Metabolically active microbial communities in uranium-contaminated subsurface sediments

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Abstract

In order to develop effective bioremediation strategies for radionuclide contaminants, the composition and metabolic potential of microbial communities need to be better understood, especially in highly contaminated subsurface sediments for which little cultivation-independent information is available. In this study, we characterized metabolically active and total microbial communities associated with uranium-contaminated subsurface sediments along geochemical gradients. DNA and RNA were extracted and amplified from four sediment-depth intervals representing moderately acidic (pH 3.7) to near-neutral (pH 6.7) conditions. Phylotypes related to *Proteobacteria* (*Alpha-*, *Beta-*, *Delta-* and *Gammaproteobacteria*), *Bacteroidetes*, *Actinobacteria*, *Firmicutes* and *Planctomycetes* were detected in DNA- and RNA-derived clone libraries. Diversity and numerical dominance of phylotypes were observed to correspond to changes in sediment geochemistry and rates of microbial activity, suggesting that geochemical conditions have selected for well-adapted taxa. Sequences closely related to nitrate-reducing bacteria represented 28% and 43% of clones from the total and metabolically active fractions of the microbial community, respectively. This study provides the first detailed analysis of total and metabolically active microbial communities in radionuclide-contaminated subsurface sediments. Our microbial community analysis, in conjunction with rates of microbial activity, points to several groups of nitrate-reducers that appear to be well adapted to environmental conditions common to radionuclide-contaminated sites.

Introduction

Uranium contamination is widespread in subsurface sediments at mining and milling sites across North America, South America, and Eastern Europe (Abdelouas *et al.*, 1999). In the US alone, the Department of Energy (DOE) is responsible for the remediation of 7280 km^2 of soils and groundwater contaminated as a result of processes associated with uranium extraction for the production of nuclear weapons (Riley & Zachara, 1992; NABIR, 2003). As a result of waste-disposal practices, subsurface sediments at these sites are often cocontaminated with nitric acid and toxic metals (Brooks, 2001; Moon *et al.*, 2006). Oxidized uranium, U(VI), is highly soluble and toxic, and thus is a potential contaminant to local drinking-water reservoirs (NABIR, 2003). A promising strategy for *in situ* uranium bioremediation is immobilization through the biological reduction of U(VI) to insoluble U(IV) by indigenous microbial communities (Lovley, 1995; Truex *et al.*, 1997).

A phylogenetically diverse assemblage of respiratory and fermentative microbial groups were demonstrated to catalyse U(VI) reduction (Lovley *et al.*, 2004; DiChristina, 2005). These communities are broadly distributed in subsurface environments (Lovley, 1995); however, their metabolism is believed to be limited by labile carbon, acidic pH, and cocontaminants such as nitrate and toxic metals (Al, Ni) (Anderson *et al.*, 2003; Istok *et al.*, 2004). High nitrate concentrations inhibit the reduction of U(VI) by serving as a competing and more energetically favourable terminal electron acceptor for microorganisms (DiChristina, 1992; Finneran *et al.*, 2002). Therefore, in order to design appropriate U(VI) bioremediation strategies, the overall phylogenetic diversity and the impact of geochemical conditions, including pH and nitrate concentrations, on indigenous microbial communities must be assessed (Lovley *et al.*, 1991; NABIR, 2003; Istok *et al.*, 2004).

In situ bioremediation experiments have successfully employed carbon substrate amendments to stimulate the

reduction and immobilization of U(VI) by indigenous microbial communities (Senko *et al.*, 2002; Anderson *et al.*, 2003; Istok *et al.*, 2004; North *et al.*, 2004). To date, cultivation-independent studies have begun to describe the microbial communities present in uranium-rich subsurface environments by focusing primarily on groundwater (Chang *et al.*, 2001; Anderson *et al.*, 2003; Peacock *et al.*, 2004; Reardon *et al.*, 2004; Fields *et al.*, 2005), sediment incubations in the laboratory (Holmes *et al.*, 2002; Wan *et al.*, 2005), or environments manipulated during bioremediation experiments (Senko *et al.*, 2002; Istok *et al.*, 2004; North *et al.*, 2004). However, the optimization of bioremediation strategies is dependent on knowing the *in situ* microbial populations within the subsurface available for bioremediation, that is, the bioremediation potential prior to biostimulation. In addition, by characterizing the metabolically active fraction of the microbial communities within these subsurface environments, the taxa currently able to survive and remain active within the contaminant plume can be identified and potentially targeted for bioremediation (Whiteley & Bailey, 2000).

Our overall objective was to elucidate the community composition and metabolic potential of microbial communities along spatial geochemical gradients in radionuclide-contaminated subsurface sediments. We hypothesized that contaminants, such as nitric acid and radionuclides, within the subsurface act as a selective pressure, altering the microbial community composition across small spatial scales. This study was conducted in parallel with a series of microcosm experiments designed to measure the potential metabolic rates of sediment-associated microbial communities (Edwards *et al.*, 2006). Molecular techniques targetting bacterial small subunit (SSU) rRNA genes (DNA) and SSU rRNA (RNA) enabled us to compare the composition and diversity of the total and metabolically active microbial communities. Specifically, clonal analysis revealed an abundance of *Proteobacteria*-related sequences closely related to known nitrate-reducing taxa. In addition, a comparison of DNA- and RNA-derived libraries indicated that the nitrate-reducing taxa available for bioremediation changed across the contamination gradient studied.

Materials and methods

Site and sample description

The Oak Ridge Field Research Center (ORFRC), located adjacent to the Y-12 industrial complex within the Oak Ridge National Laboratory (ORNL) reservation in Oak Ridge, Tennessee, was designated in 2000 by the US Department of Energy for the Environmental Remediation Sciences Program (ERSP). Waste products from uranium-enrichment processes at the Y-12 complex, including but not limited to uranium and nitric acid, were collected and stored in three unlined ponds until 1988, when the ponds were pumped and capped by a parking lot (Brooks, 2001). Subsurface groundwater flow created a contaminant plume originating from the pond site that currently extends *c.* 7 km along a geological strike east and west of the ponds to a depth of > 150 m (Brooks, 2001). For a detailed site description refer to the ORFRC webpage (http://www.esd.ornl.gov/nabirfrc/).

Sediments were sampled from borehole FB61, 2–6 m below the surface and within the saturated zone of ORFRC Area 1 on 10 July 2003 using a Geoprobe equipped with PVC-80 sleeves lining the corer. Cores (0.083 m in diameter, 0.61 m in length) were aseptically sectioned under strictly anoxic conditions in a Coy anaerobic chamber. Four core sections from distinct depths (Table 1) were subsectioned and either frozen on dry ice for cultivation-independent analysis or stored anaerobically at 4 °C for microcosm studies prior to overnight shipment to Florida State University.

