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Application of a High-Density Oligonucleotide Microarray Approach To Study Bacterial Population Dynamics during Uranium Reduction and Reoxidation†

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Reduction of soluble uranium U(VI) to less-soluble uranium U(IV) is a promising approach to minimize migration from contaminated aquifers. It is generally assumed that, under constant reducing conditions, U(IV) is stable and immobile; however, in a previous study, we documented reoxidation of U(IV) under continuous reducing conditions (Wan et al., Environ. Sci. Technol. 2005, 39:6162–6169). To determine if changes in microbial community composition were a factor in U(IV) reoxidation, we employed a high-density phylogenetic DNA microarray (16S microarray) containing 500,000 probes to monitor changes in bacterial populations during this remediation process. Comparison of the 16S microarray with clone libraries demonstrated successful detection and classification of most clone groups. Analysis of the most dynamic groups of 16S rRNA gene amplicons detected by the 16S microarray identified five clusters of bacterial subfamilies responding in a similar manner. This approach demonstrated that amplicons of known metal-reducing bacteria such as *Geothrix fermentans* (confirmed by quantitative PCR) and those within the *Geobacteraceae* were abundant during U(VI) reduction and did not decline during the U(IV) reoxidation phase. Significantly, it appears that the observed reoxidation of uranium under reducing conditions occurred despite elevated microbial activity and the consistent presence of metal-reducing bacteria. High-density phylogenetic microarrays constitute a powerful tool, enabling the detection and monitoring of a substantial portion of the microbial population in a routine, accurate, and reproducible manner.

Uranium contamination is a persistent legacy of the cold war era. When uranium mining and processing for nuclear weapons and fuel were at their peak, uranium-containing wastes accumulated, resulting in a multitude of contaminated sites worldwide. In the United States specifically, there are more than 120 uranium contaminated sites, containing approximately 6.4 trillion liters of waste (33). The dominant uranium isotope in this waste, ^{238}U, has a half-life of approximately 4.5 billion years; additionally, uranium is a heavy metal and as such is toxic to cellular function. Uranium remediation strategies in recent years have focused on containment, to minimize migration of uranium in groundwater and prevent infiltration into surrounding water courses and potable water supplies. A promising approach to minimizing uranium migration is to catalyze the reduction of soluble U(VI) to the less-soluble U(IV) (31, 39). This process can be accelerated by the action of indigenous microorganisms fueled through the addition of exogenous carbon. Organic carbon addition stimulates biomass and microbial activity in these typically nutrient-poor environments and has a profound impact on microbial community composition (18, 35).

Although a wide range of bacteria are capable of uranium reduction (27), the reduction of U(VI) to U(IV) typically coincides with an increase in populations of metal-reducing bacteria such as members of the *Geobacteraceae* and others within the *Deltaproteobacteria* (18, 22, 28, 54). A recent pilot scale field application has suggested that uranium reduction is transient, with remobilization apparently associated with shifts to microbial populations possibly less capable of U(VI) reduction (2). While it has been shown that reoxidation of U(IV) can occur in the presence of molecular O_2, NO_3^-, and denitrification intermediates (NO_2^- and N_2O) (15), it is generally assumed that reduced U is stable under constant low redox conditions.

In an effort to monitor the long-term stability of bioreduced U(IV), we carried out flowthrough column incubations for more than 500 days using soil from Area 2 of the uranium-contaminated NABIR Field Research Center (FRC) at Oak Ridge, Tenn. (60). U(VI) reduction and immobilization appeared to be successful for the first 100 days; however, following this period of net U(VI) reduction, significant reoxidation of U(IV) and remobilization of U(VI) occurred. Surprisingly, during the period of net U(IV) reduction, columns were under constant reducing (methanogenic) conditions. To determine if the remobilization of U(VI) was associated with alterations in microbial populations, we used a high-density oligonucleotide microarray-based approach (10) which permits simultaneous monitoring of the population dynamics of almost 9,000 distin-

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guishable prokaryotic taxa/units (operational taxonomic units [OTUs]). To validate this approach, we also analyzed identical samples using a more routine clone library approach in addition to confirmatory tests using quantitative PCR. Microarrays targeting functional genes have been employed for analysis of biodegradative capabilities in contaminated sites (43), while 16S rRNA gene microarrays have been used successfully to differentiate bacteria in specific groups, such as *Enterococcus* (26), *Cyanobacteria*, (4), nitrifying bacteria (24), fish pathogens (61), and human colon microflora (37). Here we report the first application of high-density array technology in profiling the complex microbial communities of soils or sediments.

MATERIALS AND METHODS

Column setup and analyses. The experiment was conducted on soils heavily contaminated from past U waste disposal (U concentration of 206 mg kg^{-1}), obtained from the NABIR Field Research Center (FRC) at Oak Ridge National Laboratory (Tennessee) (http://www.esd.ornl.gov/nabirfrc/). Wet soils were passed through a 5.6-mm sieve, homogenized, packed into columns (200-mm length, 32-mm inner diameter [ID]) to a bulk density of 1.35 Mg m^{-3} and permeated with 10.7 mM Na-lactate solution (32 mM organic carbon [OC], pH 7.2) to stimulate U bioreduction by the native microbial community. The solution was supplied at a pore fluid velocity of 13 mm day^{-1} for 215 days, and thereafter decreased to 10 mm day^{-1}. Uranium reoxidation and remobilization was noted after 100 days, 115 days prior to the reduction in fluid pore velocity. Throughout the experiment, OC concentrations in the column effluents did not decline below 1 mM, demonstrating a nonlimiting supply of OC despite decreased pore fluid velocity. Further details are presented by Wan et al. (60).

