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Identification and Isolation of a *Castellaniella* Species Important during Biostimulation of an Acidic Nitrate- and Uranium-Contaminated Aquifer▽

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Immobilization of uranium in groundwater can be achieved through microbial reduction of U(VI) to U(IV) upon electron donor addition. Microbial community structure was analyzed in ethanol-biostimulated and control sediments from a high-nitrate (>130 mM), low-pH, uranium-contaminated site in Oak Ridge, TN. Analysis of small subunit (SSU) rRNA gene clone libraries and polar lipid fatty acids from sediments revealed that biostimulation resulted in a general decrease in bacterial diversity. Specifically, biostimulation resulted in an increase in the proportion of *Betaproteobacteria* (10% of total clones in the control sediment versus 50 and 79% in biostimulated sediments) and a decrease in the proportion of *Gammaproteobacteria* and *Acidobacteria*. Clone libraries derived from dissimilatory nitrite reductase genes (*nirK* and *nirS*) were also dominated by clones related to *Betaproteobacteria* (98% and 85% of total *nirK* and *nirS* clones, respectively). Within the *nirK* libraries, one clone sequence made up 59 and 76% of sequences from biostimulated sediments but only made up 10% of the control *nirK* library. Phylogenetic analysis of SSU rRNA and *nirK* gene sequences from denitrifying pure cultures isolated from the site indicate that all belong to a *Castellaniella* species; nearly identical sequences also constituted the majority of biostimulated SSU rRNA and *nirK* clone libraries. Thus, by combining culture-independent with culture-dependent techniques, we were able to link SSU rRNA clone library information with *nirK* sequence data and conclude that a potentially novel *Castellaniella* species is important for in situ nitrate removal at this site.

Due to the Cold War legacy, uranium has become an important groundwater contaminant in the United States, thus mandating remediation by the U.S. Department of Energy (DOE). Soluble U(VI) can be biologically reduced to U(IV), which is insoluble, thus immobilizing the radionuclide and posing less of a threat to drinking water wells located near sources of contamination (24, 44). It has been suggested that bacteria capable of U(VI) reduction are ubiquitous in the environment (1), and recent field experiments have shown that the addition of electron donors (glucose, ethanol, or acetate) into injection wells will result in the stimulation of endogenous microorganisms in the subsurface to grow and reduce U(VI) (3, 12, 36, 54, 60, 64).

Microbial communities stimulated to reduce U(VI) via electron donor addition have been studied using both in situ and microcosm experiments. Members of the *Geobacteraceae* family have been stimulated during uranium reduction in contaminated sediments from Shiprock, NM (33), Rifle, CO (3, 12), and Oak Ridge, TN (51, 54). From studies done with sediment from Oak Ridge, *Anaeromyxobacter* was also stimulated under metal-reducing conditions (51, 55). In other studies, sulfate-reducing bacteria have been linked to uranium reduction (1, 13, 49, 52, 61). Of these, two studies have also found *Clostridium* to be associated with U(VI) reduction (52, 61), and another found that *Pseudomonas* was also stimulated upon uranium removal in high-salinity sediment (49).

At the DOE Field Research Center (FRC) in Oak Ridge, TN, where groundwater contains >130 mM nitrate and micromolar concentrations of uranium, addition of a biodegradable electron donor results in denitrification as the primary terminal electron-accepting process (36). Because nitrate serves as a more energetically favorable electron acceptor, uranium reduction has been shown to occur only after nitrate has been depleted to low levels (17, 23, 36, 48, 60). Thus, at sites such as the FRC, denitrifying bacteria are likely to play a critical role in uranium bioremediation. A recent phylogenetic survey of sediment from the FRC revealed several potential nitrate-reducing bacteria (2), but it remains unclear what species are involved in nitrate removal upon biostimulation.

The goal of this study was to characterize changes in the in situ microbial community structure of uranium- and nitrate-contaminated subsurface sediments upon stimulation with ethanol and to identify denitrifying bacteria that may be important during the in situ removal of nitrate. While other molecular studies have identified mainly sulfate and metal reducers in uranium-contaminated sediments, it was hypothesized in this study that electron donor addition to high-nitrate subsurface sediments cocontaminated with low levels of uranium would result mainly in the stimulation of denitrifying bacteria. Because denitrification is not a phylogenetically conserved function, numerous methods were used to analyze the microbial community structure of biostimulated and control sediments, including functional gene (*nirK* and *nirS*) clone libraries, small

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TABLE 1. Summary of initial groundwater chemistry, push-pull test results, and sediment core characteristics

Groundwater well (corresponding sediment core)	Initial groundwater chemistry[c]			Push-pull test result[b] (C/C_0)[d]			Sediment core data (following biostimulation)[e]			
	NO_3^- (mM)	U(VI) (μM)	pH	NO_3^-	EtOH	U(VI)	NO_3^- (mM)	NO_2^- (mM)	Biomass (10^6 cells/g)	% U as U(IV)
FW028 (FB064)	167.2	2.22	4.4	0.327	0.358	15.2	126.5	17.7	204	67.1
FW034 (FB067)	0.769	0.475	6.79	0.640	0.528	0.924	39.77	14.8	18.5	75.7
FW016 (FB066)[a]	11.4	2.58	3.92				2.612	9.98	5.58	4.6
FW021[b]	142.3	5.80	3.05							

[a] FW016 served as a control well.
[b] FW021 groundwater was used for the injection solution for push-pull tests in FW028 and FW034.
[c] Data publicly available at http://public.ornl.gov/nabirfrc/frcsite3.cfm.
[d] These data represent analyte concentrations (C) on the first date of the extraction phase (on or near the date of sediment core sampling) compared to initial concentrations in the injection solution (C_0) and have been adjusted to account for loss due to dilution and dispersion, as determined by loss of bromide.
[e] Sediment core FB064 was obtained 5 days after the injection phase began and 3 days prior to the start of the extraction phase for well FW028; FB067 was sampled 31 days after the injection phase began and on the same day of the start of the extraction phase.

subunit (SSU) rRNA gene clone libraries, polar lipid fatty acid (PLFA) analysis, and cultivation of nitrate-reducing bacteria from FRC sediments. Results of this study show that biostimulation of high-nitrate subsurface sediments with ethanol results in a decrease in bacterial diversity and enriches for members of the class *Betaproteobacteria*, namely, members of the newly described genus *Castellaniella* (formerly *Alcaligenes defragrans*), which are capable of nitrate reduction.

MATERIALS AND METHODS

Field site description. The field site in this study is the DOE's Environmental Remediation Sciences Program FRC, which is located near the western edge of the Y-12 national security complex at the Oak Ridge Reservation (Oak Ridge, TN). The source of the contamination plume in the shallow unconfined aquifer at the FRC comes from the former S-3 waste disposal ponds. These ponds received acidic (pH, <2) liquid waste containing nitric acid, uranium, technetium, other dissolved metals, and organic contaminants from 1951 to 1983; the ponds were neutralized in 1984 and capped in 1988. Several monitoring wells have been installed within the Area 1 field plot (just south of the former S-3 ponds), and groundwater within Area 1 has been described as acidic (pH ranging from 3.0 to 6.8), with high concentrations of nitrate (up to 168 mM), U(VI) (up to 5.8 μM), Tc(VII) (up to 12,000 pCi/liter), and <1 mM sulfate (36). Table 1 shows nitrate, uranium, and pH data from four monitoring wells before push-pull experiments began. Other contaminants in Area 1 include aluminum, nickel, tetrachloroethylene, and other chlorinated hydrocarbons. A more detailed description of the site as well as groundwater and sediment geochemical data can be found at the URL http://www.esd.ornl.gov/nabirfrc/index.html.