Groundwater and solid-phase chemical constituents [pH, nitrate, Fe(II), and Fe(III)] were analysed in sediment core and microcosm samples according to previous methods (Petrie *et al.*, 2003; North *et al.*, 2004). A detailed description of the sediment microcosm experiments is presented in Edwards *et al.* (2006). In brief, for this study sediments from two of the four core sections, representing the lowest and highest sediment pH (sections 61-01-00 and 61-03-25, respectively; Table 1), were homogenized inside a Mecaplex anaerobic chamber (100% N_2 atmosphere). Microcosms were constructed using 30 g of homogenized sediment and 60 mL of deionized water in gas-tight anaerobic serum bottles. Treatments included amendments with ethanol or glucose to 20 mM final concentration and control bottles to which no carbon substrate was added (Table 2). To examine the impact of pH on potential rates, a second set of incubations was constructed in which acidic sediments from core 61-03-25 were neutralized with bicarbonate. Bottles were sealed with butyl rubber stoppers, purged with sterile argon, incubated statically at 28 °C in the dark, and sampled over a 2-month period. Potential rates of microbial activity

Table 1. Geochemical parameters of sediment cores collected with increasing depth below surface in the saturated subsurface of ORFRC Area 1 in borehole FB61

Sample	Depth (m)	pH	Nitrate*	Fe(III)*,†
61-01-00	2.4–3.1	6.7	0.6	31.5
61-01-24	3.1–3.7	6.1	0.1	17.0
61-03-00	4.9–5.5	3.9	17.8	17.3
61-03-25	5.5–6.1	3.7	40.1	18.6

*Units in μmoL g^{-1}.

†Fe(III) measured using the oxalate extraction method.

Table 2. Initial conditions and potential rates of microbial activity measured in sediment microcosms of two sections of borehole FB61

Sample	Treatment	Nitrate (μmoL g^{-1})*	Microcosm pH†	Nitrate reduction‡	Fe(II) production‡
61-01-00	Control	60.8	5.7	0.26	ND§
	Ethanol	60.8	5.7	2.70	1.36
	Glucose	60.8	5.7	2.84	1.44
61-03-25	Control	22.6	4.3	0.13	ND
	Ethanol	22.6	4.3	0.28	ND
	Glucose	22.6	4.3	0.26	ND
61-03-25+$NaHCO_3$¶	Control	22.7	7.2	0.18	ND
	Ethanol	22.7	7.2	2.82	0.04
	Glucose	22.7	7.2	1.68	0.28

*Values are averages of initial concentrations in μmoL g^{-1}.

†Average of initial pH measured in all treatment bottles. Variations between replicate bottles were < 0.5 pH units.

‡Potential rate of activity in μmoL g^{-1} d^{-1}. Values are averages from triplicate microcosm incubations.

§None detected.

¶Incubations of 61-03-25 neutralized with bicarbonate.

in microcosms were calculated by regression of the change in concentration of chemical constituents with time.

Nucleic acid extraction and amplification

Prior to nucleic acid extraction, potentially contaminating RNases were removed from solutions and solids as described previously in Mills *et al.* (2004). Total nucleic acids were extracted from 2 g aliquots of ORFRC sediments as described by Hurt *et al.* (2001). RNA and DNA were separated and purified using the QIAGEN RNA/DNA Midi Kit according to the manufacturer's instructions (Qiagen, Valencia, CA). Residual DNA was removed from RNA extracts with 5 U of RQ1 RNase-free DNase (Promega, Madison, WI) according to the manufacture's instructions, with the addition of RNasin ribonuclease inhibitor (Promega, Madison, WI).

Aliquots of purified DNA were PCR-amplified using the *Bacteria* domain-specific SSU rRNA gene primers 27F (5′-AGR CTT TGA TCM TGG CTC AG-3′) (Johnson, 1994) and 1392R (5′-ACG GGC GGT GTG TAC-3′) (Wilson *et al.*, 1990). The PCR mixture contained 10 to 50 ng of DNA, 1 × PCR buffer (10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$; TaKaRa Mirus Bio, Madison, WI), 200 μM deoxynucleoside triphosphates, 0.5 μM of each forward and reverse primer, and 0.03 U μL^{-1} *rTaq* polymerase (TaKaRa Mirus Bio). Thermocycling was performed with a 95 °C incubation for 5 min, followed by 35 cycles of 95 °C for 1 min, 55 °C for 1 min and 72 °C for 1 min, with a final extension step at 72 °C for 10 min.

Aliquots of rRNA were reverse-transcribed to cDNA using Moloney murine leukemia virus reverse transcriptase (M-MLV RT) and *Bacteria* domain-specific SSU rRNA reverse primer 518R (5′-CGT ATT ACC GCG GCT GCT GG-3′) (Nogales *et al.*, 1999) or 1392R (Wilson *et al.*, 1990) according to the manufacturer's instructions (Promega, Madison, WI). The 10 to 50 ng of cDNA was then used in a standard PCR reaction using *Bacteria* domain-specific primers 27F (Johnson, 1994) and 518R (Nogales *et al.*, 1999) or 1055F (5′-ATG GCT-GTC GTC AGC T-3′) (Amann *et al.*, 1995) and 1392R (Wilson *et al.*, 1990). PCR amplification included an initial denaturing step of 95 °C for 5 min, followed by 30 cycles of denaturation at 95 °C for 30 s, annealing at 50 °C for 27F/518R or 53 °C for 1055F/1392R for 30 s and elongation at 72 °C for 30 s, with a final extension step at 72 °C for 10 min. DNA contamination of RNA extracts was routinely monitored by PCR amplification of RNA extracts that had not been reverse-transcribed. No contaminating DNA was detected in any of these reactions. Amplicons from both DNA- and RNA-based reactions were visualized by gel electrophoresis on 0.7% agarose gels, stained with ethidium bromide, and UV-illuminated.

