subdivision would constitute a substantial proportion of the sedimentary microbial communities after in situ biostimulation. We further proposed that the overall change in microbial community composition during biostimulation must also be understood to place the metal-reducing organisms into the context of competing heterotrophs.

With a combination of push-pull activity tests, solid-phase geochemistry, and cultivation-independent analysis of microbial communities, we examined the in situ change in microbial community composition in subsurface sediments cocontaminated with U(VI) and nitrate during biostimulation. Through quantification of the in situ activity and community composition of metal-reducing microorganisms likely to catalyze U(VI) sorption and precipitation, we provide important inputs for reaction-based biogeochemical models that greatly aid in the development of in situ U(VI) bioremediation strategies.

MATERIALS AND METHODS

Site and sample description. This study focused on contaminated subsurface sediments collected from the U.S. Department of Energy FRC, located at the Y-12 complex within the Oak Ridge National Laboratory in Oak Ridge, Tenn. It is centered on groundwater plumes that originate from former waste disposal ponds at the Y-12 plant. Contaminated acidic subsurface sediments were sampled from area 1 in the saturated zone of shallow residuum overlying Nolichucky shale, where elevated concentrations of U(VI) and nitrate have been observed. Other contaminants include additional radionuclides (plutonium, technetium), toxic metals (nickel, aluminum, barium, chromium, mercury), chelating agents (EDTA), chlorinated hydrocarbons (trichloroethylene [TCE] and perchloroethylene), polychlorinated biphenyls, and fuel hydrocarbons (toluene, benzene) (http://www.lbl.gov/NABIR/). The "background area" is a pristine site ~163 ha in size, located in West Bear Creek Valley, approximately 2 km away from the S-3 ponds (http://www.lbl.gov/NABIR/). Subsurface sediments of the background area contain a parent rock mineralogy and sediment characteristics similar to those of contaminated sites of the FRC (http://www.lbl.gov/NABIR/).

Sediment cores were sampled prior to biostimulation, on 30 August 2001, and after biostimulation (following the final push-pull test), on 16 December 2002, with an Acker Drill Co. (TBD-II) Holegator track drill equipped with polyurethane sleeves lining the corer. The ranges of core depths sampled before and after biostimulation experiments were 3.0 to 5.7 and 2.1 to 3.6 m for contaminated area 1 and the background areas, respectively. Cores (0.42 m in diameter, 1.83 m in length) were immediately transferred to a Coy anaerobic chamber adjacent to the field sites, where they were subsampled under aseptic and strictly anoxic conditions with alcohol-rinsed equipment. Intact subsamples were sealed under argon, frozen in liquid nitrogen, and shipped to Florida State University

on dry ice via Fed-Ex priority overnight. Before sediment biostimulation, cores FB32, FB33, and FB34 were sampled adjacent to injection wells from the push-pull experiment (42). After biostimulation, cores FB45, FB46, FB47, and FB49 were sampled adjacent to wells corresponding to the same sites where cores FB32 (corresponding to both FB45 and FB46), FB33, and FB34 were sampled, respectively.

Chemical analysis. The sediment pH was determined by diluting 2 ml of sediment with 2 ml of deionized water. The 1:1 dilution was shaken for 1 h and centrifuged, and then the pH of the supernatant was measured with a calibrated digital pH meter (36). Nitrate was extracted from sediments for 1 h with a 1:1 dilution of sediment sample-deionized water, followed by centrifugation, and the supernatant was analyzed for dissolved nitrate after reduction with V(III) to NO with a chemiluminescence detector (4). Poorly crystalline Fe oxide minerals were quantified by a 1-h 0.5 M HCl extraction (23).

In situ biostimulation. Push-pull tests for in situ bioremediation activity were conducted as described in a companion paper by Istok et al. (20). Briefly, wells were successively tested five times with approximately 1 to 2 months between treatments. Test solutions consisted of site groundwater that was neutralized with 100 mM sodium bicarbonate, made anoxic by sparging with 80% N_2–20% CO_2, and amended with either ethanol or glucose to a final concentration of 400 mM. Approximately 200 liters of test solution was injected into each well for each test with a siphon over a period of 1 to 2 days. Following injection, groundwater samples were periodically collected from the same well for up to 1,000 h. Samples were filtered in the field with 0.2-μm-pore-size syringe filters and stored at 4°C until analyzed. Groundwater was analyzed for nitrate by ion chromatography, for U(VI) with a kinetic phosphorescence analyzer (Chemcheck, Richland, Wash.) (5), for Fe(II) by the ferrozine assay (52), for ethanol by gas chromatography with flame ionization detection, and for glucose by the phenol-sulfuric acid method (8). Rates of donor utilization and nitrate and U(VI) reduction were calculated by plotting dilution-adjusted concentrations versus time (see reference 20 for further information).

DNA extraction and 16S rRNA gene analysis. Microbial community DNA was extracted directly from the sediment by a method adapted from the simultaneous RNA-DNA extraction protocol described previously (19). The DNA was purified twice with a Wizard column (Promega, Madison, Wis.) and resuspended in 50 μl of sterile water. Sterile-water aliquots also extracted by this method served as negative controls. All of the 16S rRNA gene sequences obtained from negative controls were considered laboratory contaminants. Community DNA from uncontaminated sediment was extracted with the Ultra Clean Soil DNA kit (Mo Bio Laboratories, Solana Beach, Calif.) in accordance with the manufacturer's instructions. Immediately after extraction, aliquots (1 μl) of DNA were added to a mixture of PCR reagents for cloning and sequencing. Bacterial 16S rRNA genes were amplified from community DNA with primers 8F (5′-AGA GTT TGA TCM TGG CTC AG-3′) and 1392R (5′-ACG GGC GGT GTG TRC-3′) as previously described (10). The PCR products were cloned with a TOPO TA Cloning kit (Invitrogen, Carlsbad, Calif.) and screened by digestion with restriction enzyme HaeIII (New England Biolabs, Beverly, Mass.) as previously described (11). Clone libraries were generated from three contaminated sediment cores, four biostimulated sediment cores, and one pristine sediment core. Cloned inserts with unique restriction patterns were amplified with M13 primers from whole cells and purified for sequencing with QIAquick PCR purification columns (QIAGEN, Valencia, Calif.). Sequencing was performed with an Applied Biosystems 3100 genetic analyzer with primers G and H (27) at the Florida State University sequencing facility. Sequences were aligned against close relatives from the Ribosomal Database Project with the ARB software package (54) and rRNA secondary-structure diagrams. Dendrograms were constructed with ARB and PAUP* 4.0s (Sinauer Associates, Sunderland, Mass.) with only unambiguously aligned nucleotides. The possibility of chimera formation was analyzed by submitting the sequences to the CHIMERA_CHECK program in the Ribosomal Database Project database (7).