In situ biostimulation of subsurface sediments. Single-well, push-pull tests were done in wells FW028 and FW034 in Area 1 as previously described (36, 65, 66). Test solutions for push-pull tests were prepared using high-nitrate (>130 mM) Area 1 groundwater (from well FW021) amended with 300 mM ethanol, 50 to 100 mM sodium bicarbonate, and 1.25 mM Br^- as a conservative tracer. Sediment cores were sampled adjacent to wells FW028 and FW034 (cores FB064 and FB067, respectively) approximately 1 week after injection of test solutions; the injection phase lasted only a few hours for FW028 but lasted approximately 3 weeks for FW034, due to differences in well flow characteristics due to past push-pull experiments. Thus, FB064 and FB067 were sampled 5 and 31 days, respectively, after the beginning of the injection phase. One sediment core (FB066) was also taken adjacent to an Area 1 donor control well FW016, which has never been biostimulated in push-pull tests. Sediment sampling and handling procedures followed those previously described (66) in order to keep sediment material anoxic. Core sizes were all approximately 1 meter in length and were sampled from the following depths below the surface: 6.1 to 7.0 m, 3.4 to 4.3 m, and 3.0 to 4.0 m for cores FB064, FB067, and FB066, respectively. Intact subsections of cores, approximately 10 cm in length, were frozen at −80°C and were later shipped on dry ice to the University of Oklahoma for molecular analysis. A subsection of another core from borehole FB064, taken from 5.2 to 5.7 m below the surface, was stored at 4°C and shipped to the University of Oklahoma on ice for enrichment and isolation of denitrifying bacteria.

Enrichment and isolation of denitrifying pure cultures. Medium for enrichment of dissimilatory nitrate-reducing microorganisms was prepared anaerobically (5) with the following components (per liter): 10 ml vitamin solution (47), 5 ml metals solution (47), 0.1 g NaCl, 0.1 g NH_4Cl, 10 mg KCl, 3 mg KH_2PO_4, 40 mg $MgCl_2 \cdot 6H_2O$, 40 mg $CaCl_2 \cdot 2H_2O$, 11.9 g HEPES, 11.7 g morpholineethanesulfonic acid (MES), and 8.5 g $NaNO_3$. The pH of the medium was adjusted to either 4.5 or 7.5 using HCl or NaOH and dispensed into serum tubes under an N_2 headspace. Ethanol was added from a sterile, anoxic stock solution to reach a final concentration of 100 mM.

Anaerobic nitrate-reducing enrichment cultures were set up in an anaerobic glovebag by adding 1 g of homogenized biostimulated sediment from borehole FB064 to 10 ml nitrate-reducing liquid medium at both pH 4.5 and 7.5. Headspace of enrichment cultures was exchanged three times with N_2 and incubated in the dark at room temperature. Upon observable growth and removal of nitrate, enrichments were serially diluted and plated onto solid nitrate-reducing media both with and without ethanol at either pH 4.5 or 7.5, depending on the pH of the enrichment culture. Nitrate-reducing solid medium had the same composition as the liquid media except it contained 1.5% agar and 1.7 g/liter $NaNO_3$. After autoclaving, the medium was dispensed into plates and dried overnight. Plates were placed in an anaerobic glovebag (Coy Instruments) overnight. Subsequently, a piece of sterile filter paper was placed in the lid of each petri dish and saturated with 500 μl of a sterile, anoxic 1 M ethanol solution. All plates were incubated at room temperature in an anaerobic glovebag. Colonies from plates containing ethanol that differed in morphology from colonies on ethanol-free plates were further reisolated and transferred to nitrate-reducing liquid medium at pH 4.5 or 7.5. In total, 24 colonies were obtained from pH 7.5 enrichment cultures and 22 from pH 4.5 enrichment cultures.

DNA extraction. DNA was extracted from frozen soil cores from boreholes FB064, FB067, and FB066 (from depths of 6.4, 4.6, and 3.6 m below the surface, respectively) using the FastDNA SPIN kit for soil (QBiogene, Irvine, CA), which involves a silica and ceramic bead-beating method to achieve cell lysis. Manufacturer's instructions were followed, except nuclease-free water was used as the eluent. In order to increase DNA yield and to account for heterogeneity of the cores, 10 DNA extractions using 0.3 g sediment were done from each core. The 10 DNA samples were then pooled and concentrated by using a Centrivap at 45°C. DNA samples were stored at −20°C.

DNA was extracted from pure cultures by boiling late-log-phase washed cells for 5 minutes; samples were centrifuged to remove cell debris, and supernatants were transferred to clean, sterile 1.5-ml microcentrifuge tubes and stored at −20°C for use as DNA template for PCRs.

PCR, cloning, and sequencing. Partial SSU rRNA genes from sediment community DNA and denitrifying isolates were amplified using 2 μl of DNA template in a 50-μl PCR mixture (<100 ng/μl, final concentration) containing the following components: 1× PCR buffer (Invitrogen Corp., Carlsbad, CA), 2.5 mM $MgCl_2$, 100 μM each deoxynucleoside triphosphate, 10 pmol/ml each primer (uni8f and eubac805r) (19), and 1.5 U of Platinum *Taq* DNA polymerase (Invitrogen). Amplification of partial SSU rRNA genes was carried out in a GeneAmp PCR system 9700 (Applied Biosystems, Foster City, CA) using the following parameters: initial denaturation at 94°C for 5 min; 35 cycles of 95°C for 30 s, 50°C for 60 s, and 72°C for 90 s; and a final extension step at 72°C for 20 min. Near-complete SSU rRNA genes of two denitrifying isolates (4.5A2 and 7.5A2) were amplified in the same manner, only using universal primers 27F and 1492R and an annealing temperature of 45°C.

Amplification of *nirK* and *nirS* genes from sediment community DNA and denitrifying isolates used the same PCR mixture as described above, except that

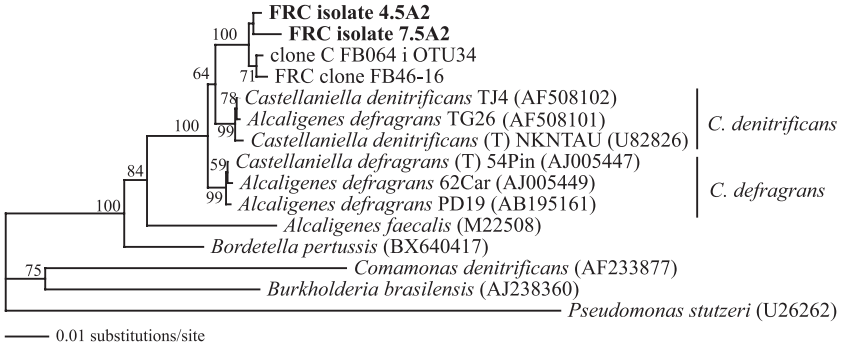

FIG. 1. Distance phylogram based on near-full-length SSU rRNA gene sequences (approximately 1,490 bp) from FRC isolates (in bold), FRC sediment clone sequences (clone C FB064 I OTU34 was identified from the FRC biostimulated sediment in this study), and other members of *Castellaniella* as well as related organisms in the order *Burkholderiales* (accession numbers are shown in parentheses). Bootstrap values are based on 1,000 replicates and are shown for branches with bootstrap support of >50%.

primer concentrations were 12.5 pmol/ml, *nirK* primers were nirK1F and nirK5R, and *nirS* primers were nirS1F and nirS6R (9). PCR parameters were as follows: 94°C for 5 min; 35 cycles of 94°C for 30 s, 54°C for 45 s, and 72°C for 45 s; and a final extension at 72°C for 20 min.

PCR products were cloned using the TOPO TA cloning kit (Invitrogen Corp., Carlsbad, CA) either directly from the PCR product or after a gel purification step using a commercially available kit (QBioGene). Sequencing of inserts was performed by the Advanced Center for Genome Technology at the University of Oklahoma (Norman) or the Oklahoma Medical Research Foundation (Oklahoma City, OK).