Environmental clone library construction and phylogenetic analysis

DNA- and RNA-derived SSU rRNA amplicons were pooled from three to five PCR or reverse transcriptase-PCR (RT-PCR) reactions, respectively, and purified using a QIAquick PCR purification kit (QIAGEN, Valencia, CA). Purified PCR product was cloned into the TOPO TA cloning vector pCR 2.1 according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). RNA-derived clone libraries from depth intervals 61-01-00 and 61-01-24, amplified with primers 1055F/1392R, were designated with 'RR'. RNA-derived libraries from depth intervals 61-01-00, 61-03-00 and 61-03-25, amplified with 27F/518R, were designated with an 'R'. Cloned inserts were PCR-amplified using the vector-specific primers (M13F/R) and digested with the restriction enzymes *Hae*III (0.25 U μL^{-1}) (New England Biolabs, Beverly, MA) and *Msp*I (1 U μL^{-1}) (Promega) for 2 h at 37 °C. Clones were grouped into phylotypes according to restriction fragment length polymorphism (RFLP) banding patterns, and

representative clones were sequenced bidirectionally at the Florida State University Sequencing Facility using a Big-Dye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA) on an Applied Biosystems 3100 genetic analyzer with capillary electrophoresis. RFLP grouping of phylotypes containing multiple members was verified by sequencing two or more representative clones for each phylotype. Sequences were assembled using Sequencher v4.5 (Gene Codes Corp., Ann Arbor, MI). Prior to comparative phylogenetic analysis, vector sequences flanking the SS rRNA gene or cDNA inserts were removed. Previously identified sequences with high sequence similarity to the clones obtained in this study were determined using the BLAST algorithm against the GenBank database available from National Center for Biotechnology Information (NCBI) (Altschul *et al.*, 1990). Clone sequences were checked for chimeras using the program Chimera Check from the Ribosomal Database Project II (Cole *et al.*, 2003). All clone sequences and reference sequences were aligned in the arb software package using the Fast Aligner algorithm, incorporating ribosomal secondary structure data (Strunk & Ludwig, 1997). Neighbour-joining trees incorporating a Jukes–Cantor distance correction were created from the alignments using the ARB software package (Strunk & Ludwig, 1997). Bootstrap data represented 1000 samplings.

Statistical analysis

Statistical analyses were used to determine the sampling efficiencies and diversity differences within and between clone libraries based upon RFLP analysis. Rarefaction curves were calculated using Analytic Rarefaction 1.3 (Heck *et al.*, 1975; Holland, 2003). EstimateS (Colwell *et al.*, 2004) was used to estimate species richness nonparametrically with Chao1 and to calculate the Shannon–Wiener and the reciprocal of Simpson's ($1/D$) indices. Percent coverage was calculated using a standard equation (Begon *et al.*, 1990). Clone library sequence data were used to compare phylogenetic diversity between samples. Clone sequence diversity indices for gene and nucleotide diversity (Nei, 1987), and $\theta(\pi)$ (Tajima, 1983) were calculated using Arlequin (Schneider *et al.*, 2000).

Nucleotide sequence accession numbers

The 74 SSU rRNA gene and SSU rRNA sequences presented in this study have been deposited in the GenBank database under accession numbers DQ316797–DQ316870.

Results

Sediment geochemistry and potential rates of microbial metabolism

The sediment pH in core sections of borehole FB61 ranged from moderately acidic (pH 3.7) to near-neutral (pH 6.7; Table 1). Nitrate concentration was inversely proportional to sediment pH and was over two orders of magnitude higher in acidic pH (17.8–40.1 μmoL g^{-1}) than it was in neutral pH (0.1–0.6 μmoL g^{-1}) samples. Iron mineral content varied by a factor of two between samples but did not show any trend with pH or nitrate concentration (Table 1). Current biomass estimates from contaminated subsurface sediments of the ORFRC site are *c.* 10^3–10^4 cells g^{-1} (E.L. Brodie, pers. commun.; P.A. Sobecky, pers. commun.). Although we recognize that nitrate and pH vary with depth below surface, we henceforth refer to sediment pH when comparing and contrasting microbial communities across spatial gradients.

Nitrate reduction rates remained low, and no Fe(III) reduction activity was observed in microcosms at acidic pH even after the addition of glucose or ethanol (Table 2) (Edwards *et al.*, 2006). In contrast, rapid nitrate and Fe(III) reduction rates were observed in sediment microcosms at neutral pH and in those neutralized with bicarbonate. Low nutrient levels limited microbial activity, as determined by nitrate reduction rates being an order of magnitude higher in carbon-amended treatments than in no-carbon-amended controls. Iron(III) reduction activity was only observed after nitrate was depleted at neutral pH. Porewater manganese, sulfide and methane levels remained below detection in all microcosms during the incubation period.

RFLP and statistical analysis of clone libraries

From four depth intervals of borehole FB61, total nucleic acids were successfully extracted and clone libraries were constructed from amplified SSU rRNA gene (DNA-derived; 337 clones) and SSU rRNA (RNA-derived; 159 clones) targets (Tables S1 and S2). RFLP analysis of the DNA-derived clones indicated 42 distinct phylotypes, with only two phylotypes containing representative clones in all four libraries analysed (Table S1). Although rarefaction curves from each DNA-derived library (Fig. 1a) did not indicate saturation, that is, the slope was greater than zero (Heck *et al.*, 1975), percent coverage ranged from 88.3% for 61-01-24 to 97.3% for 61-03-25 (Table 3). Although additional sampling of clones would be necessary to describe the overall diversity fully, numerically dominant RFLP groups from multiple lineages were obtained (Table 3). Species richness and Shannon–Wiener diversity indices, based on RFLP clone data, indicated higher diversity in the DNA-derived clone libraries from neutral pH sediments compared with those from acidic pH sediments (Table 3). However, a comparison of DNA-derived clone sequence data indicated that the acidic pH sediment sample 61-03-25 had the highest diversity, as demonstrated by gene and nucleotide diversity, $\theta(\pi)$, and Simpson's diversity ($1/D$) index (Table 3).

A total of five RNA-derived clone libraries were generated for the four depth intervals of borehole FB61. The 159 clones from these RNA-derived clone libraries grouped into 33 distinct phylotypes, with 12 phylotypes identified in RR61-01-00 and RR61-01-24 and 21 phylotypes identified in R61-01-00, R61-03-00 and R61-03-25 (Table S2). Rarefaction curves from RNA-derived clone libraries from all depth intervals and primer sets indicated saturation of sampling, that is, the slope neared a value of zero (Heck *et al*., 1975), with the exception of library R61-03-00 (Fig. 1b). Owing to the utilization of two primer sets for RNA clone library construction, cross-library comparisons with robust statistical analysis were not possible. Sequences of RNA-derived phylotypes generated with the two primer sets were compared with common relatives and were shown to be greater than 92% similar to either the 5′- or 3′-end of the SSU rRNA gene sequence. Comparative analysis of DNA- and RNA-derived libraries indicated that a total of 33% of phylotype sequences obtained from RNA-derived clone libraries had greater than 92% sequence similarity to DNA-derived phylotype sequences, with 15% greater than 95% similar (data not shown). Although inherent biases are associated with the molecular techniques used in this study, we are confident that the techniques used provide a valid indication of the overall composition of sediment-associated microbial communities in borehole FB61.