Quantitative PCR analysis. The relative abundances of 16S rRNA gene sequences closely related to *Geobacter* sp., *Anaeromyxobacter* sp., *Paenibacillus* sp., and *Brevibacillus* sp. were determined from DNA directly extracted from subsurface sediment by an MPN-PCR technique as previously described (14). Serial 10-fold dilutions of extracted DNA were made in sterile water, and 1-μl aliquots were used as the template in the PCR. Primer sets for *Anaeromyxobacter* and *Paenibacillus-Brevibacillus* were designed with the probe design function in the ARB software package (54) (Table 1). Optimum temperature and cycling parameters were determined to be an initial denaturation step of 94°C for 10 min, followed by 35 cycles of 95°C (30 s), 56.5°C (30 s), and 72°C (45 s), with a final extension step of 72°C for 10 min. To test primer specificity, each primer set was compared to the sequences available in the Ribosomal Database Project and GenBank databases with the PROBE_CHECK (Ribosomal Database Project)

TABLE 1. Primer pairs and amplicon lengths for use in MPN-PCR

Target organism(s)	Primer name	Primer sequence (5′–3′)	Amplicon length (bp)	Reference
Anaeromyxobacter sp.	60F 461R	CGA GAA AGC CCG CAA GGG T ATT CGT CCC TCG CGA CAG T	401	This study
Paenibacillus Brevibacillus spp.	160F 334R	TGA GTA ACA CGT AGG CAA CCT TAA TGC GCC GCA GGC CCA T	174	This study
Geobacter sp.	494F 825R	AGG AAG CAC CGG CTA ACT CC TAC CCG CRA CAC CTA GTC T	331	18

and BLAST algorithms (1). Clone libraries produced from each primer set were also analyzed to ensure primer specificity. Each primer set produced cloned sequences closely related to their intended target organisms. PCR products were analyzed by gel electrophoresis in 1% agarose gels, stained in an ethidium bromide bath, and visualized by UV transillumination. The highest dilution that yielded a product was noted, and a standard three-tube MPN chart was consulted in order to determine the number of 16S rRNA gene copies per gram of sediment extracted (2). A test with known amounts of template DNA was conducted to verify the accuracy of the 16S rRNA gene abundances determined through MPN-PCR. The accuracy test revealed that the numbers of 16S rRNA gene copies per gram of sediment may be slightly overestimated but are well within the standard error.

Nucleotide sequence accession numbers. The nucleotide sequences reported here were submitted to the GenBank database under accession numbers AY527742 to AY527816.

RESULTS

In situ push-pull tests for microbial activity. Microbial activity was quantified by analysis of electron acceptor and donor utilization in groundwater samples collected over regular intervals (Table 2; Fig. 1). Prior to testing, groundwater was aerobic and products of microbial respiration [nitrite, Fe(II)] were not detectable, indicating that microbial activity in the contaminated aquifer was carbon substrate limited (http://www.lbl.gov/NABIR/). Carbon substrate limitation was corroborated by control tests conducted with no added electron donor in which no microbial activity was detected (20).

Extensive microbial reduction of available electron acceptors [nitrate, Fe(III), U(VI)] and utilization of electron donors (glucose, ethanol) were indicated at all of the sites where in situ tests were conducted at the FRC. A representative plot of activity is shown for well FB34 in Fig. 1, and a summary of the rates obtained from push-pull activity tests at all of the wells is provided in Table 2. Although rates were initially highly variable, substrate utilization was induced upon repeated treatment with an electron donor, such that nearly complete reduction of available nitrate and U(VI) was observed upon com-

pletion of the field experiment (Fig. 1). During the later stages of successive push-pull treatment, utilization of electron acceptors and the electron donor occurred simultaneously in a parallel, linear relationship (Fig. 1).

Results from groundwater testing were supported by geochemical analysis of subsurface sediment cores collected before and after biostimulation experiments immediately adjacent to the wells treated by push-pull testing. Sediment pH generally increased by 1 to 2 pH units (the only exception being borehole FB45, with the small decrease in pH possibly due to other simultaneously occurring microbial processes, such as fermentation), and extractable nitrate concentrations decreased by up to 1 to 2 orders of magnitude in treated sediments (Table 3). Additionally, the reduction of amorphous Fe(III) minerals by metal-reducing bacteria was indicated by the accumulation of Fe(II) to between 59 and 88% of the total HCl-extractable Fe (Table 3). Each sediment sample used for DNA extraction in our study came from the same cores as those used for geochemical analysis.

Clone library analysis. In contaminated FRC sediment before biostimulation, common 16S rRNA gene sequences detected through cloning and sequencing (>10% of the contaminated clone libraries) included species within the α-, β-, and γ-*Proteobacteria* subdivisions (Fig. 2, 3, and 4). The most abundant (23%) 16S rRNA gene sequences were 98% similar to the *Methylobacterium* genus within the α-*Proteobacteria* subdivi-

TABLE 2. In situ biostimulation data showing effect of electron donor on nitrate and U(VI) reduction rates

Well	Treatment	Avg rate (SD)[a]		
		Electron donor use (mM/h)	Nitrate reduction (mM/h)	U(VI) reduction (μM/h)
FB32	Glucose	1.135 (1.980)	0.281 (0.229)	0.017 (0.017)
FB33	Glucose	1.116 (1.816)	0.950 (1.180)	0.028 (0.011)
FB34	Ethanol	1.774 (3.271)	0.915 (1.193)	0.009 (0.010)

[a] Values are average rates obtained from five successive push-pull activity tests at each site.
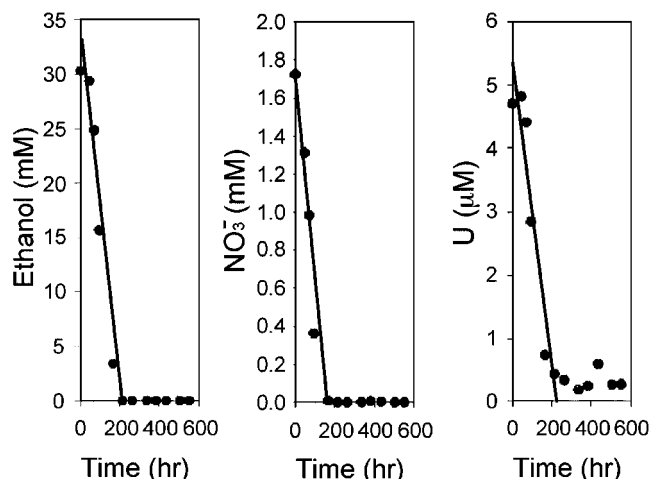

FIG. 1. Rates of ethanol utilization and nitrate and U(VI) reduction in contaminated well FB34.

TABLE 3. Changes in pH, nitrate concentration, and percentage of Fe(II) in total Fe after biostimulation of contaminated FRC sediment