Phylogenetic analysis. SSU rRNA gene sequences were aligned using ClustalX (62). Sequences with similarities of ≥97% were placed into the same operational taxonomic unit (OTU); also, sequences with ≥93% similarity were placed into the same genus-level taxonomic group (GLTG). Possible chimera within our libraries were identified using Bellerophon (34) and by manual inspection. Chimeric sequences made up approximately 10% of total sequences and were removed from further phylogenetic analyses. Initial phylogenetic placement of each SSU rRNA gene OTU was determined using the Ribosomal Database Project's Classifier program (14). Closely related sequences and sequences identified from this site in previous studies were downloaded from GenBank and aligned with our sequences using ClustalX; the multiple alignment was imported into PAUP 4.01b10 for final phylogenetic analysis. Evolutionary distance-based trees were generated using the neighbor-joining algorithm and Jukes-Cantor corrections. Bootstrap values were determined using 1,000 replicates.

The Shannon-Weiner diversity index, Simpson's dominance index, and species evenness were calculated as previously described (57). A limitation of these indices is that each OTU is considered equivalent, regardless of the degree of sequence divergence (46). To ameliorate this bias, diversity indices were calculated at both the OTU level as well as the GLTG level; also, average nucleotide divergence was calculated for each clone library (46). Calculations of percent coverage were done as described elsewhere (58) at both the OTU and GLTG levels.

A chi-square test for an $r \times k$ contingency table was done to determine whether the population distribution in biostimulated samples differed from the unstimulated sample. Rows (r) were phylum affiliation, and columns (k) were different samples (biostimulated and unstimulated). Expected frequencies for each phylum in each sample (E) were calculated by the equation E = (row total) × [(column total)/(grand total)]. A chi-squared value was determined by the equation $\chi^2 = \Sigma(O - E)^2/E$ (O = observed frequency). The critical χ^2 value was chosen with nine degrees of freedom and with a P value of 0.05.

Phylogenetic analysis of *nirK* and *nirS* genes was done similarly to that of the SSU rRNA genes described above. Sequences were grouped into OTUs based on ≥98% nucleotide sequence similarity, and the closest relatives were identified and downloaded using BLAST. Other reference *nirK* and *nirS* sequences were downloaded from the Functional Gene Pipeline/Repository (http://flyingcloud.cme.msu.edu/fungene/). Neighbor-joining trees were constructed from translated amino acid sequences. Similarity values reported in the results are based on amino acid similarity.

PLFA extraction and analysis. Lyophilized sediment from each core was extracted with the single-phase chloroform-methanol-buffer system (8), as later modified (67). The total lipid extract was fractionated into neutral lipids, glycolipids, and polar lipids by silicic acid column chromatography (29). PLFA analysis was conducted as previously described (56). Biomass (cells/g of sediment) was calculated from total PLFA/g of sediment using the conversion 2.5×10^4 cells per pmol PLFA (6). Shannon-Weiner diversity indices for sediment samples were also calculated based on PLFA (31).

Analytical methods. Uranium speciation from sediment cores FB064, FB067, and FB066 was determined by sequential extractions of total U(VI) (soluble and solids associated) and U(IV) from triplicate 0.5-g sediment subsamples using sodium bicarbonate and nitric acid, respectively (18). Uranium from each extraction was measured by kinetic phosphorescence analysis (KPA-11; Chemcheck Instruments, Richland, WA). Nitrate and nitrite from nitrate-reducing enrichments and sediment-associated pore water were measured by ion chromatography (model DX500 fitted with an AS-4A column; Dionex Corp., Sunnyvale, CA). Push-pull groundwater analysis was done at Oregon State University as previously described (36).

Nucleotide sequence accession numbers. SSU rRNA, *nirK*, and *nirS* sequences from this study were deposited with GenBank and can be retrieved with accession numbers EF175318 to EF175380 and EF177768 to EF177803.

RESULTS

Isolation and phylogenetic analysis of denitrifying pure cultures. From nitrate-reducing enrichments using biostimulated sediment as the inoculum, all 46 pure cultures, once restreaked for purity, shared the same colony morphology on nitrate-reducing medium: colonies were convex, round, small (<1 mm in diameter), and white, with smooth margins. Upon inoculation into liquid medium at pH 4.5 and pH 7.5, all pure cultures were capable of growth (to a final optical density of approximately 0.4 at 600 nm) using nitrate and ethanol as the sole electron acceptor and donor, respectively; gas production indicated that the organisms coupled growth to denitrification rather than reduction of nitrate to nitrite or ammonium. Because of the similar colony morphologies and growth characteristics, 10 of the pure cultures were chosen at random for phylogenetic analysis; SSU rRNA gene sequences of these isolates were 97.6 to 100% similar to each other with an average similarity of 99.4%, suggesting these isolates belong to the same species within the family *Alcaligenaceae* and the class *Betaproteobacteria*.

Two strains, 4.5A2 and 7.5A2 (isolated at pH 4.5 and 7.5, respectively), which had 99.9% SSU rRNA gene sequence similarity, were chosen for further phylogenetic analysis. Isolates 4.5A2 and 7.5A2 were 99.4 and 99.7% similar to clone FB46-16, which was identified from biostimulated FRC sediments in a previous study (51). The closest cultured relative

TABLE 2. Descriptive diversity statistics based on SSU rRNA gene clone library data from two ethanol-stimulated sediments and one control sediment

Sample (condition)	No. of clones	No. of OTUs[a]	No. of GLTGs[b]	Avg. nucleotide diversity	Diversity index based on OTUs			Diversity index based on GLTGs		
					Shannon-Weiner	Simpson's	Evenness	Shannon-Weiner	Simpson's	Evenness
FB064 (stimulated)	58	23	13	0.0994	2.010	0.3181	0.641	1.234	0.5333	0.481
FB067 (stimulated)	64	21	18	0.1518	2.173	0.2266	0.714	2.012	0.2427	0.696
FB066 (control)	51	21	16	0.1767	2.512	0.1272	0.825	2.222	0.1572	0.801

[a] Similarity cutoff of 97% (OTUs).
[b] Similarity cutoff of 93% (GLTGs).

was *Alcaligenes* sp. strain AMS10, which was isolated from a polycyclic aromatic hydrocarbon-degrading consortium (GenBank accession no. AY635901). The closest validly described relatives belong to the genus *Castellaniella*, which consists of two described species, *C. defragrans* and *C. denitrificans*, both of which were previously identified as *Alcaligenes defragrans* (40). Isolates 4.5A2 and 7.5A2 were 98.3 and 98.5% similar to *C. defragrans* 54Pin, which was isolated from activated sludge on nitrate and α-pinene (25), and 98.4% similar to *C. denitrificans* TJ4, a phenol-degrading, denitrifying bacterium (4). Neighbor-joining analysis and bootstrap values supported that FRC isolates 4.5A2 and 7.5A2 may not belong to either of the previously described species of *Castellaniella* and could represent a novel species within the genus *Castellaniella* (Fig. 1). However, further physiological tests are needed to prove this.

While the *nirS* gene was not detected by PCR in any of the 10 isolates, all contained a *nirK* gene, which provides evidence that these strains are denitrifying bacteria. All *nirK* partial gene sequences from these isolates were 99.0 to 100% similar to each other, reaffirming that these isolates are likely different strains among the same species. Furthermore, translated amino acid sequences of NirK from isolates 4.5A2 and 7.5A2 were 100% identical to each other, 84.8% identical to NirK of a clone identified from acetate-fed activated sludge (clone NR2-819K1; GenBank accession no. BAD36891), and 81.8% identical to NirK from *Alcaligenes* sp. strain N, isolated from a denitrifying reactor (20).