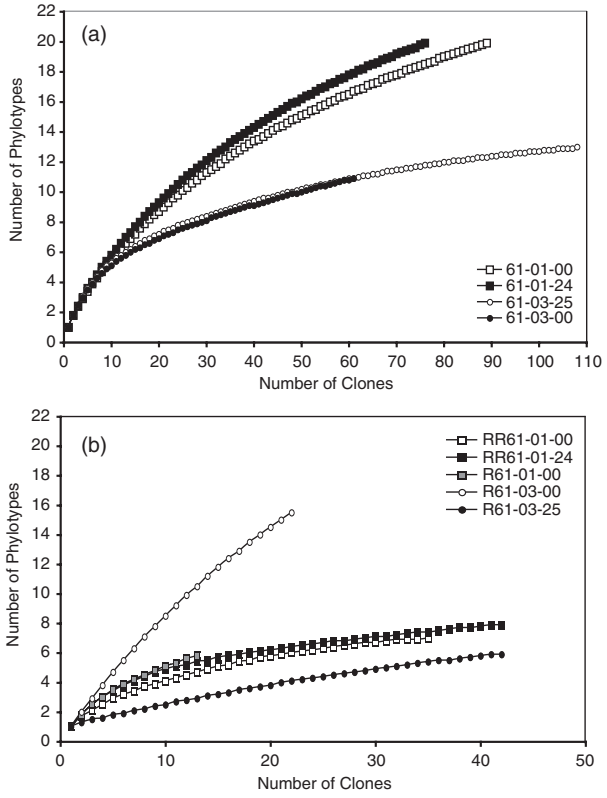

Fig. 1. Rarefaction curves determined for the various phylotypes of (a) SSU rRNA gene (DNA-derived) and (b) SSU rRNA (RNA-derived) clones from four FB61 sediment samples. Phylotypes were defined as distinct RFLP patterns resulting from digestion of clone sequences with restriction endonucleases *Hae*III and *Msp*I. Rarefaction analysis was performed using equations reported by Heck *et al*. (1975).

Phylogenetic analysis of the clone libraries

Sequenced DNA- and RNA-derived clones from FB61 were most closely related to members of the *Proteobacteria* (classes *Alpha-*, *Beta-*, *Delta-* and *Gammaproteobacteria*), *Bacteroidetes*, *Actinobacteria*, *Firmicutes,* and *Planctomycetes* lineages. In the DNA-derived clone libraries, the most frequently detected lineage was *Alphaproteobacteria* (31% of all clones) with *Beta-* and *Gammaproteobacteria* each comprising 21% of the total DNA-derived clones (Fig. 2). Phylotypes related to the class *Alphaproteobacteria* were only detected in neutral pH sediment-derived clone libraries, and represented 59% and 67% of the 61-01-00 and 61-01-24 DNA-derived clone libraries, respectively (Fig. 2). Clones related to the classes *Beta-* and *Gammaproteobacteria* were detected more frequently in the acidic pH sediment DNA-derived clone libraries than in the neutral pH sediment DNA-derived libraries (Table S1; Fig. 2). In the RNA-derived clone libraries, the *Proteobacteria*-related clones represented 83% of the clones and grouped within the classes *Alphaproteobacteria* (3% of total), *Betaproteobacteria* (44% of total), *Deltaproteobacteria* (1% of total), and *Gammaproteobacteria* (34% of total) (Fig. 2). RNA-derived *Alpha-* and *Betaproteobacteria*-related phylotypes had greater than 92% sequence similarity to phylotypes detected in DNA-derived clone libraries (data not shown).

Table 3. Statistical analyses of SSU rRNA gene clone libraries using ecological and molecular estimates of phylotype diversity

Samples	No. of clones	No. of phylotypes	Species richness	Shannon–Wiener	1/*D*	Percent coverage	θ(π)	Nucleotide diversity	Gene diversity
61-01-00	90	20	29 (22, 56)*	2.09	4.22	90.0	172.8 ± 82.8†	0.15 ± 0.07	0.76 ± 0.04
61-01-24	77	20	27 (22, 49)	2.19	4.72	88.3	167.4 ± 80.3	0.14 ± 0.07	0.79 ± 0.05
61-03-00	62	11	21 (13, 63)	1.86	5.25	91.9	172.3 ± 82.9	0.15 ± 0.07	0.81 ± 0.03
61-03-25	109	13	14 (13, 21)	1.98	5.78	97.3	204.3 ± 97.6	0.18 ± 0.08	0.83 ± 0.02

*The numbers in parentheses are 95% confidence intervals.
†Mean ± SD.