Core (carbon source added)	Corresponding unstimulated core	pH		Nitrate concn (mM)		% of Fe(II) in total Fe	
		Before biostimulation	After biostimulation	Before biostimulation	After biostimulation	Before biostimulation	After biostimulation
FB45 (glucose)	FB32	4.4	4.1	8.6	1.5	38.6	58.7–85.5
FB46 (glucose)	FB32	4.4	6.6	8.6	2.2	38.6	58.7–85.5
FB47 (glucose)	FB33	3.6	4.5	154.3	6.8	9.60	66.8
FB49 (ethanol)	FB34	3.8	4.9	36.9	0.1	5.10	87.8

sion, including *Methylobacterium radiotolerans*, *M. dichloromethanicum*, and *M. mesophilicum*. *M. dichloromethanicum* is able to use dichloromethane as its sole source of carbon and energy (21), and *M. mesophilicum* is within a group of bacteria capable of degrading EDTA-metal complexes (58). Almost

one-fourth of the cloned 16S rRNA genes before biostimulation had 98% sequence identity to organisms cultured from low-nutrient environments, such as *Caulobacter leidyi* and *Brevundimonas vesicularis* (22). Also, 18% of retrieved sequences were greater than 95% similar to a cloned 16S rRNA gene
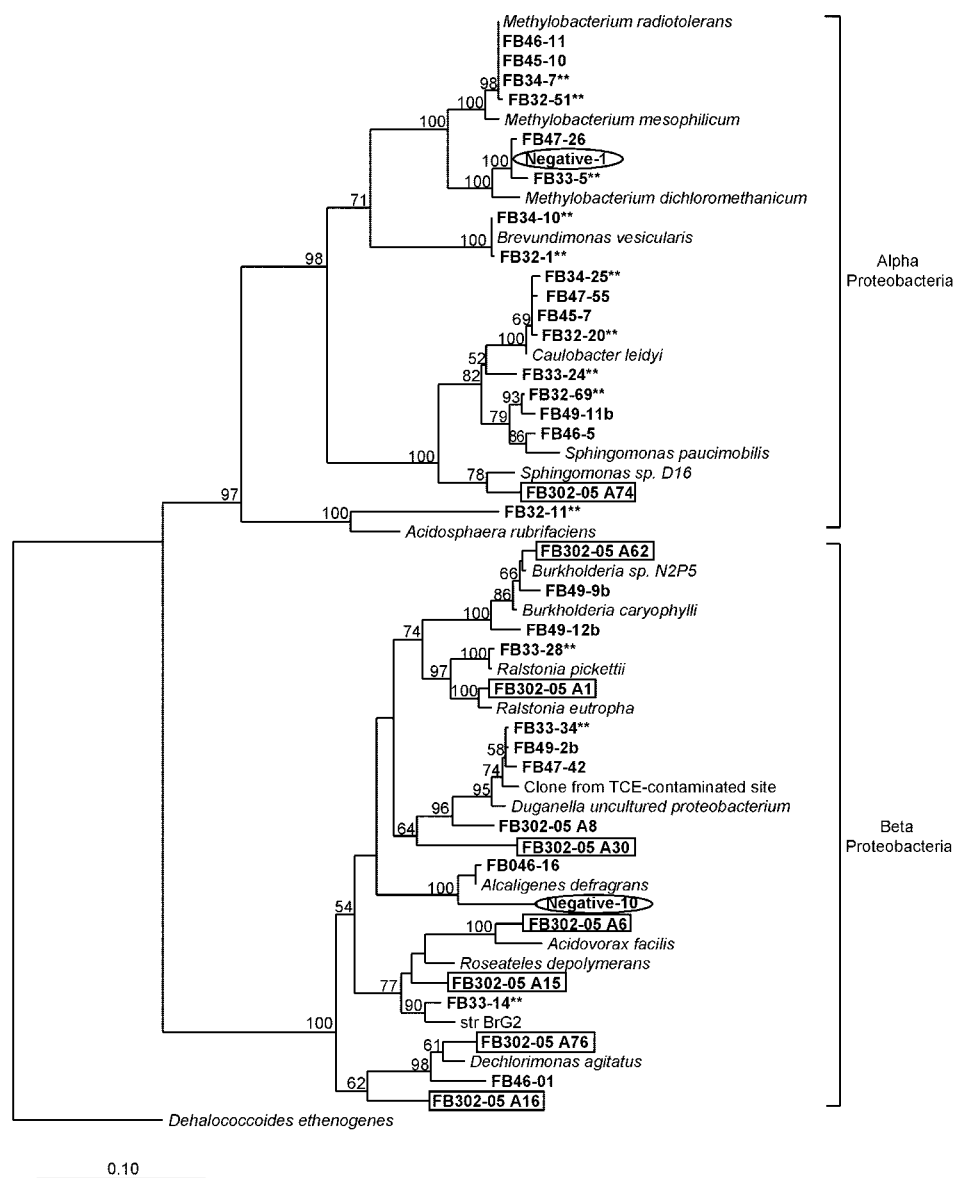

FIG. 2. Phylogenetic tree of α- and β-proteobacterial 16S rRNA gene sequences cloned from contaminated sediment. Asterisks indicate a phylotype detected before biostimulation, while no asterisks indicates a phylotype detected after biostimulation. Circled clones represent negative controls, and boxed clones are from the uncontaminated site.

FIG. 3. Phylogenetic tree of δ- and γ-proteobacterial and gram-positive 16S rRNA gene sequences cloned from contaminated sediment. Asterisks indicate a phylotype detected before biostimulation, while no asterisks indicates a phylotype detected after biostimulation. Circled clones represent negative controls, and boxed clones are from the uncontaminated site.
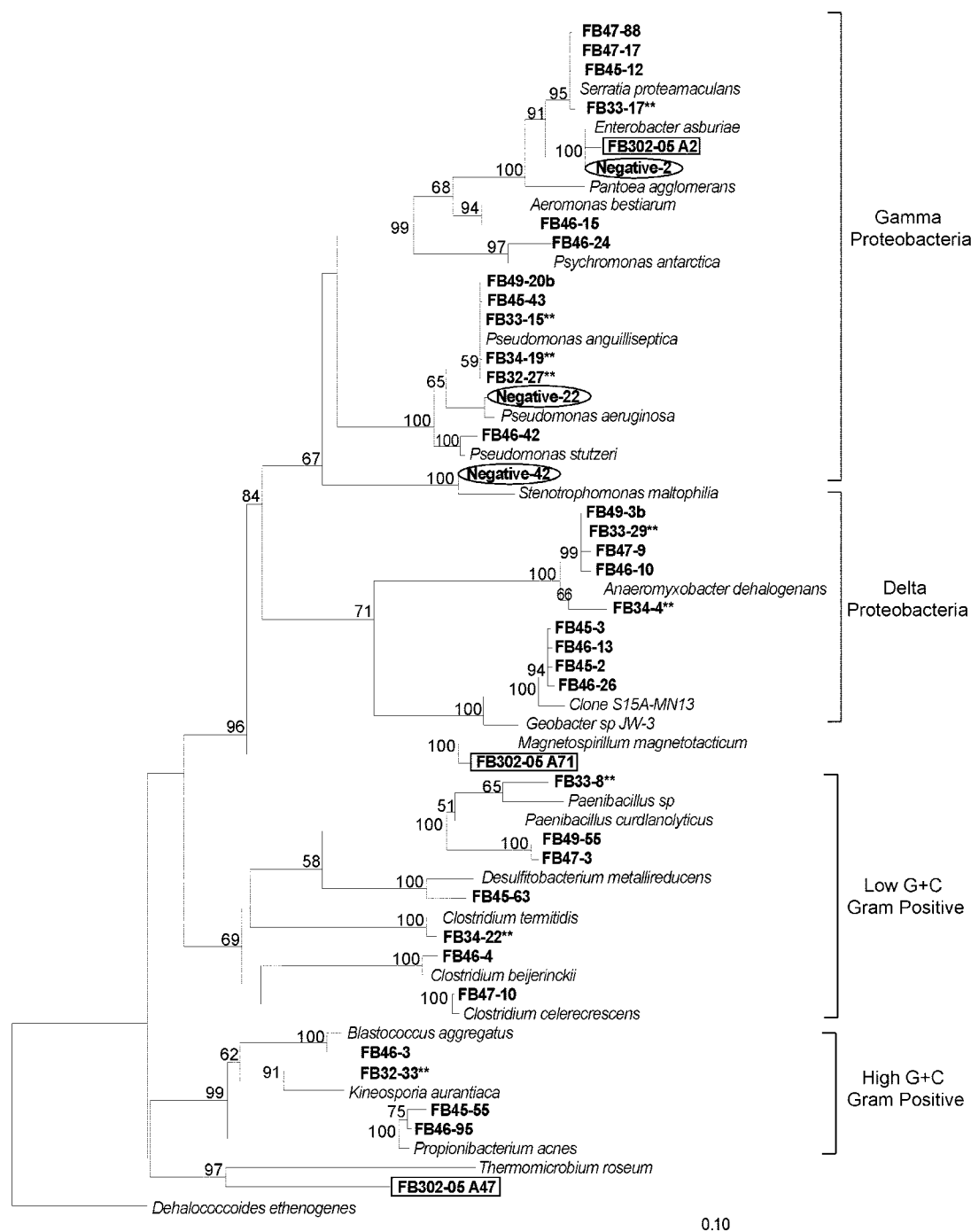
sequence within the β-*Proteobacteria* subdivision initially detected within a TCE-contaminated site undergoing in situ bioremediation treatment (A. B. Carroll and S. H. Zinder, unpublished data). Ten percent of the contaminated clone libraries were composed of 16S rRNA gene sequences related to *Pseudomonas anguilliseptica*, a γ-*Proteobacteria* subdivision member previously detected in U(VI) waste piles (55).