In situ biostimulation of contaminated subsurface sediments and reduction of U(VI). Push-pull tests were done with ethanol-amended, high-nitrate (142.3 mM) FW021 groundwater (neutralized with bicarbonate) in two wells, FW028 and FW034. Prior to biostimulation, the groundwater from FW028 contained high levels of nitrate (167.2 mM) and uranium (2.2 μM) and was more acidic than FW034, which contained <1 mM nitrate and 0.475 μM uranium (Table 1). The control well, FW016, was also acidic but contained 11.4 mM nitrate (Table 1). Following injection of ethanol-amended FW021 groundwater into FW028 and FW034, push-pull data showed nitrate and ethanol loss in both test wells by the time of sediment sampling and U(VI) accumulation in FW028, suggesting U(IV) oxidation may have occurred in this well (Table 1). However, analysis of uranium from bicarbonate- and nitric acid-extractable fractions from sediment cores showed that the majority of the uranium in both cores adjacent to ethanol-stimulated wells (FB064 and FB067, corresponding to wells FW028 and FW034, respectively) was reduced, whereas only 4.6% of the total uranium from the control core FB066 (adjacent to FW016) was reduced (Table 1), suggesting that the U in stimulated cores remained fairly reduced, compared to the control, which has never been biostimulated. Some of the U(IV) in biostimulated cores may have been due to previous push-pull tests performed in adjacent wells (36). Biomass estimates based on total PLFA from sediment cores following in situ biostimulation showed that FB064 and FB067 had approximately 37- and 3-fold higher biomass than the control core, FB066 (Table 1). Pore water nitrate concentrations from the three cores varied, which can be explained by the differences in initial nitrate concentrations of the three sites. Nitrite was present at high concentrations (≥10 mM) in all three (Table 1), indicating that nitrate reduction was not complete in these sediment cores.

Differences in bacterial community structure between ethanol-stimulated and unstimulated sediment samples. (i) Diversity statistics. According to all diversity indices calculated from SSU rRNA gene clone library data (at both the OTU and GLTG levels), both biostimulated sediments, FB064 and FB067, were less diverse than the control sediment, FB066 (Table 2). The percent coverage was 64, 78, and 71% (at the OTU level) and 83, 83, and 80% (at the GLTG level) for sediment samples FB064, FB067, and FB066, respectively. There was a significant negative linear correlation between log biomass of the sediments and average nucleotide divergence ($r = -0.999$, $P = 0.01$), indicating that genetic diversity decreased with increasing biomass. Similarly, when diversity indices were calculated based on GLTGs, there were negative correlations between log biomass versus Shannon-Weiner di-

TABLE 3. Summary of phylogenetic distributions of SSU rRNA clones from samples FB064, FB067, and FB066

Phylum or candidate division	% of total clones		
	FB064 (stimulated)	FB067 (stimulated)	FB066 (control)
Proteobacteria	93.1	84.4	56.9
Betaproteobacteria	79.3	50.0	9.8
Deltaproteobacteria	0.0	6.3	0.0
Gammaproteobacteria	12.1	26.6	47.1
Unclassified	1.7	1.6	0.0
Bacteroidetes	1.7	0.0	2.0
Firmicutes	3.4	3.1	2.0
Actinobacteria	0.0	1.6	2.0
Acidobacteria	0.0	1.6	27.5
Candidate division WD272_C2	0.0	6.3	9.8
Candidate division TM7	1.7	0.0	0.0
Candidate division ZB1	0.0	1.6	0.0
Termite group I	0.0	1.6	0.0

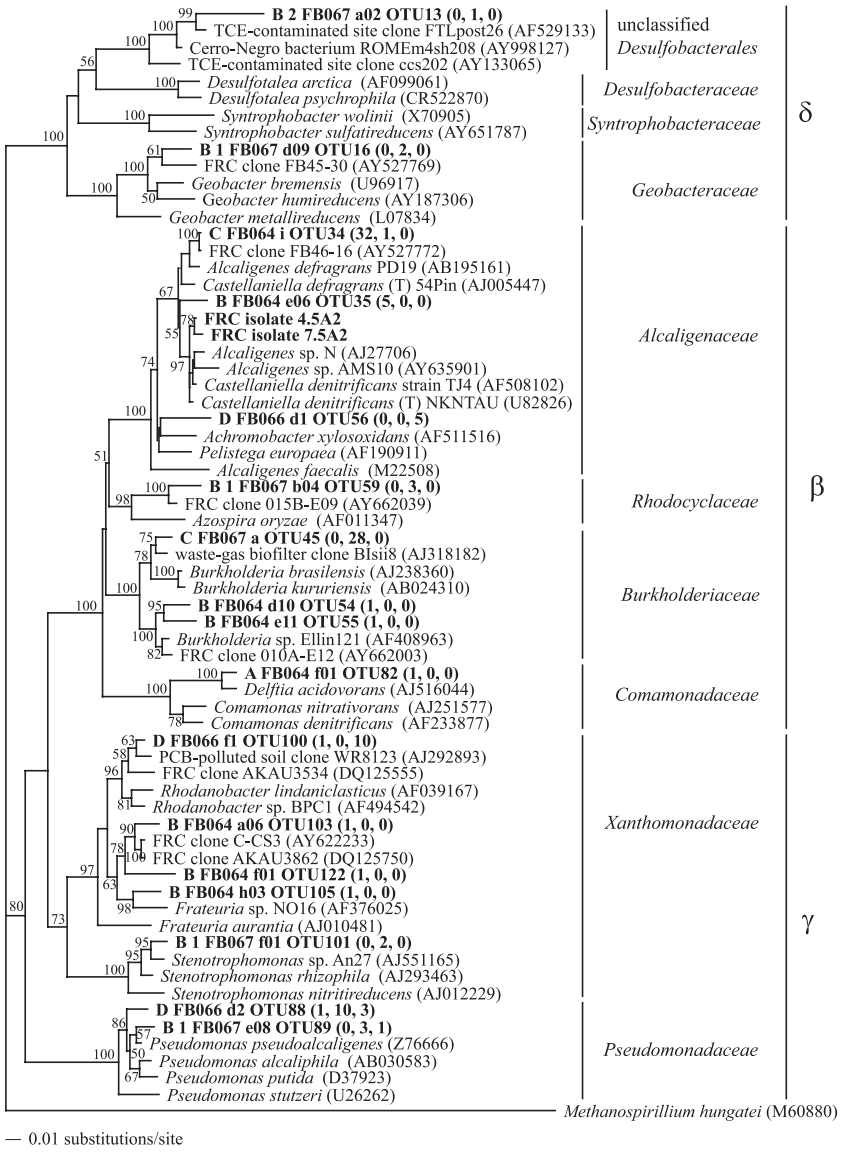

FIG. 2. Distance phylogram of *Proteobacteria* partial SSU rRNA gene sequences (approximately 800 bp). Bootstrap values are based on 1,000 replicates and are shown for branches with bootstrap support of >50%. Selected OTUs from this study as well as FRC isolate sequences are in bold, and numbers in parentheses indicate the number of clones belonging to that OTU from sediments FB064, FB067, and FB066, respectively. Accession numbers of sequences from GenBank are in parentheses.

versity index ($r = -0.992$, $P < 0.05$) and log biomass versus evenness ($r = -0.999$, $P = 0.01$). In addition, there was a positive correlation between log biomass and Simpson's dominance index at both the OTU level ($r = 0.977$, $P < 0.1$) and the GLTG level ($r = 0.993$, $P < 0.04$), indicating that increasing biomass resulted in the selection of one dominant species or genus. Correlations between log biomass and diversity indices were more significant when using GLTGs rather than OTUs; this was due to the high number of OTUs in sample FB064 that belonged to the same GLTG. Taking all diversity indices into account, biostimulation may have led to an overall decrease in bacterial diversity and an increase in dominance of one species or genus. Past push-pull biostimulation experiments performed in injection wells FW028 and FW034 (36) may have also contributed to this effect.