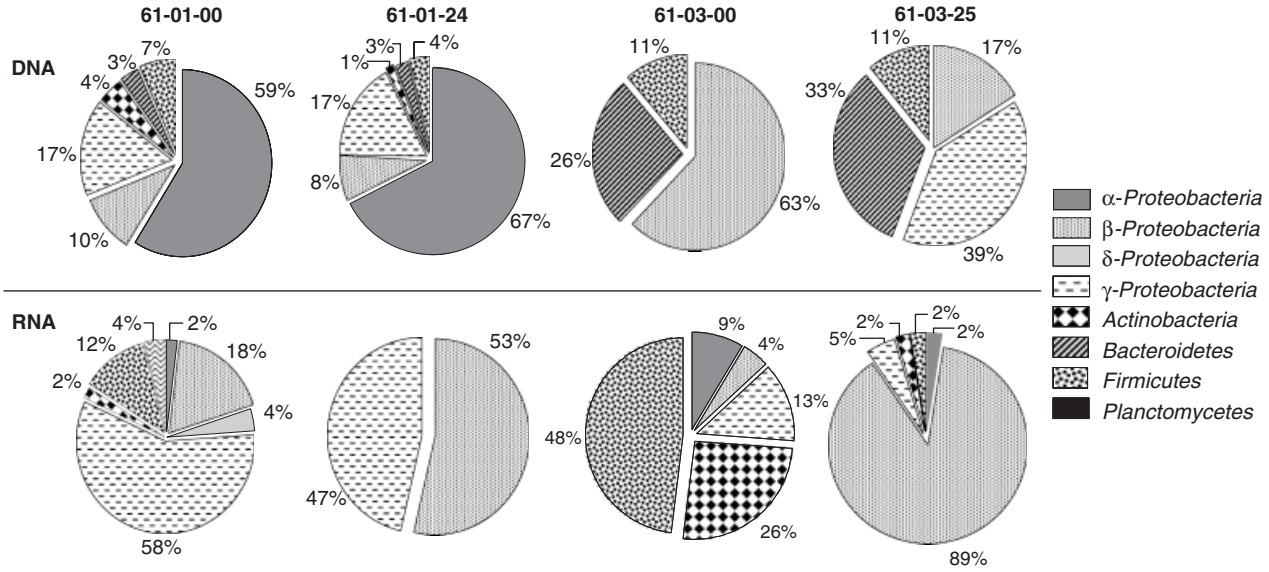

Fig. 2. Frequencies of bacterial phylogenetic lineages detected in SS rRNA gene and SS rRNA clone libraries derived from four depth intervals of borehole FB61. Calculations were made based on the total number of clones associated with phylotypes of sequenced representatives.

Adaptations to low-nutrient environments, nitrate reduction, and metal resistance are characteristics of lineages within the *Alpha-*, *Beta-*, and *Gammaproteobacteria* that were frequently detected in this study. Phylotypes within the *Alphaproteobacteria* were only detected in neutral pH sediment-derived clone libraries, and clustered within three families: *Caulobacteraceae*, *Methylobacteriaceae*, and *Sphingomonadaceae* (Fig. 3). *Sphingomonadaceae*-related phylotypes were detected in both RNA- and DNA-derived clone libraries (Tables S1 and S2; Fig. 3), and members of this group are capable of growth in low-nutrient environments and of nitrate reduction (Balkwill *et al.*, 2003). The genus *Methylobacterium*, adapted for growth in low-nutrient environments (Kayser *et al.*, 2002), is closely related to the most frequently detected DNA-derived phylotype (76 out of a total of 337 clones screened) (Table S1; Fig. 3).

A total of 71 *Betaproteobacteria*-related clones, which grouped into 12 phylotypes within the *Oxalobacteraceae* and *Comamonadaceae* families, were detected in DNA-derived clone libraries. The *Comamonadaceae* family included the most frequently detected *Betaproteobacteria*-related phylotype, 61-05-22c311 (26 clones), which was closely related to the denitrifying, low-nutrient-adapted *Acidovorax* genus (Wen *et al.*, 1999; Khan *et al.*, 2002) (Table S1; Fig. 3). Interestingly, this lineage also included a phylotype derived from the acidic pH sediment clone library, 61-03-25, which was 95% similar to ORFRC clone 005C-F01, retrieved from a previous study of contaminated groundwater at the ORFRC site (Fields *et al.*, 2005) (Table S1; Fig. 3). Nine phylotypes, representing 44% of the total RNA-derived clones, were related to the class *Betaproteobacteria* and grouped into two families, *Alcaligenes* and *Burkholderiaceae*, both characterized by nitrate reduction and metal resistance capabilities (Goris *et al.*, 2001; Konstantinidis *et al.*, 2003; Mergeay *et al.*, 2003; Busse & Stolz, 2004). Phylotypes related to *Alcaligenes* sp. Ho-11 (unpublished; GenBank accession number AB166879) (Table S2) represented the most frequently detected clones in the 61-01-24 RNA-derived library (14 clones). In contrast, the most frequently detected phylotype in the acidic pH sediment RNA-derived library of 61-03-25 (86% of the 61-03-25 clones) was most closely related (99%) to the metal-resistant, nitrate-reducing sediment isolate *Ralstonia* sp. 13A (Goris *et al.*, 2001; Konstantinidis *et al.*, 2003; Mergeay *et al.*, 2003) (Table S2; Fig. 3).

Phylotypes related to the class *Gammaproteobacteria* in the DNA-derived clone libraries grouped into four families (*Moraxellaceae*, *Pseudomonadaceae*, *Xanthomonadaceae* and *Pasteurellaceae*), whereas those detected in RNA-derived clone libraries clustered mainly within the family *Xanthomonadaceae*. Phylotypes related to the metal-resistant, nitrate-reducing group *Acinetobacter* (Dhakephalkar & Chopade, 1994; Boswell *et al.*, 2001) were detected in both acidic pH and neutral pH sediment-derived libraries (Table S1; Fig. 3).

Phylotypes related to the *Bacteroidetes, Firmicutes* and *Actinobacteria* phyla were detected less frequently than *Proteobacteria*-related phylotypes in DNA- and RNA-derived clone libraries. The *Bacteroidetes* represented 17% of the total DNA-derived clones, with greater abundance in the acidic pH sediment clone libraries (Fig. 2). Interestingly, acidic pH sediment-derived clones related to the