In the biostimulated sediments, the most common 16S rRNA

gene sequences (>10% of the biostimulated clone libraries) detected included species within the α-, β-, δ-, and γ-*Proteobacteria* subdivisions (Fig. 2, 3, and 4). Forty percent of the sequences retrieved in the biostimulated clone libraries were related to members of the δ-*Proteobacteria* subdivision. Nearly 25% of the 16S rRNA gene sequences in the biostimulated clone libraries were 96% similar to the δ-*Proteobacteria* subdivision species *Anaeromyxobacter dehalogenans*, a known dissim-
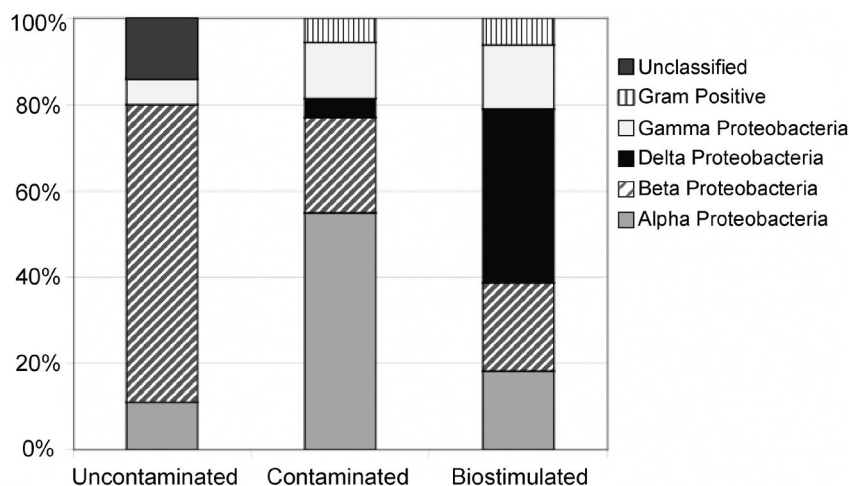
FIG. 4. Percentages of 16S rRNA gene sequences cloned from contaminated, biostimulated, and uncontaminated sediment.

ilatory Fe(III)-reducing organism (16). Members of the metal-reducing *Geobacteraceae* family within the δ-*Proteobacteria* subdivision were also detected, as several cloned 16S rRNA gene sequences were 93% similar to species in this family. Organisms within the δ-*Proteobacteria* subdivision represented the greatest increase in frequency of 16S rRNA gene sequences retrieved after biostimulation of FRC sediment, increasing from less than 5% to 37% of the clone libraries. Almost 15% of the biostimulated clone libraries were composed of sequences closely related to polycyclic aromatic hydrocarbon-degrading organisms (38), *Burkholderia* sp. strain N2P5 and *Sphingomonas paucimobilis*, within the β- and α-*Proteobacteria* subdivisions, respectively. Clones closely related to members of the *Methylobacterium* genus, including *M. dichloromethanicum*, *M. radiotolerans*, and *M. mesophilicum*, were still present (11% of the clone library) after biostimulation experiments but were not as common as in the initial contaminated clone libraries.

To compare sequences from contaminated FRC sediment to sequences from pristine sediment with similar geochemistry, DNA was also extracted from a pristine background site. In the background sediment, a lower diversity of retrieved sequences was observed in comparison with the contaminated and biostimulated clone libraries (Fig. 2, 3, and 4). The most common 16S rRNA gene sequences (>10% of the clone library) were either within the β-*Proteobacteria* subdivision or were not closely related to any previously identified organisms. Almost 70% of the 16S rRNA gene clones retrieved from the background sediment were within the β-*Proteobacteria* subdivision. Similar results were obtained in a recent study of a U(VI)-contaminated aquifer in Rifle, Colo., with β-proteobacterial sequences predominating in pristine samples from an upgradient control well (3). Thirty percent of the clones from the background site were 92% similar to an uncultured member of the β-*Proteobacteria* subdivision most closely related to *Duganella zoogloeoides*, an aerobic chemoorganotroph isolated from wastewater and activated sludge (44). Almost one-fifth of cloned sequences were greater than 98% similar to the β-*Proteobacteria* subdivision species *Ralstonia eutropha*, a well-known facultative chemolithotroph (22). Also within the β-*Proteobacteria* subdivi-

sion, sequences closely related to the species *Dechloromonas agitata* and *Acidovorax facilis* were detected in 14% of the clone library.

To ensure that retrieved sequences were not laboratory contaminants, 16S rRNA gene clones generated from sterile-water extractions were used as negative controls (Fig. 2 and 3). About 70% of the cloned sequences in the negative-control library were closely related to members of the γ-*Proteobacteria* subdivision, in contrast to sediment libraries, where less than 20% of retrieved sequences were related to members of the γ-*Proteobacteria* subdivision. More than one-third of the negative-control clones were greater than 98% similar to the γ-*Proteobacteria* subdivision member *Enterobacter asburiae*, a species recently detected in contaminated agricultural water (25). Sequences related to the γ-proteobacterial species *Pseudomonas aeruginosa* and *Stenotrophomonas maltophilia*, two organisms previously identified in a study documenting laboratory contaminants, were also found (56).

MPN-PCR. Before the in situ addition of carbon substrates (glucose or ethanol) to contaminated FRC sediments, *Anaeromyxobacter*-related 16S rRNA gene sequences were more abundant than *Geobacter*- or *Paenibacillus-Brevibacillus*-related sequences (Table 4). One to 2 orders of magnitude fewer *Geobacter*-related 16S rRNA gene copies per gram of wet sediment were detected, and in all boreholes but one, *Paenibacillus-Brevibacillus*-related 16S rRNA genes were the least abundant (Table 4). After biostimulation, the number of *Geobacter*-related 16S rRNA genes increased by 2 orders of magnitude in two of the four boreholes (one stimulated with glucose and one stimulated with ethanol) (Table 4, borehole sets A and D) and remained relatively stable in the other two (Table 4, borehole sets B and C). In three of the four boreholes (Table 4, borehole sets A, B, and D), the number of *Anaeromyxobacter*-related and *Paenibacillus-Brevibacillus*-related 16S rRNA genes decreased by 1 to 2 and 1 to 3 orders of magnitude, respectively. In the fourth borehole (Table 4, borehole set C), the number of *Anaeromyxobacter*-related 16S rRNA genes remained constant and the number of *Paenibacillus-Brevibacillus*-related 16S rRNA genes increased.