(ii) Community composition. The majority of clones from SSU rRNA gene clone libraries from the biostimulated sediment cores, FB064 and FB067, belonged to the beta, delta, and gamma subdivisions of *Proteobacteria* (88.5%); the remaining clones belonged to *Bacteroidetes*, *Firmicutes*, *Actinobacteria*, *Acidobacteria*, and candidate divisions TM7, ZB1, termite group I, and WD272_C2 (Table 3 and Fig. 2 and 3). In the SSU rRNA gene clone library from the unstimulated sediment core (FB066), *Proteobacteria* (beta and gamma subdivisions) made up only 56.9% of the total clones, while other clones were affiliated with *Acidobacteria* (27.5% of total clones), *Bacteroidetes*, *Firmicutes*, and candidate division WD272_C2 (Table 3 and Fig. 2 and 3). By performing chi-square tests based on $r \times k$ contingency tables of frequencies of each phylum, it was found that the community structures of the two biostimu-

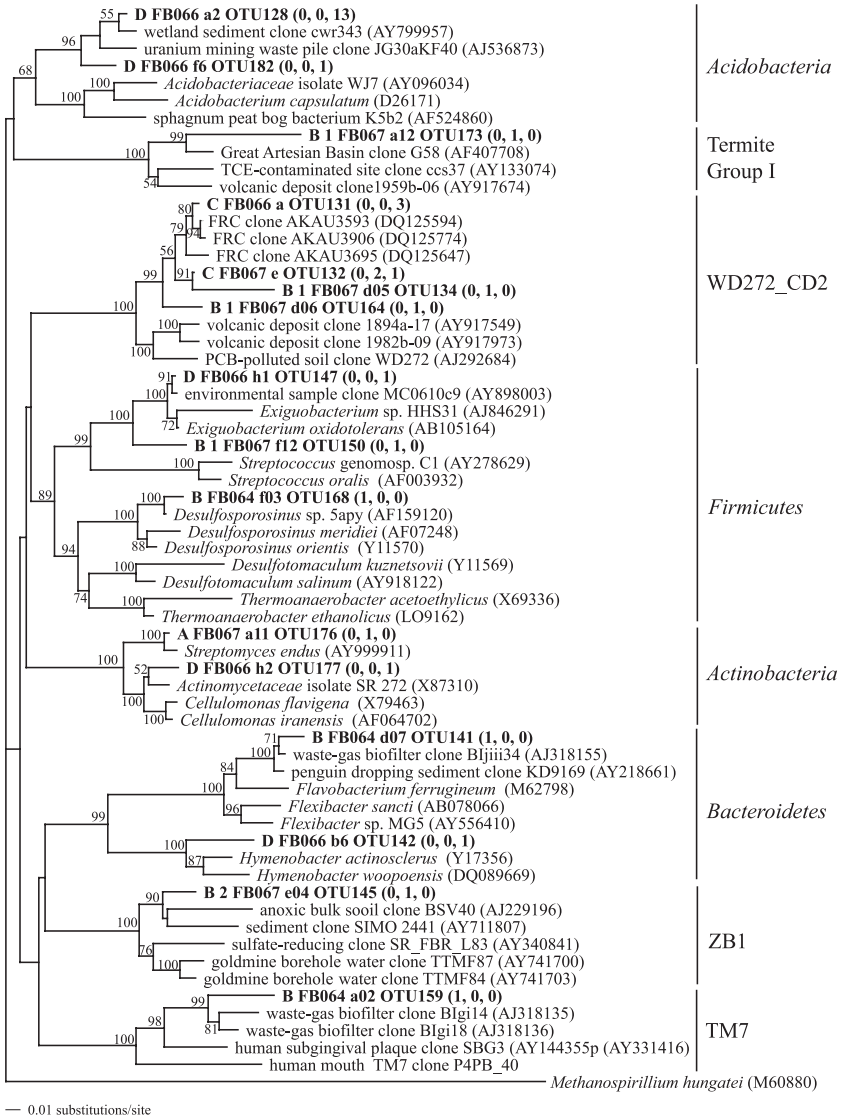

FIG. 3. Distance phylogram of non-*Proteobacteria* partial SSU rRNA gene sequences (approximately 800 bp). Bootstrap values are based on 1,000 replicates and are shown for branches with bootstrap support of >50%. Selected OTUs from this study are in bold, and numbers in parentheses indicate the number of clones belonging to that OTU from sediments FB064, FB067, and FB066, respectively. Accession numbers of sequences from GenBank are in parentheses.

lated samples (FB064 and FB067) did not differ significantly ($P > 0.2$), whereas community structures of the biostimulated versus unstimulated samples were significantly different ($P < 0.001$). Thus, biostimulation of subsurface sediments with ethanol-amended groundwater significantly impacted the subsurface microbial community structure at the phylum/division level. Most noticeably, these differences may have been due to the frequencies of *Proteobacteria* and *Acidobacteria* OTUs in the biostimulated versus the control clone libraries (Table 3).

Biostimulation resulted in an increase in the proportion of *Betaproteobacteria* sequences in the SSU rRNA gene clone libraries (9.8% of total clones in FB066 compared to 79.3% and 50.0% in FB064 and FB067, respectively) (Table 3). As biomass of the samples increased (Table 1), so did the percent of clones that belong to *Betaproteobacteria* (Table 3). Of the *Betaproteobacteria* clones from the core with the highest biomass, FB064, 69.6% belonged to OTU 34 and 10.7% belonged to OTU 35. Both OTUs 34 and 35 grouped with members of the genus *Castellaniella* (Fig. 2) and were 100% and 97.6% similar to FRC isolate 7.5A2, respectively. Only one clone from FB067 belonged to OTU 34; rather, 87.5% of *Betaproteobacteria* clones from FB067 belonged to OTU 45, whose closest relative was clone BIsii8 (97.8% similarity), which was identified from an industrial waste gas biofilter (26). Its two closest cultured relatives were *Burkholderia brasilensis*, an N_2-fixing bacterium (GenBank accession no. AJ238360), and *Burkholderia kururiensis*, a trichloroethylene-degrading bacterium isolated from a trichloroethylene-contaminated aquifer (72).

Unlike the effect observed on the class *Betaproteobacteria*, biostimulation resulted in a decrease in the proportion of *Gammaproteobacteria* sequences in the SSU rRNA gene clone

libraries (Table 3). In the control clone library (FB066), 47.1% of total clones were affiliated with *Gammaproteobacteria*, and of these, the majority (70.8%) belonged to the family *Xanthomonadaceae*, while others were affiliated with *Pseudomonadaceae*. The dominant *Gammaproteobacteria* OTU from the control FB066 (OTU 100) belonged to the genus *Rhodanobacter* and was closely related to other sequences identified from unstimulated contaminated sites, including groundwater from the FRC (Fig. 2).

Similarly, biostimulated sediments contained a decreased proportion of *Acidobacteria* clones compared to the control sediment (Table 3). The dominant OTU from the control sediment sample FB066 (OTU 128) belonged to *Acidobacteria* and clustered with other environmental *Acidobacteria* clones (Fig. 3); however, only one *Acidobacteria*-affiliated sequence was detected in the biostimulated libraries (Table 3).

(iii) Novel bacterial diversity identified in SSU rRNA gene clone libraries. From the three SSU rRNA gene clone libraries generated in this study, 7.5% of all clones belonged to divisions with no cultivated representatives. Three clones belonged to candidate divisions TM7, termite group I, and ZB1 (Table 3; Fig. 3). Nine clones from FB066 and FB067 (belonging to five OTUs) clustered with each other and with other clones, belonging to the candidate division WD272_C2, from the FRC (Fig. 3). The closest non-FRC relatives of these clones came from volcanic ash and polychlorinated biphenyl-polluted soil; bootstrap values from Fig. 3 support that these clones likely belong to the same division as these novel FRC sequences. This candidate division, based on Hugenholtz taxonomy (16), may represent either a novel division or a novel lineage within the *Firmicutes* (Fig. 3).