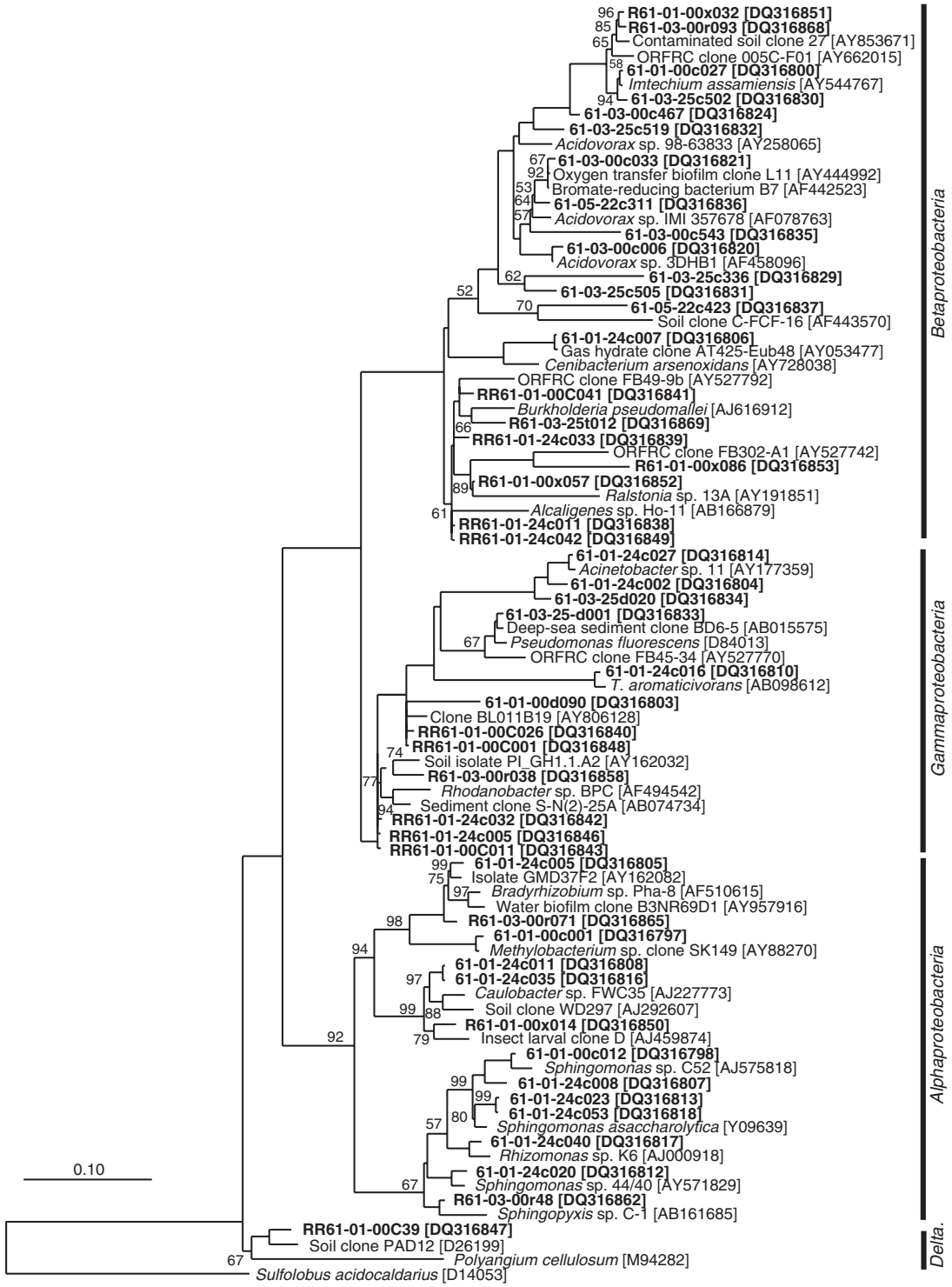

Fig. 3. Phylogenetic tree of *Proteobacteria*-related SS rRNA gene and SS rRNA clone sequences (indicated by boldface type), as determined by neighbour-joining methods incorporating Jukes–Cantor distance correction, from borehole FB61 sediment samples, selected cultured isolates, and environmental clone reference sequences. *Sulfolobus acidocaldarius* was used as the outgroup. Clones whose designations include 61-01-00 and 61-01-24 represent sequences derived from neutral pH sediment libraries, whereas those with 61-03-00, 61-03-25, and 61-05-22 were derived from acidic pH sediment libraries. Clones whose designations include 'R' and 'RR' represent sequences derived from SS rRNA clone libraries. One thousand bootstrap analyses were conducted, and percentages greater than 50% are indicated at the nodes. Scale bar = 0.1 change per nucleotide position.