TABLE 4. MPN-PCR assay results

Borehole set	Organism(s) probed	No. of copies of 16S RNA gene/g of sediment (95% confidence limits)[a]	
		Before biostimulation	After biostimulation
A	*Geobacter* sp.	5.38×10^3 (4.23×10^3–2.63×10^4)	2.63×10^5 (2.19×10^5–8.50×10^5)
B	*Geobacter* sp.	5.38×10^3 (4.23×10^3–2.63×10^4)	1.50×10^3 (1.06×10^3–7.38×10^3)
C	*Geobacter* sp.	2.25×10^3 (1.80×10^3–8.85×10^3)	1.50×10^3 (1.06×10^3–7.38×10^3)
D	*Geobacter* sp.	6.50×10^2 (4.25×10^2–1.30×10^3)	2.88×10^4 (2.38×10^4–1.50×10^5)
A	*Anaeromyxobacter* sp.	2.63×10^4 (2.10×10^4–1.03×10^5)	1.16×10^3 (9.75×10^2–5.91×10^3)
B	*Anaeromyxobacter* sp.	2.63×10^4 (2.10×10^4–1.03×10^5)	1.13×10^2 (1.00×10^2–5.63×10^2)
C	*Anaeromyxobacter* sp.	3.45×10^3 (2.25×10^3–1.46×10^4)	5.38×10^3 (4.50×10^3–1.79×10^4)
D	*Anaeromyxobacter* sp.	4.25×10^4 (3.18×10^4–1.65×10^5)	1.16×10^3 (9.75×10^2–5.91×10^3)
A	*Paenibacillus-Brevibacillus* spp.	1.16×10^3 (9.75×10^2–5.91×10^3)	1.43×10^2 (9.3×10^1–6.07×10^2)
B	*Paenibacillus-Brevibacillus* spp.	1.16×10^3 (9.75×10^2–5.91×10^3)	1.00×10^0
C	*Paenibacillus-Brevibacillus* spp.	1.00×10^0	6.98×10^3 (5.85×10^3–3.55×10^4)
D	*Paenibacillus-Brevibacillus* spp.	2.35×10^4 (1.65×10^4–7.85×10^4)	1.88×10^2 (1.5×10^2–7.38×10^2)

[a] Quantitative PCR assay results obtained before and after biostimulation of contaminated sediment with glucose (comparing FB32 to nearby FB45 [borehole set A], FB32 to nearby FB46 [borehole set B], and FB33 to nearby FB47 [borehole set C]) and ethanol (comparing FB34 to nearby FB49 [borehole set D]) are shown.

DISCUSSION

Few previous studies have applied cultivation-independent approaches for in-depth characterization of metal-reducing communities in subsurface sediment (18, 46, 47, 51). In order to develop effective bioremediation strategies for contaminant metals, the in situ microbial communities likely to catalyze metal reduction need to be understood. The change in the general bacterial community composition brought on by biostimulation must also be understood to place the metal-reducing organisms into the context of competing heterotrophs. We conducted a field experiment on the in situ bioremediation of subsurface environments at the U.S. Department of Energy Natural and Accelerated Bioremediation Research FRC, Oak Ridge, Tenn., where subsurface sediments are cocontaminated with high levels of U(VI) and nitrate. The dominant contaminants in FRC sediment include radionuclides [U(VI), technetium], toxic metals (nickel, aluminum, barium, chromium, mercury), chelating agents (EDTA), chlorinated hydrocarbons (TCE, perchloroethylene), polychlorinated biphenyls, and fuel hydrocarbons (toluene, benzene) (http://www.lbl.gov/NABIR/). This combination of a low pH and high concentrations of nitrate, radionuclides, and organic contaminants in an aerobic subsurface environment is representative of many sites within the U.S. nuclear weapon complex managed by the Department of Energy (39, 45). Therefore, our results are not only important for bioremediation research at the FRC but can also be applied to other sites.

Cloning and sequencing techniques provided measurements of the bacterial 16S rRNA gene compositions of FRC sediments before and after biostimulation experiments. Before biostimulation, the contaminated FRC subsurface was likely carbon limited (20) and contained an extremely high concentration of nitrate (Table 3) (http://www.lbl.gov/NABIR/). Over a 3.5-month period during the in situ biostimulation experiment, the microbial populations responded to an increased pH (Table 3) and increased carbon substrate availability. The environment within the FRC sediment was no longer inhibited by acidic pH or carbon limitations, and sedimentary organisms were able to compete for electron donors and acceptors.

Before sediment biostimulation, microorganisms within the α-*Proteobacteria* subdivision made up 57% of clone libraries, with almost half of those sequences closely related to *Methylobacterium* species (Fig. 2 and 4). As previously mentioned, *M. mesophilicum* was detected within a group of bacteria with the ability to degrade EDTA-metal complexes (58). EDTA forms stable, water-soluble complexes with metals, including U(VI), hindering their adsorption to sediment surfaces. Thus, another approach for limiting the migration of complexed U(VI) at the FRC may be to biodegrade co-occurring organic ligands (15). Owing to cocontamination of sediments with synthetic chelators, EDTA may have been one of the few carbon sources available before biostimulation experiments, thus encouraging the growth of microorganisms capable of EDTA degradation. Chlorinated compounds were also available, perhaps supporting the presence of dechlorinating organisms, such as *M. dichloromethanicum*. Thus, the dominance of *Methylobacterium*-type sequences may have been due to their ability to survive by using the limited growth substrates available in the contaminated sediment. In fact, before biostimulation, 43% of the clone libraries were made up of sequences related to dechlorinating organisms and 22% of the sequences were related to nitrate-reducing organisms (Table 5). This could be expected, as both nitrate and halogenated compounds are common contaminants present at elevated concentrations in the subsurface of the FRC (http://www.lbl.gov/NABIR/). Not surprisingly, sequences related to organisms that have adapted to survival in low-nutrient environments were also present in FRC sediment before biostimulation, comprising about 22% of the clone libraries. Almost all of the sequences within the clone library before biostimulation were related to organisms with the ability to reduce nitrate, dechlorinate, or survive in low-nutrient environments. It should be noted that the above-mentioned sequences may be located within less acidic microsites of the contaminated sediment, as our measurement reflects the bulk pH of the contaminated sediment.