(iv) PLFA analysis of sediment samples. In accordance with clone library data, PLFA data (Table 4) showed that community structure was more diverse and evenly distributed in the unstimulated sample (FB066) compared to the two biostimulated sediment samples (FB064 and FB067). Shannon-Weiner (*H*) indices calculated from PLFA data further confirm that the unstimulated sediment was less diverse ($H = 2.774$) than the stimulated sediments, FB064 ($H = 1.908$) and FB067 ($H = 2.461$). As with clone library data, there was a significant negative linear correlation between log biomass and Shannon-Weiner diversity index based on PLFA data ($r = -0.992$, $P < 0.05$).

As the biomass of the samples increased (Table 1), so did the percentage of monounsaturates (Table 4), which are generally indicative of gram-negative bacteria (68). Furthermore, biostimulated samples contained a smaller percentage of terminal branched saturates compared to the control (Table 4). Terminal branched saturates are generally indicative of gram-positive bacteria; however, other microorganisms may contain these fatty acids as well (68).

Table 4 shows that the dominant PLFAs from the genus *Castellaniella* ($C_{16:0}$, $C_{16:1}\omega7c$, $C_{17:0}$ cyclo, and $C_{18:1}\omega7c$) (40) were higher in the biostimulated samples than in the control. Although other microorganisms can contain these particular PLFAs, it is likely that some or most of these fatty acids that increased with biomass were derived from *Castellaniella* species, given that species of this genus were dominant in biostimulated clone libraries.

TABLE 4. PLFA analysis of samples FB064, FB067, and FB066[a]

PLFA group and name	% of total PLFA		
	FB064 (stimulated)	FB067 (stimulated)	FB066 (control)
Total normal saturates	27.30	34.39	28.92
14:0	1.02	1.34	0.00
15:0	0.18	0.00	0.00
16:0	**25.70**	**26.37**	**20.33**
17:0	0.10	0.52	0.70
18:0	0.30	6.16	7.08
20:0	0.00	0.00	0.33
22:0	0.00	0.00	0.48
Total mid-chain branched saturates	0.38	0.00	12.07
i10me16	0.16	0.00	1.27
10Me16:0	0.18	0.00	3.84
12me16:0	0.04	0.00	0.65
i10me17:0	0.00	0.00	4.90
10Me18:0	0.00	0.00	1.40
Total terminal branched saturates	3.67	11.18	22.59
i14:0	0.12	0.17	0.00
i15:0	1.09	4.00	5.69
a15:0	1.05	1.45	4.44
i16:0	0.59	0.73	3.30
i17:0	0.66	3.35	6.51
a17:0	0.15	1.49	2.65
Total branched monounsaturates	1.54	6.05	7.66
br16:1a	0.02	0.00	0.00
br16:1b	0.04	0.00	0.00
i17:1a	0.41	1.48	1.97
i17:1b	0.00	0.00	0.00
br18:1	0.98	4.57	5.69
br19:1	0.09	0.00	0.00
Total monounsaturates	66.96	47.07	28.76
16:1w9c	0.07	0.33	0.00
16:1w7c	**10.21**	**4.56**	**1.83**
16:1w7t	0.56	1.81	0.00
16:1w5c	0.22	0.76	0.00
cy17:0	**31.79**	**7.40**	**4.96**
17:1	0.17	0.00	0.00
18:1w9c	0.11	14.34	9.66
18:1w7c	**7.44**	**8.25**	**5.79**
18:1w7t	0.59	3.87	2.44
18:1w5c	0.22	0.00	0.00
cy19:0	15.52	5.75	4.09
19:1	0.07	0.00	0.00
Total polysaturates	0.00	1.31	0.00

[a] Data shown in bold represent the dominant PLFAs from described species among the genus *Castellaniella* (40).

Denitrifying community composition based on *nirK* and *nirS* clone libraries. From the three *nirK* clone libraries, 67 clones were sequenced and 10 OTUs were identified. From all three *nirK* libraries, 98.5% of clones had closest cultured relatives that are *Betaproteobacteria* (Table 5). Ethanol stimulation resulted in an increase in proportion of total sequences within *nirK* clone libraries that belong to OTU1K (Table 5; Fig. 4). Clones belonging to OTU1K made up 76 and 59.4% of total clones from libraries derived from biostimulated cores FB064 and FB067, respectively, but only 20% of the total clones from

TABLE 5. Summary of distributions of *nirK* OTUs from samples FB064, FB067, and FB066 and *nirS* OTUs from samples FB064 and FB067

OTU group and name	No. of clones per OTU			Closest cultured relative(s)[b]	% Similarity[c]
	FB064 (stimulated)	FB067 (stimulated)	FB066[a] (control)		
nirK OTUs					
1K	19	19	2	FRC isolates 4.5A2 and 7.5A2	100.0
2K	1	0	0	*Nitrosomonas* sp. strain TA92liNH$_4$	84.8
4K	0	0	1	*Ochromobactrum* sp. strain 4FB14	93.5
7K	1	11	7	*Alcaligenes* sp. strain DSM 30128	81.7
8K	0	1	0	FRC isolate 4.5A2	84.7
9K	0	1	0	FRC isolate 4.5A2	94.2
10K	1	0	0	FRC isolate 4.5A2	92.5
11K	1	0	0	FRC isolate 4.5A2	98.0
12K	1	0	0	FRC isolate 4.5A2	87.5
13K	1	0	0	FRC isolate 4.5A2	100.0
nirS OTUs					
1S	38	0		*Dechloromonas aromatica*	90.5
9S	0	7		*Thiobacillus denitrificans*	75.9
10S	0	1		*Ralstonia eutropha*	75.1
11S	0	1		*Magnetospirillum magnetotacticum*	85.1
14S	0	10		*Ralstonia metallidurans*	78.5
18S	0	1		*Ralstonia eutropha*	82.2
19S	0	3		*Ralstonia eutropha*	82.7
20S	11	0		*Ralstonia metallidurans*	74.1
21S	6	0		*Azoarcus tolulyticus*	81.5
22S	1	0		*Azoarcus tolulyticus*	84.3
23S	1	0		*Azoarcus tolulyticus*	94.6
24S	1	0		*Azoarcus tolulyticus*	87.0
25S	1	0		*Azoarcus tolulyticus*	86.6
26S	0	2		*Ralstonia metallidurans*	84.4
27S	0	8		*Magnetospirillum magnetotacticum*	86.6
28S	0	3		*Magnetospirillum magnetotacticum*	82.8
34S	0	5		*Magnetospirillum magnetotacticum*	85.2
35S	4	0		*Magnetospirillum magnetotacticum*	87.9
36S	0	3		*Thauera aromatica*	80.4
				Pseudomonas stutzeri	80.4
37S	2	0		*Ralstonia metallidurans*	74.7
38S	0	10		*Dechloromonas aromatica*	88.8
39S	0	10		*Dechloromonas aromatica*	87.8
41S	0	3		*Dechloromonas aromatica*	89.8
42S	0	1		*Dechloromonas aromatica*	91.9
43S	1	0		*Dechloromonas aromatica*	96.6
44S	0	2		*Dechloromonas aromatica*	88.8

[a] An amplified PCR product using *nirS* primers could not be obtained from this sample.
[b] GenBank accession numbers for closest cultured relatives are located next to corresponding genus and species names in Fig. 4 and 5.
[c] Similarity values are based on pair-wise distance values from multiple alignment files using translated amino acid sequences.

the control clone library from FB066. Also, OTU1K was 100% similar to the *nirK* sequences from isolate 4.5A2 and 7.5A2, indicating that these genes may belong to the same *Castellaniella* species dominant in nitrate-reducing enrichments and in SSU rRNA gene clone libraries from biostimulated sediment (Fig. 4), although it is possible some of these genes belong to other species, as horizontal transfer of *nirK* genes within a site has previously been implicated (32). Seventy percent of clones from the control *nirK* clone library from FB066 belonged to OTU7K, whose closest relative was the *nirK* gene product from *Alcaligenes* sp. strain DSM30128 (81.7% similarity). Amino acid sequences derived from OTU1K and OTU7K, however, were only 77.8% similar to each other.