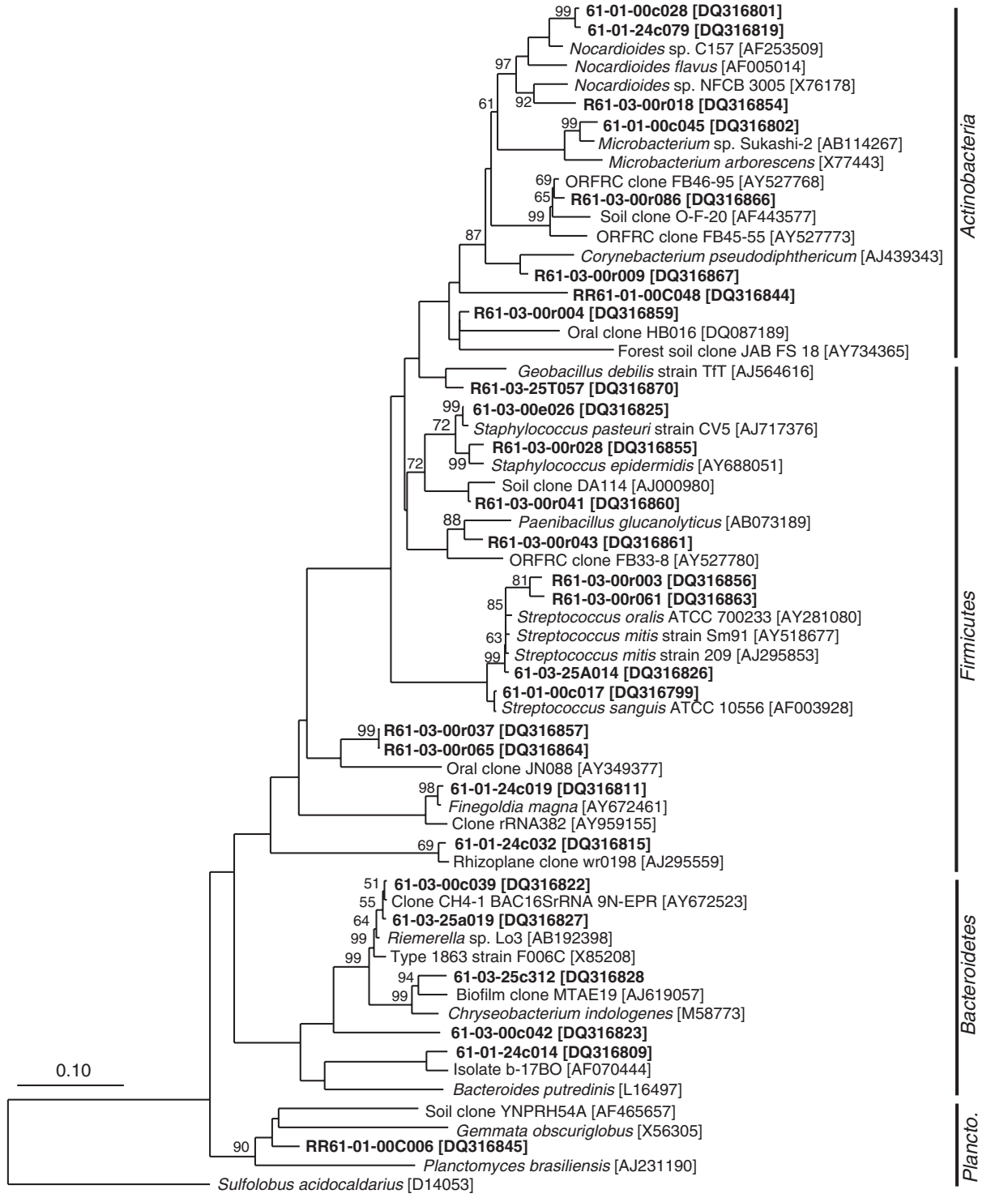

Fig. 4. Phylogenetic tree of SS rRNA gene and SS rRNA clone sequences (indicated by boldface type) related to the phyla *Actinobacteria, Bacteroidetes, Firmicutes,* and *Planctomycetes*, as determined by neighbour-joining methods incorporating Jukes–Cantor distance correction, from borehole FB61 sediment samples, selected cultured isolates, and environmental clone reference sequences. *Sulfolobus acidocaldarius* was used as the outgroup. Clones whose designations include 61-01-00 and 61-01-24 represent sequences derived from neutral pH sediment libraries, whereas those with 61-03-00, 61-03-25, and 61-05-22 were derived from acidic pH sediment libraries. Clones whose designations include 'R' and 'RR' represent sequences derived from SS rRNA clone libraries. One thousand bootstrap analyses were conducted, and percentages greater than 50% are indicated at the nodes. Scale bar = 0.1 change per nucleotide position.

Bacteroidetes grouped into the family *Flavobacteraceae*, whereas clones derived from the neutral pH sediments were related to the family *Bacteroidaceae* (Fig. 4). Two gram-positive phyla, *Firmicutes* and *Actinobacteria*, together represented 10% of the total DNA-derived clones (Fig. 2). *Firmicutes*-related phylotypes were detected in all four DNA-derived clone libraries analysed (< 11% of total clones for each library), while in contrast *Actinobacteria*-related phylotypes were detected only in DNA-derived clone libraries from neutral pH sediment samples (Fig. 2). With the exception of the *Firmicutes* (12%), no phylum detected in the RNA-derived clone libraries represented more than 5% of the total clones. *Firmicutes*-related phylotypes were the most frequently detected in the 61-03-00 acidic pH sediment-derived library (Table S2; Fig. 2).

Discussion

In order to develop effective strategies for the bioremediation of radionuclide contaminants, a better understanding of the composition and metabolic potential of microbial communities in highly contaminated subsurface sediments using cultivation-independent methods is required. Therefore, using molecular techniques targetting both DNA and RNA, we have described the subsurface microbial communities of the US Department of Energy's (DOE) Oak Ridge Field Research Center (ORFRC), where contaminants include nitric acid, radionuclides (uranium and technicium) and other metals (nickel, aluminum, barium, chromium, mercury) (Brooks, 2001; Moon *et al.*, 2006). The combination of low pH with the above-mentioned contaminants in the shallow subsurface is representative of many sites within the US nuclear weapons complex managed by the DOE, as well as of radionuclide-contaminated sites worldwide (Riley & Zachara, 1992; Abdelouas *et al.*, 1999; NABIR, 2003). Thus, our results are not only applicable to bioremediation research at the ORFRC but also have implications for widespread radionuclide contamination across the globe.

Bioremediation efforts can be complicated by spatial heterogeneity in both the composition and metabolic activity of indigenous microbial communities (Whiteley & Bailey, 2000; NABIR, 2003). Previous studies have assessed the differences in microbial community composition over relatively broad spatial scales in subsurface environments contaminated with radionuclides, with the majority of these studies focusing on groundwater or manipulated sediments during bioremediation experiments (Chang *et al.*, 2001; Petrie *et al.*, 2003; North *et al.*, 2004; Peacock *et al.*, 2004; Reardon *et al.*, 2004; Fields *et al.*, 2005; Wan *et al.*, 2005). A caveat is that primer bias, cell lysis, and nucleic acid extraction and recovery can contribute to an underestimation of overall microbial diversity. Nucleic acid extractions from ORFRC sediments were not always successful in previous attempts (Reardon *et al.*, 2004) owing to the limitations of conventional nucleic acid extraction techniques, which often result in only 1–10% recovery of the total nucleic acids available (Chandler *et al.*, 1998). Hurt *et al.* (2001) reported a modified nucleic acid extraction technique for soils that could yield 40% more DNA than previously published methods and 68% more than commercial bead-milling techniques. By utilizing the Hurt *et al.* (2001) method, we provide the first study to simultaneously extract and amplify both DNA and RNA from low-biomass ($< 10^4$ cells g^{-1}) subsurface sediments. Therefore, we are able to describe in detail the variations in total and metabolically active fractions of the *in situ* microbial communities across vertical subsurface geochemical gradients. To support detected variations in community composition, a suite of statistical indices were applied to clone library sequence data, and potential rates of microbial activity were measured in parallel under near *in situ* conditions (Edwards *et al.*, 2006).

Change in subsurface activity and microbial diversity across spatial contaminant gradients

The activity and composition of Area 1 ORFRC sediment-associated microbial communities were hypothesized to be limited by low pH and a paucity of carbon substrates. Nitrate and Fe(III) are the most abundant electron acceptors available for microbial metabolism in ORFRC subsurface sediments (Petrie *et al.*, 2003; Istok *et al.*, 2004). We therefore determined the potential rates of nitrate and Fe(III) reduction in microcosm incubations under near *in situ* sediment conditions. Our hypothesis was supported, as microbial activity was minimal at pH 4 and in the absence of added carbon substrate. Activity was stimulated by an order of magnitude upon pH neutralization and with the addition of carbon substrates, suggesting that acidity and nutrient limitation are important variables controlling microbial metabolism in contaminated ORFRC sediments (Table 2; Edwards *et al.*, 2006). As expected based on thermodynamic considerations (Chapelle, 2000) and in agreement with previous studies (Finneran *et al.*, 2002; Senko *et al.*, 2002; Petrie *et al.*, 2003; Istok *et al.*, 2004; North *et al.*, 2004), metal reduction in the acidic subsurface did not occur as long as abundant nitrate was present. Building upon previous work focused on neutral pH environments, our results indicate that nitrate-reducing communities are present in acidic subsurface sediments and become active upon pH neutralization.

The composition and diversity of sediment-associated microbial communities changed in parallel with the potential rates of microbial activity and contamination gradients in the ORFRC core sections studied. The diversity of SS rRNA gene clones, as indicated by species richness and the Shannon–Wiener index, was significantly lower in

sediments that had a lower pH and contained higher levels of cocontaminants, implying that these factors are a selective pressure on the total microbial community. Similarly, previous studies of groundwater samples at the ORFRC observed reduced microbial community diversity under acidic conditions (Reardon *et al.*, 2004; Fields *et al.*, 2005), but these studies did not investigate microbial activity or sedimentary environments in detail. Selective events are thought to minimize diversity through the survival of a few species (Martin, 2002), and the lower total species diversity observed in acidic pH sediments may result in a lower potential for bioremediation owing to the presence of fewer surviving taxa and metabolic groups.