We hypothesized that metal-reducing members of the δ-*Proteobacteria* subdivision would make up a substantial proportion of sedimentary microbial communities after in situ biostimu-

TABLE 5. FRC contaminants and potential bioremediating organisms

Contaminant	Physiological potential	Potential bioremediating organism (reference)	Clone library	
			% Before	% After
U(VI)	Reduction and immobilization by FeRB	*Geobacter* sp. (30) *Anaeromyxobacter dehalogenans* (16) *Desulfitobacterium metallireducens* (12)	4.5	37.0
	Reduction and immobilization by fermentative FeRB	*Clostridium beijerinckii* (55) *Serratia proteamaculans* (30)	5.7	10.5
Nitrate	Reduction	*Pseudomonas stutzeri* (39) *Alcaligenes defragrans* (17) *Ralstonia pickettii* (41) *Anaeromyxobacter dehalogenans* (48) Denitrifying Fe-oxidizing clone (53) *Paenibacillus* sp. (50)	22.0	27.1
Chlorinated hydrocarbons	Dechlorination	*Methylobacterium dichloromethanicum* (21) *Anaeromyxobacter dehalogenans* (48) Clone from TCE-contaminated site (A. B. Carroll and S. H. Zinder, unpublished data) *Dechloromonas* sp. (30)	42.5	34.4
Polychlorinated biphenyls	Dechlorination	*Acidosphaera rubrifaciens* (40) *Caulobacter leidyi* (40)	14.9	2.2
Fuel hydrocarbons	Degradation	*Burkholderia* sp. N2P5 (38) *Sphingomonas paucimobilis* (38)	5.7	14.9

lation. Our hypothesis was upheld, as clone libraries were dominated by members of the δ-*Proteobacteria* subdivision, which made up about 40% of the 16S rRNA gene sequences after in situ biostimulation (Fig. 4). Cloned sequences within the δ-*Proteobacteria* subdivision were all closely related to members of the *Geobacteraceae* family or the *Anaeromyxobacter* genus. Interestingly, the δ-*Proteobacteria* subdivision sequences that were found to increase considerably after biostimulation are closely related species that have the ability to use several types of electron acceptors. Certain members of the *Geobacteraceae* family have been known to reduce both nitrate and Fe(III) (29). *Anaeromyxobacter* species can also utilize several electron acceptors, including nitrate, Fe(III), and chlorinated hydrocarbons (16, 48). It appears that organisms with the ability to utilize multiple FRC contaminants as electron donors or acceptors are able to outcompete other organisms in the acidic subsurface. Other than members of the δ-*Proteobacteria* subdivision, sequences related to species such as *Serratia proteamaculans* and *Clostridium beijerinckii*, fermentative Fe(III)-reducing organisms (30, 55), increased from 5.7 to 10.5% of the clone libraries after biostimulation (Table 5). Sequences related to both dissimilatory and fermentative FeRB showed the largest increase (10.2 to 47.5%) of sequences identified through cloning and sequencing after biostimulation. The second largest increase (5.7 to 14.9%) was that of sequences related to *Sphingomonas paucimobilis* and *Burkholderia* sp. strain N2P5, two species capable of degrading polycyclic aromatic hydrocarbons in fuel hydrocarbons (38), another significant contaminant at the FRC. Thus, a qualitative change in microbial community structure was observed after biostimulation, as the dominance of sequences related to members of the

α-*Proteobacteria* subdivision in contaminated sediment shifted to sequences related to members of the δ-*Proteobacteria* subdivision in biostimulated sediment.

In contrast, no sequences detected in the clone library from the uncontaminated background sediment were related to the metal-reducing members of the δ-*Proteobacteria* subdivision (Fig. 3). In fact, the most common 16S rRNA gene sequences retrieved from the background sediment were either within the β-*Proteobacteria* subdivision or not closely related to any previously identified organisms (Fig. 2 and 3). Most of the sequences retrieved from the background sediment were related to chemoorganotrophic and chemolithotrophic organisms.

We sought to quantify structure-function relationships of metal-reducing microbial groups in the FRC sediment. Although it would be difficult to state absolute quantification of *Geobacteraceae* species in the FRC sediment, it is clear that a large increase in 16S rRNA gene sequences closely related to this family did occur in one-half of the sites studied (Table 4) over a 3.5-month period in parallel with extensive nitrate and metal reduction (Table 3; Fig. 1). 16S rRNA gene sequences closely related to members of the *Geobacteraceae* family have been detected in several types of contaminated environments, including the subsurface (3, 18, 46, 47). For example, *Geobacter* sp. was identified in the Fe(III)-reducing zones of a petroleum-contaminated site and a landfill leachate-polluted aquifer (46, 47). Other studies involving U(VI)-contaminated aquifer sediments at neutrophilic pH revealed an enrichment of *Geobacteraceae* in carbon-amended sediment incubations (18) and during groundwater treatment (3), suggesting that members of the family *Geobacteraceae* were responsible for substantial Fe(III) and U(VI) reduction in the subsurface. In the presence

of sufficient carbon substrate, the *Geobacteraceae* group appears to be important in a variety of contaminated subsurface environments, perhaps because of its ability to utilize the abundant electron acceptors present. A close correspondence between microbial activity and an increase in 16S rRNA gene sequences related to members of the *Geobacteraceae* family suggests that *Geobacter*-type organisms are important metal reducers in the acidic subsurface of the FRC. Although *Geobacteraceae* sequences increased in half of the cores tested, other microbial groups must have been involved in bioremediation at the two remaining boreholes. Thus, it will be important to expand our studies to other organisms responding to biostimulation in acidic subsurface sediments.

We hypothesized that electron acceptor utilization was limited by low pH and a paucity of carbon substrates in the contaminated FRC subsurface. Our hypothesis was confirmed, as the abundance and diversity of organisms changed after biostimulation, when the subsurface was neutralized and carbon was added. Both qualitative and quantitative shifts in microbial communities were detected in our investigation. Results from cloning and sequencing of community DNA paralleled those of quantitative PCR to show that stimulation of members of the δ-*Proteobacteria* subdivision represented the largest change in the microbial community structure. However, in contrast to the MPN-PCR results (Table 4), clone libraries indicated that *A. dehalogenans* was a prominent member of the biostimulated microbial community (Fig. 3). This could be due to cloning bias as a result of the nucleotide composition of *Anaeromyxobacter*-type sequences. Yet, *A. dehalogenans* sequences were also the dominant sequences detected in Fe(III)-reducing enrichments in a previous study of contaminated FRC sediment (42). Both geochemical and microbial community analyses demonstrated that the FRC subsurface sediments contain a large amount of heterogeneity, even within cores. Thus, we cannot rule out the importance of this newly described metal reducer during biostimulation.

Future studies should focus on the active members of microbial populations that are stimulated through in situ carbon source addition. By using RNA (rather than DNA) as a nucleotide template, the active indigenous microbial groups involved in bioremediation processes could be better resolved.