Clone libraries from *nirS* genes were constructed from biostimulated samples FB064 and FB067 but not from the control core, FB066, since *nirS* PCR product could not be obtained from this sample. While the overwhelming majority of *nirK* clones seemed to belong to *Castellaniella*, *nirS* clone libraries were more diverse than *nirK* clone libraries (Table 5); although the reason for this difference in diversity is unknown, the inverse relationship between *nirS* and *nirK* diversity in groundwater at the FRC has previously been observed (69). From the *nirS* clone libraries constructed from the two biostimulated samples, FB064 and FB067, 136 clones were sequenced and 26 OTUs were identified. In accordance with *nirK* libraries, the majority of clones from the *nirS* libraries had closest cultured relatives that are *Betaproteobacteria* (84.6% of total clones); these clones, however, were related to families other than *Alcaligenaceae* (Table 5). The dominant OTU from FB064 was OTU1S (57.6% of total clones), which was closely related to the *nirS* gene product from the anaerobic benzene-degrading *Dechloromonas aromatica* (90.5% similarity) (Fig. 5). OTU20S made up 16.7% of the *nirS* clone library from FB064 (Table 5), and its closest relative was clone R2-s02 (77.6% similarity),

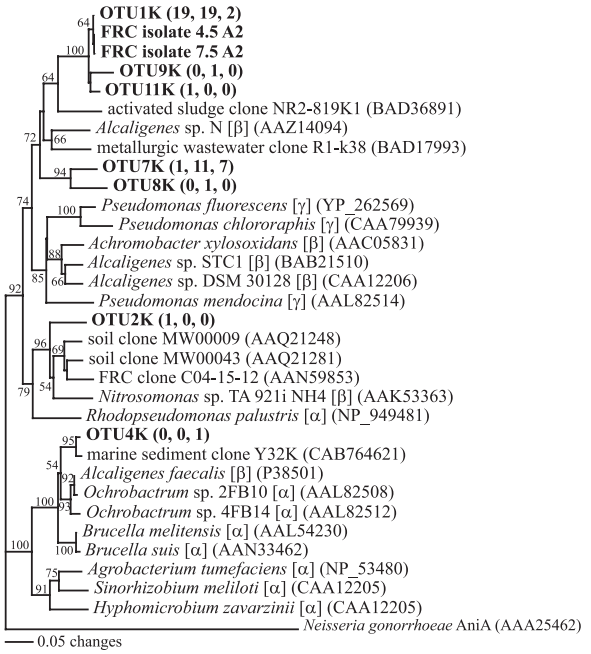

FIG. 4. Distance phylogram of partial *nirK* gene product sequences. Bootstrap values are based on 1,000 replicates and are shown for branches with bootstrap support of >50%. Selected OTUs from this study are in bold, and numbers in parentheses indicate the number of clones belonging to that OTU from sediments FB064, FB067, and FB066, respectively. Accession numbers of sequences downloaded from GenBank are in parentheses.

identified from a metallurgic wastewater treatment system (71) (Fig. 5). NirS sequences from FB067 were more diverse (Table 5), and the most abundant OTUs clustered with *D. aromatica* (OTUs 38S and 39S), *Ralstonia metallidurans* and *R. eutropha* (OTUs 14S, 18S, and 19S), and *Magnetospirillum magnetotacticum* (OTUs 27S, 28S, and 34S) (Fig. 5).

DISCUSSION

By using a combination of PLFA analysis, SSU rRNA and functional gene (*nirK* and *nirS*) clone libraries, and a cultivation approach, we were able to examine the effect of biostimulation on microbial community structure and identify and isolate a microorganism that likely plays a role in nitrate removal in an acidic aquifer cocontaminated with nitrate and uranium. The use of PCR and cloning methods for microbial community analysis is qualitative or "semiquantitative," due to several well-recognized limitations (30). In this study, PCR and cloning biases may have affected the frequency in which some OTUs and GLTGs in clone libraries were detected. Also, the limited number of clones analyzed may have led to underestimated levels of diversity and detection of only the most abundant species and genera. The percent coverage in each library ranged from 64 to 78% at the OTU level and 80 to 83% at the GLTG level. The use of PLFA analysis, however, as a quantitative measure helped demonstrate the inverse relationship between biomass and diversity, while the cultivation approach confirmed the dominance of *Castellaniella* in sediment from FB064 and its ability to grow on ethanol and nitrate. However, variations in numbers of specific organisms or groups were only semiquantitative, as they were based on clone library data; a quantitative approach, such as real-time PCR or fluorescent in situ hybridization using group-specific primers/probes, would help determine whether the numbers of organisms within these samples were different.

Several studies have documented impacts of radionuclide, heavy metal, and hydrocarbon contamination on microbial community structure, and the general consensus is that pollution decreases microbial diversity (22, 28, 39, 43, 45, 57). Two previous studies done on microbial community structures of pristine versus contaminated areas of the aquifer at the FRC have found that contamination resulted in a decrease in microbial diversity and selected for *Betaproteobacteria* species related to or belonging to *Azoarcus* (22) and *Alcaligenaceae* (57). Furthermore, *Betaproteobacteria* were found to be abundant in other contaminated environments, including polychlorinated biphenyl-contaminated soil (50), a waste gas biofilter (26, 27), metal- and petroleum-contaminated soil (39), heavy metal-amended soil microcosms (45), and metallurgic wastewater (70). Similarly, our results show that *Betaproteobacteria* SSU rRNA clones, primarily those affiliated with *Alcaligenaceae* and *Burkholderiaceae*, are present in contaminated sediment samples from the FRC (Fig. 2). Also, the majority of *nirK* and *nirS* clones in this study shared similarity to *nirK* and *nirS* gene products from cultured *Betaproteobacteria* belonging to the families *Alcaligenaceae* and *Burkholderiaceae* as well as *Rhodocyclaceae* (Table 5; Fig. 4 and 5), suggesting that several of the *Betaproteobacteria* genera detected in SSU clone libraries may also be capable of denitrification at this site. In a recent phylogenetic survey of bacterial populations from FRC sediment, SSU rRNA clones belonging to *Alcaligenaceae* and *Burkholderiaceae* were found to be dominant as well as metabolically active (2). These results, along with the results of this study, suggest that the enrichment of *Betaproteobacteria* in sediments observed in this study could be due to growth of *Betaproteobacteria* already widespread and/or active in the aquifer prior to biostimulation that have adapted to the groundwater contaminants at the FRC, which include nitrate, heavy metals, radionuclides, and hydrocarbons.

While our SSU rRNA gene clone libraries showed an abundance of *Betaproteobacteria* clones in biostimulated sediments, multiple lines of evidence suggest the dominance of a *Castellaniella* species in biostimulated sediments and its role in nitrate removal in situ. While several studies have proven successful in using molecular approaches to identify bacteria important in bioremediation (12, 33, 61), very few studies have both identified and isolated microorganisms responsible for in situ bioremediation. In one study, organisms were cultivated that had been identified by DGGE from 2,4-dichlorophenoxyacetic acid-degrading enrichments; these isolates were capable of 2,4-dichlorophenoxyacetic acid degradation, suggesting their importance in bioremediation in contaminated environments (42). Another study used stable isotope probing of RNA to show that *Azoarcus* was involved in benzene degradation in groundwater incubations under denitrifying conditions and further isolated organisms belonging to the same phylotype, showing that they could oxidize benzene to CO_2 (41). These two studies, however, do not prove the importance of the isolated organisms for in situ bioremediation. In a different