The composition of the metabolically active fraction of the total microbial community was assessed by comparing RNA- and DNA-derived clone libraries from sediment total nucleic acid extracts. Although numerous sequences obtained in the RNA-derived library were closely related to sequences in the DNA-derived library, distinct lineages were unique to a single library. This lack of direct overlap between DNA- and RNA-derived libraries has been observed in several other studies (Nomura *et al.*, 1984; Kerkhof & Kemp, 1999; Nogales *et al.*, 1999, 2001; Mills *et al.*, 2005). In undercharacterized environments, the proportion of DNA and RNA concentrations is not well known (Morita, 1993; Jeffrey *et al.*, 1996; Binder & Liu, 1998; Kerkhof & Kemp, 1999; Griffiths *et al.*, 2003). However, previous studies have shown that an increased proportion of SSU rRNA molecules to SSU rRNA genes per cell can be observed in highly metabolically active cells (Nomura *et al.*, 1984). Therefore, highly active taxa with low cell counts may be underrepresented or not detected in DNA-derived clone libraries because SSU rRNA gene targets are at or below detection limits (Dell'Anno *et al.*, 1998; Nogales *et al.*, 2001; Mills *et al.*, 2005). The opposite would be observed if the cells were numerous but had low or no metabolic activity. Further quantitative molecular- and culture-based analyses will be required to determine RNA/DNA ratios in these sediments. Such discrepancies between SSU rRNA gene and SSU rRNA clonal analysis demonstrates the necessity of generating both DNA- and RNA-derived libraries when possible to perform a more representative analysis of the extant microbial communities.

Phylogenetic composition of microbial communities in radionuclide-contaminated subsurface sediments

The phyla detected in ORFRC contaminated subsurface sediment clone libraries include groups commonly found in pristine surficial soils; however, the proportion of phylotypes detected within these phyla differs substantially from those in previously published studies (Buckley & Schmidt, 2002). Our study detected families within the phyla *Proteobacteria*, *Firmicutes, Actinobacteria* and *Bacteroidetes* in DNA- and RNA-derived clone libraries are frequently associated with contaminated environments (i.e. taxa known to reduce nitrate and heavy metals, be resistant to heavy metal toxicity, and degrade polyaromatic compounds and polychlorinated biphenyls) (Grimes & Morrison, 1975; Dhakephalkar & Chopade, 1994; Boswell *et al.*, 2001; Goris *et al.*, 2001; Nogales *et al.*, 2001; Kanaly *et al.*, 2002; Bodour *et al.*, 2003; Konstantinidis *et al.*, 2003; Mergeay *et al.*, 2003; Petrie *et al.*, 2003; Fields *et al.*, 2005). Contrasts in community composition in comparison with pristine surficial soils may be explained by the drastically different geochemical conditions present in the contaminated ORFRC subsurface. The majority of surficial soils are typically near neutral pH and rich in organic matter, whereas ORFRC subsurface sediments are lower in organic carbon content, have higher nitrate concentrations, exhibit a wider range in pH, and have higher toxic metal concentrations (Brooks, 2001; Jardine *et al.*, 2003; NABIR, 2003; Istok *et al.*, 2004; Moon *et al.*, 2006). Phylotypes closely related to taxa adapted for growth in low-nutrient environments (i.e. *Methylobacterium, Caulobacter, Sphingomonas, Acidovorax,* and *Ralstonia*) were frequently detected in the ORFRC subsurface. In corroboration with our results from unamended sediments, sequences of these genera were also detected in abundance during bioremediation experiments (North *et al.*, 2004) and in groundwater at the ORFRC (Palumbo *et al.*, 2004; Reardon *et al.*, 2004). Thus, our clonal analysis indicates a microbial community adapted for these and potentially other radionuclide-contaminated sites.

Denitrification is believed to be mediated by a group of facultative anaerobes that display a wide range in phylogenetic affiliation and metabolic capabilities. In pristine soils, nitrate concentrations are typically too low to select for large populations of denitrifying organisms, and denitrifiers are thought to rely on aerobic heterotrophy rather than on their denitrification capacity (Tiedje, 1988). In contrast, the abundance of nitrate in the ORFRC subsurface provides more selective pressure in favour of denitrifiers and soil microorganisms that tolerate an acidic, nutrient-starved environment (Yan *et al.*, 2003; Palumbo *et al.*, 2004; Fields *et al.*, 2005). In agreement with the high nitrate concentrations observed in ORFRC subsurface sediments, sequences related to nitrate-reducing bacteria, such as members of the *Proteobacteria* (including the genera *Sphingomonas*, *Acidovorax*, *Acinetobacter*, *Alcaligenes,* and *Ralstonia*), showed a high relative abundance in the total and metabolically active fractions of the microbial community. Interestingly, members of the *Ralstonia* and *Acinetobacter* groups are typically resistant to metals, i.e. Cu, Ni, Cd and Zn (Dhakephalkar & Chopade, 1994; Brim *et al.*, 1999; Boswell *et al.*, 2001; Goris *et al.*, 2001; Mergeay *et al.*, 2003), suggesting that the

abundance of toxic metals in the ORFRC subsurface may have selected for these lineages.

In order to create conditions favourable for microbially mediated U(VI) reduction, current bioremediation strategies are directed towards reducing nitrate concentrations by stimulating nitrate-reducing microbial communities (Finneran *et al*., 2002; Senko *et al*., 2002; Istok *et al*., 2004; North *et al*., 2004). Our study points to several groups of metabolically active bacterial lineages with nitrate-reducing capabilities that are adapted to the pH range, low-nutrient, and high toxic metal concentrations common to radionuclide-contaminated subsurface sediments. Our microbial community analysis in conjunction with potential rates of microbial activity suggests that these groups have a high potential for bioremediation and should be explored further.

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Supplementary material

The following supplementary material is available for this article online:

Table S1. Summary of phylogenetic affiliation and distribution of SSU rRNA gene sequences from four FB61 clone libraries.

Table S2. Summary of phylogenetic affiliation and distribution of SSU rRNA sequences from four FB61 clone libraries.

This material is available as part of the online article from: http://www.blackwell-synergy.com/doi/abs/10.1111/j.1574-6941.2006.00203.x (This link will take you to the article abstract).

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