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REFERENCES

1. **Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman.** 1990. Basic local alignment search tool. J. Mol. Biol. **215:**403–410.
2. **American Public Health Association.** 1969. Standard methods for the examination of water and wastewater, including bottom sediments and sludge, p. 604–609. American Public Health Association, Washington, D.C.
3. **Anderson, R. T., H. A. Vrionis, I. Ortiz-Bernad, C. T. Resch, P. E. Long, R. Dayvault, K. Karp, S. Marutzky, D. R. Metzler, A. Peacock, D. C. White, M. Lowe, and D. R. Lovley.** 2003. Stimulating the in situ activity of *Geobacter* species to remove uranium from the groundwater of a uranium-contaminated aquifer. Appl. Environ. Microbiol. **69:**5884–5891.
4. **Braman, R. S., and S. A. Hendrix.** 1989. Nanogram nitrate and nitrate determination in environmental and biological materials by vanadium(III) reduction with chemiluminescence detection. Anal. Chem. **61:**2715–2718.
5. **Brina, R., and A. G. Miller.** 1992. Direct detection of trace levels of uranium by laser-induced kinetic phosphorimetry. Anal. Chem. **64:**1413–1418.
6. **Chang, Y., A. D. Peacock, P. E. Long, J. R. Stephen, J. P. McKinley, S. J. Macnaughton, A. K. M. Hussain, A. M. Saxton, and D. C. White.** 2001. Diversity and characterization of sulfate-reducing bacteria in groundwater at a uranium mill tailings site. Appl. Environ. Microbiol. **67:**3149–3160.
7. **Cole, J. R., B. Chai, T. L. Marsh, R. J. Farris, Q. Wang, S. A. Kulam, S. Chandra, D. M. McGarrell, T. M. Schmidt, G. M. Garrity, and J. M. Tiedje.** 2003. The Ribosomal Database Project (RDP-II): previewing a new autoaligner that allows regular updates and the new prokaryotic taxonomy. Nucleic Acids Res. **31:**442–443.
8. **Daniels, L., R. S. Hanson, and J. A. Phillips.** 1994. Chemical analysis, p. 512–554. *In* P. Gerhardt, R. G. E. Murray, W. A. Wood, and N. R. Krieg (ed.) Methods for general and molecular bacteriology, American Society for Microbiology, Washington, D.C.
9. **DiChristina, T. J.** 1992. Effects of nitrate and nitrite on dissimilatory iron reduction by *Shewanella putrefaciens* 200. J. Bacteriol. **174:**1891–1896.
10. **Dollhopf, S. L., S. A. Hashsham, F. B. Dazzo, R. F. Hickey, C. S. Criddle, and J. M. Tiedje.** 2001. The impact of fermentative organisms on carbon flow in methanogenic systems under constant low substrate conditions. Appl. Microbiol. Biotech. **56:**531–538.
11. **Fernández, A., S. Huang, S. Seston, J. Xing, R. Hickey, C. Criddle, and J. Tiedje.** 1999. How stable is stable? Function versus community composition. Appl. Environ. Microbiol. **65:**3697–3704.
12. **Finneran, K. T., H. M. Forbush, C. Gaw VanPraagh, and D. R. Lovley.** 2002. *Desulfitobacterium metallireducens* sp. nov., an anaerobic bacterium that couples growth to the reduction of metals and humic acids, as well as chlorinated compounds. Int. J. Syst. Evol. Microbiol. **52:**1929–1935.
13. **Finneran, K. T., M. E. Housewright, and D. R. Lovley.** 2002. Multiple influences of nitrate on uranium solubility during bioremediation of uranium-contaminated subsurface sediment. Environ. Microbiol. **4:**510–516.
14. **Fredslund, L., F. Ekelund, C. S. Jacobsen, and K. Johnsen.** 2001. Development and application of a most-probable-number–PCR assay to quantify flagellate populations in soil samples. Appl. Environ. Microbiol. **67:**1613–1618.
15. **Ganesh, R., K. G. Robinson, G. D. Reed, and G. S. Sayler.** 1997. Reduction of hexavalent uranium from organic complexes by sulfate- and iron-reducing bacteria. Appl. Environ. Microbiol. **63:**4385–4391.
16. **He, Q., and R. A. Sanford.** 2003. Characterization of Fe(III) reduction by chlororespiring *Anaeromyxobacter dehalogenans*. Appl. Environ. Microbiol. **69:**2712–2718.
17. **Heyen, U., and J. Harder.** 1998. Cometabolic isoterpinolene formation from isolimonene by denitrifying *Alcaligenes defragrans*. FEMS Microbiol. Lett. **169:**67–71.
18. **Holmes, D. E., K. T. Finneran, R. A. O'Neil, and D. R. Lovley.** 2002. Enrichment of members of the family *Geobacteraceae* associated with stimulation of dissimilatory metal reduction in uranium-contaminated aquifer sediments. Appl. Environ. Microbiol. **68:**2300–2306.
19. **Hurt, R. A., X. Qiu, L. Wu, Y. Roh, A. V. Palumbo, J. M. Tiedje, and J. Zhou.** 2001. Simultaneous recovery of RNA and DNA from soils and sediments. Appl. Environ. Microbiol. **67:**4495–4503.
20. **Istok, J. D., J. M. Senko, L. R. Krumholz, D. Watson, M. A. Bogle, A. Peacock, Y.-J. Chang, and D. C. White.** 2004. *In situ* bioreduction of technetium and uranium in a nitrate-contaminated aquifer. Environ. Sci. Technol. **38:**468–475.
21. **Kayser, M. F., Z. Ucurum, and S. Vuilleumier.** 2002. Dichloromethane metabolism and C₁ utilization genes in *Methylobacterium* strains. Microbiology **148:**1915–1922.
22. **Kersters, K. P., P. De Vos, M. Gillis, J. Swings, P. VanDamme, and E. Stackebrandt.** 28 March 2003. Introduction to the proteobacteria. *In* K. P. Kersters et al. (ed.), The prokaryotes: an evolving electronic resource for the microbiological community. 3rd ed, release 3.12. [Online.] Springer-Verlag, New York, N.Y. http://link.springer-ny.com/link/service/books/10125/.
23. **Kostka, J. E., and G. W. Luther III.** 1994. Partitioning and speciation of solid phase iron in saltmarsh sediments. Geochim. Cosmochim. Acta **58:**1701–1710.
24. **Kostka, J. E., D. D. Dalton, H. Skelton, S. L. Dollhopf, and J. W. Stucki.** 2002. Growth of iron(III)-reducing bacteria on clay minerals as the sole electron acceptor and comparison of growth yields on a variety of oxidized iron forms. Appl. Environ. Microbiol. **68:**6256–6262.
25. **Laukova, A., P. Jurisce, Z. Vasilkova, and I. Papajova.** 2000. Treatment of sanitary-important bacteria by bacteriocin substance V24 in cattle dung water. Lett. Appl. Microbiol. **30:**402–405.
26. **Liger, E., L. Charlet, and P. Van Chappellen.** 1999. Surface catalysis of uranium(VI) reduction by iron(II). Geochim. Cosmochim. Acta **63:**2939–2955.
27. **Liu, Y., D. L. Balkwill, H. C. Aldrich, G. R. Drake, and D. R. Boone.** 1999. Characterization of the anaerobic propionate-degrading syntrophs *Smithella propionica* gen. nov., sp. nov. and *Syntrophobacter wolinii*. Int. J. Syst. Bacteriol. **49:**545–556.