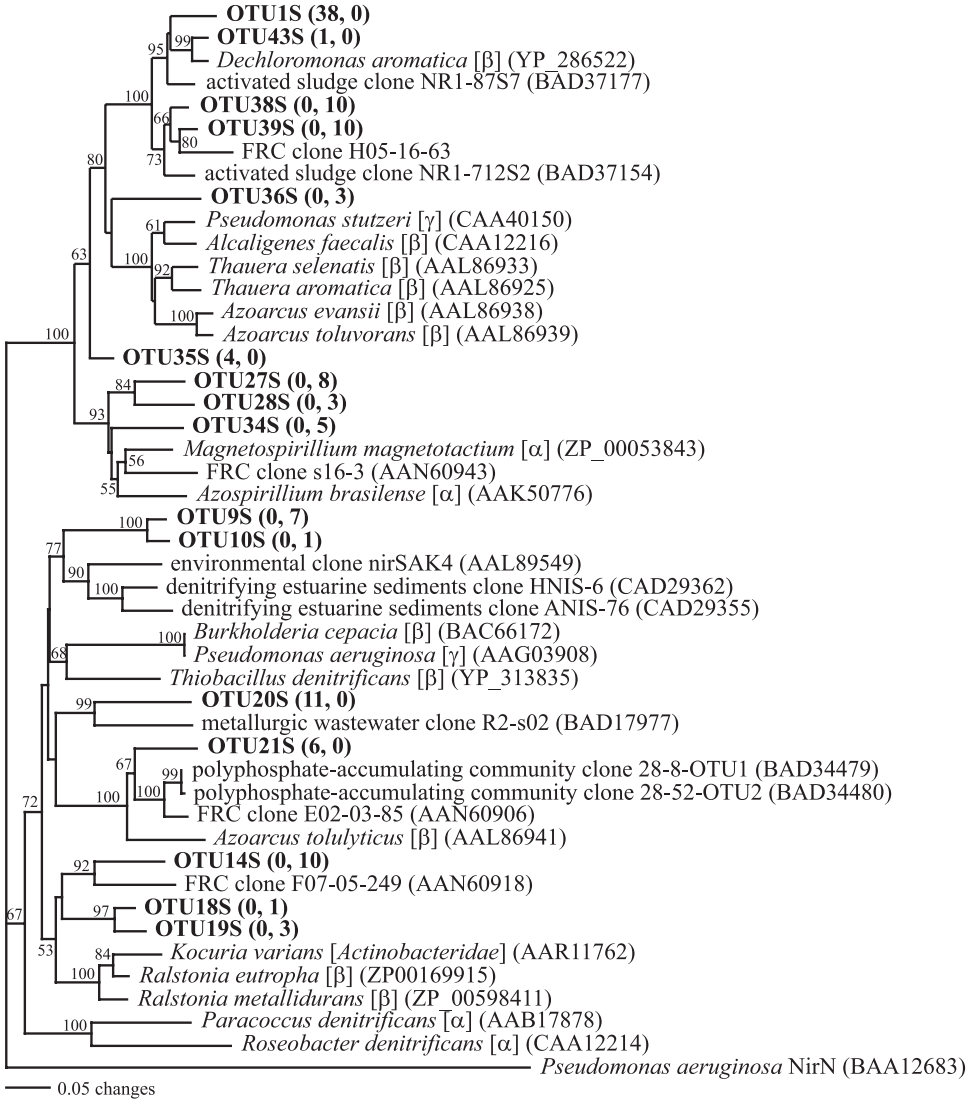

FIG. 5. Distance phylogram of partial *nirS* gene product sequences. Bootstrap values are based on 1,000 replicates and are shown for branches with bootstrap support of >50%. Selected OTUs from this study are in bold, and numbers in parentheses indicate the number of clones belonging to that OTU from sediments FB064 and FB067, respectively. Accession numbers of sequences downloaded from GenBank are in parentheses.

study, however, stable isotope probing was used to identify in situ naphthalene degraders; one dominant clone was identified, and an isolate matching this clone (belonging to the genus *Polaromonas*) was cultivated and shown to also contain a naphthalene dioxygenase gene also detected in the site sediment (38). Similarly, in this study, isolates belonging to the genus *Castellaniella* were cultivated that matched dominant clones from both SSU rRNA gene and *nirK* clone libraries generated from biostimulated sediment where nitrate reduction was occurring. Furthermore, PLFA analysis from sediment samples showed an increase in fatty acids common to the genus *Castellaniella* were associated with biomass increase. Both *Castellaniella* sp. strains 4.5A2 and 7.5A2 contained *nirK* and were capable of growth on nitrate as the sole electron acceptor and producing gaseous end product, indicating these organisms are capable of denitrification; if the *Castellaniella* organisms identified in situ through SSU rRNA and *nirK* clone libraries shared similar physiology to these isolates, then *Castellaniella* might play an active role in denitrification at this site upon biostimulation with ethanol. Along with the *Polaromonas* study (38), this paper shows a relationship between microbial community structure and function through the isolation of a microorganism dominant in clone libraries while also using functional gene sequences to suggest that the microorganism is involved in the process of interest in situ.

The *Castellaniella* species identified in this study may represent a novel species (Fig. 1). Other *Castellaniella* organisms have been isolated from activated sludge and are capable of denitrification coupled to the oxidation of monoterpenes (25), taurine (15), and phenol (4). Furthermore, other *Alcaligenaceae* isolates have been implicated in the degradation of xenobiotic compounds (10) as well as in nitrate removal systems (53). FRC *Castellaniella* isolates 4.5A2 and 7.5A2 are pH tolerant and were isolated at both low and neutral pHs; thus, they

may have been able to out-compete other denitrifiers for nitrate in the acidic groundwater found in Area 1.

A similar molecular ecology study at the FRC found that electron donor addition resulted in an increase in *Gammaproteobacteria*, such as *Geobacter* and *Anaeromyxobacter*, in contaminated FRC Area 1 sediments (51). However, push-pull tests in those experiments were done with low-nitrate groundwater from well GW835 (36), and samples were taken at the end of the extraction phase. Those experiments point to an important role for Fe(III)-reducing bacteria during biostimulation. In this study, groundwater wells were injected with high-nitrate (>130 mM) groundwater from FW021, and sediment samples were taken 1 week after injection of ethanol-amended groundwater (at the beginning of the extraction phase), at which point denitrification was likely occurring (Table 1). The differences in nitrate concentrations of the injection solutions as well as the time at which sediment samples were taken could reflect the differences in community compositions based on SSU rRNA gene clone libraries. Since several terminal electron-accepting processes sequentially occur during biostimulation (36), it is likely that the results from our study provide a snapshot of the microbial community structure during the denitrification phase, while the previous study (51) provides a snapshot of the microbial community structure when geochemical conditions were more reduced. This would reflect observations of other studies that shifts in microbial community structure occur during different stages of bioremediation processes (35, 37, 73).

In this study descriptive diversity statistics are provided to describe the effect of biostimulation on in situ diversity of microbial populations. A recent study has shown that bioremediation in a fluidized bed reactor treating nitrate- and uranium-contaminated groundwater resulted in an initial decrease in bacterial diversity followed by an increase in diversity (35). In accordance with this finding, other molecular studies have also shown that biostimulation of hydrocarbon-contaminated sediments results in an initial decrease in species diversity followed by an increase in diversity (37, 59). Our results also support that biostimulation resulted in a decrease in bacterial diversity; however, it is possible that biodiversity could later increase, as observed in the above studies. The effects of fluctuations in species diversity on ecosystem function (in this case, nitrate and uranium removal from groundwater at the FRC) are unclear. While many ecological studies have linked species richness or high species diversity in natural systems or microcosms with an increase in ecosystem function and/or stability (7, 11, 63), few studies have examined the effect of bacterial species diversity on ecosystem function in engineered systems, where often one substrate is available for consumption, as opposed to natural ecosystems, where increased species richness might aid in a more productive consumption of all available resources. For example, in glucose-fed methanogenic bioreactors, it was found that a bioreactor with lower bacterial diversity, or more "flexible" microbial communities, was more functionally stable than a more species-rich bioreactor (21). Similarly, at the FRC, the desired ecosystem function (i.e., nitrate and uranium reduction) may likely be unaffected by lower diversity when a simple substrate such as ethanol is used as an electron donor.

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