28. **Lovley, D. R.** 1991. Dissimilatory Fe(III) and Mn(IV) reduction. Microbiol. Rev. **55:**259–287.
29. **Lovley, D. R.** 1995. Bioremediation of organic and metal contaminants with dissimilatory metal reduction. J. Ind. Microbiol. **14:**85–93.
30. **Lovley, D. R.** 2000. Fe(III) and Mn(IV) reduction, p. 3–30. *In* D. R. Lovley (ed.) Environmental microbe-metal interactions. ASM Press, Washington, D.C.
31. **Lovley, D. R.** 1 December 2000, posting date. Dissimilatory Fe(III)- and Mn(IV)-reducing prokaryotes. *In* D. R. Lovley et al. (ed.), The prokaryotes: an evolving electronic resource for the microbiological community, 3rd ed., release 3.4. [Online.] Springer-Verlag, New York, N.Y. http://link.springer -ny.com/link/service/books/10125/.
32. **Lovley, D. R.** 2002. Dissimilatory metal reduction: from early life to biore-mediation. ASM News **68:**231–237.
33. **Lovley, D. R., E. E. Roden, E. J. P. Phillips, and J. C. Woodward.** 1993. Enzymatic iron and uranium reduction by sulfate-reducing bacteria. Mar. Geol. **113:**41–53.
34. **Lovley, D. R., and R. T. Anderson.** 2000. Influence of dissimilatory metal reduction on fate of organic and metal contaminants in the subsurface. Hydrogeol. J. **8:**77–88.
35. **Madigan, M., J. M. Martinko, and J. Parker (ed.).** 2000. Brock biology of microorganisms, 9th ed., p. 608. Prentice-Hall, Inc., Upper Saddle River, N.J.
36. **McLean, E. O.** 1982. Soil pH and lime requirement, p. 199–209. *In* A. L. Page, R. H. Miller, and D. R. Keeney (ed.), Methods of soil analysis, part 2, chemical and microbiological properties—agronomy monograph no. 9, 2nd ed. ASA-SSSA, Madison, Wis.
37. **Mohagheghi, A., D. M. Updegraff, M. B. Goldhaber.** 1985. The role of sulfate reducing bacteria in the deposition of sedimentary uranium ores. Geomicro-biol. J. **4:**153–173.
38. **Mueller, J. G., R. Devereux, D. L. Santavy, S. E. Lantz, S. G. Willis, and P. H. Pritchard.** 1997. Phylogenetic and physiological comparisons of PAH-de-grading bacteria from geographically diverse soils. Antonie van Leeuwen-hoek **71:**329–343.
39. **Natural and Accelerated Bioremediation Research Program.** 2003. Biore-mediation of metals and radionuclides. . .what it is and how it works, p. 1–78. *In* T. C. Hazen, S. M. Benson, F. B. Metting, B. Faison, A. C. Palmisano, and J. McCullough (ed.), NABIR primer, 2nd ed. Lawrence Berkeley National Laboratory, Berkeley, Calif.
40. **Nogales, B., E. R. B. Moore, E. Llobet-Brossa, R. Rossello-Mora, R. Amann, and K. N. Timmis.** 2001. Combined use of 16S ribosomal DNA and 16S rRNA to study the bacterial community of polychlorinated biphenyl-polluted soil. Appl. Environ. Microbiol. **67:**1874–1884.
41. **Park, J., J. J. Kukor, and L. M. Abriola.** 2002. Characterization of the adaptive response to trichloroethylene-mediated stresses in *Ralstonia pick-ettii* PKO1. Appl. Environ. Microbiol. **68:**5231–5240.
42. **Petrie, L., N. N. North, S. L. Dollhopf, D. L. Balkwill, and J. E. Kostka.** 2003. Enumeration and characterization of iron(III)-reducing microbial commu-nities from acidic subsurface sediments contaminated with uranium U(VI). Appl. Environ. Microbiol. **69:**7467–7479.
43. **Rabus, R., T. Hansen, and F. Widdel.** 8 September 2000, posting date. Dissimilatory sulfate- and sulfur-reducing prokaryotes. *In* R. Rabus et al. (ed.), The prokaryotes: an evolving electronic resource for the microbiolog-ical community, 3rd ed., release 3.3. [Online.] Springer-Verlag, New York, N.Y. http://link.springer-ny.com/link/service/books/10125/.
44. **Rappe, M. S., K. Vergin, and S. J. Giovannoni.** 2000. Phylogenetic compar-isons of a coastal bacterioplankton community with its counterparts in open ocean and freshwater systems. FEMS Microbiol. Ecol. **33:**219–232.
45. **Riley, R. G., and J. Zachara.** 1992. Chemical contaminants on DOE lands and selection of contaminant mixtures for subsurface research. DOE/ER-0547T. U.S. Department of Energy, Washington, D.C.
46. **Röling, W. F. m., B. M. van Breukelen, M. Braster, B. Lin, and H. W. van Verseveld.** 2001. Relationships between microbial community structure and hydrochemistry in a landfill leachate-polluted aquifer. Appl. Environ. Mi-crobiol. **67:**4619–4629.
47. **Rooney-Varga, J. N., R. T. Anderson, J. L. Fraga, D. Ringelberg, and D. R. Lovley.** 1999. Microbial communities associated with anaerobic benzene degradation in a petroleum-contaminated aquifer. Appl. Environ. Microbiol. **65:**3056–3063.
48. **Sanford, R. A., J. R. Cole, and J. M. Tiedje.** 2002. Characterization and description of *Anaeromyxobacter dehalogenans* gen. nov., sp. nov., an aryl-halorespiring facultative anaerobic myxobacterium. Appl. Environ. Micro-biol. **68:**893–900.
49. **Senko, J. M, J. D. Istok, J. M. Suflita, and L. R. Krumholz.** 2002. In-situ evidence for uranium immobilization and remobilization. Environ. Sci. Tech-nol. **36:**1491–1496.
50. **Shida, O., H. Takagi, K. Kadowaki, L. K. Nakamura, and K. Komagata.** 1997. Transfer of *Bacillus alginolyticus*, *Bacillus chondroitinus*, *Bacillus curd-lanolyticus*, *Bacillus glucanolyticus*, *Bacillus kobensis*, and *Bacillus thiami-nolyticus* to the genus *Paenibacillus* and emended description of the genus *Paenibacillus*. Int. J. Syst. Bacteriol. **47:**289–298.
51. **Snoeyenbos-West, O. L., K. P. Nevin, R. T. Anderson, and D. R. Lovley.** 2000. Enrichment of *Geobacter* species in response to stimulation of Fe(III) re-duction in sandy aquifer sediments. Microb. Ecol. **39:**153–167.
52. **Stookey, L. L.** 1970. Ferrozine: a new spectrophotometric reagent for iron. Anal. Chem. **42:**779–781.
53. **Straub, K. L., M. Benz, B. Schink, and F. Widdel.** 1996. Anaerobic, nitrate-dependent microbial oxidation of ferrous iron. Appl. Environ. Microbiol. **62:**1458–1460.
54. **Strunk, O., and W. Ludwig.** 1997. ARB: software for phylogenetic analysis. Technical University of Munich, Munich, Germany.
55. **Suzuki, Y., S. D. Kelly, K. M. Kemner, and J. F. Banfield.** 2003. Microbial populations stimulated for hexavalent uranium reduction in uranium mine sediment. Appl. Environ. Microbiol. **69:**1337–1346.
56. **Tanner, M. A., B. M. Goebel, M. A. Dojka, and N. R. Pace.** 1988. Specific ribosomal DNA sequences from diverse environmental settings correlate with experimental contaminants. Appl. Environ. Microbiol. **64:**3110–3113.
57. **Tebo, B. M., and A. Y. Obraztsova.** 1998. Sulfate-reducing bacterium grows with Cr(VI), U(VI), Mn(IV), and Fe(III) as electron acceptors. FEMS Microbiol. Lett. **162:**193–198.
58. **Thomas, R. A. P., K. Lawlor, M. Bailey, and L. E. Macaskie.** 1998. Biodeg-radation of metal-EDTA complexes by an enriched microbial population. Appl. Environ. Microbiol. **64:**1319–1322.