Microbial activity. Dehydrogenase activity was determined by 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyl tetrazolium chloride (INT) reduction (57) under anaerobic conditions at room temperature.

DNA extraction from soil. DNA was extracted from 500 mg (wet weight) of soil using a BIO101 soil DNA extraction kit (QBiogene, Irvine, Calif.) according to the manufacturer's protocol. For samples taken prior to lactate stimulation ("Area 2" soil) and during the net U(VI) reduction phase ("Red."), 3 subsamples were taken from homogenized material and each extracted independently. For samples taken during the net U(IV) oxidation phase ("Ox."), 3 regions of the column were sampled, bottom, middle, and top, to test for variability in microbial community composition. Extracted DNA was quantified by absorption at 260 nm, and quantities are expressed in terms of μg DNA g soil (dry weight)$^{-1}$.

PCR amplification of 16S rRNA genes. 16S rRNA genes were amplified from each DNA extract in triplicate, and amplicons were pooled for each extract. PCRs were performed in a final volume of 100 μl and contained 1× Takara ExTaq PCR buffer, 2 mM $MgCl_2$, 300 μM primers (27F and 1492R) (62), 200 μM deoxynucleoside triphosphates, 50 μg bovine serum albumin, 0.5 μl of DNA extract, and 2.5 U ExTaq DNA polymerase (Takara Mirus Bio Inc., Madison, Wis.), and sterile milliQ H_2O up to 100 μl. For terminal restriction fragment length polymorphism (T-RFLP) analysis, the forward primer 27F was labeled with 6-carboxyfluorescein for detection by capillary electrophoresis. Cycling was performed using an iCycler (Bio-Rad, Hercules, Calif.) with initial denaturation at 95°C (5 min), followed by 35 cycles of 95°C (30 s), 53°C (30 s), and 72°C (1 min) using maximum temperature ramp rates, and a final extension at 72°C (7 min). Amplicons were run on 1% agarose gels. Amplicons were purified using an UltraClean PCR clean up kit (MoBio, Inc., Carlsbad, Calif.) according to the manufacturer's protocol, eluted in a final volume of 50 μl, and quantified by gel electrophoresis.

Microbial community screening by T-RFLP. Terminally labeled 16S rRNA gene amplicons were generated, purified, and quantified as described above. Approximately 400 ng of amplicon was digested overnight at 37°C in a 50-μl reaction mixture containing 2 U of restriction endonuclease AluI (NEB, Beverly, Mass.) in 1× NEB buffer 2. Digested amplicons were precipitated and desalted prior to capillary electrophoresis using glycogen as a coprecipitant and standard salt-alcohol procedures. Pellets were dried under a vacuum in the dark and resuspended in Hi-Di formamide (10 μl; Applied Biosystems, Foster City, Calif.). Aliquots (1 μl) were mixed with a GeneScan 500-Rox size standard (0.25 μl; Applied Biosystems) and 10 μl Hi-Di formamide. Immediately prior to electrophoresis, samples were denatured at 95°C for 5 min and cooled rapidly on ice. Electrophoresis was carried out using an ABI 3100 automated capillary sequencer (Applied Biosystems) run in GeneScan mode, with an electrokinetic injection time of 60 s. Terminal restriction fragments (TRFs) were sized using GeneScan v3.7 software (Applied Biosystems). All profiles consistently gave cumulative peak heights of over 10,000 fluorescence units, and only peaks of 50 fluorescence units or greater were considered further. TRFs were considered identical if they differed by <0.5 bp. Individual TRF heights were normalized as a percentage of total peak height. Principal component analysis (PCA) ordination was performed using the software package PC-Ord v.4.01 (MjM Software, Gleneden Beach, Oreg.).

Oligonucleotide probe selection and 16S PhyloChip design. The microarray probe design approach previously described for differentiating *Staphylococcaceae* (10) was applied to all known prokaryotic sequences of substantial length. Briefly, 16S rRNA gene sequences (*Escherichia coli* base pair positions 47 to 1473) were obtained from over 30,000 16S rRNA gene sequences that were at least 600 nucleotides in length in the 15 March 2002 release of the 16S rRNA gene database "Greengenes" (greengenes.lbl.gov). This region was selected because it is bounded on both ends by universally conserved segments that can be used as PCR priming sites to amplify bacterial or archaeal genomic material using only 2 to 4 primers (11). Putative chimeric sequences were filtered from the data set using the software package Bellerophon, preventing them from being misconstrued as novel organisms (19). The filtered sequences are considered to be the set of putative 16S rRNA gene amplicons. Sequences were clustered to enable each sequence of a cluster to be complementary to a set of perfectly matching (PM) probes. Putative amplicons were placed in the same cluster as a result of common 17-mers found in the sequence. The resulting 8,935 clusters, each containing approximately 0 to 3% sequence divergence, were considered OTUs representing all 121 demarcated prokaryotic orders. The taxonomic family of each OTU was assigned according to the placement of its member organisms in Bergey's Taxonomic Outline (16). The taxonomic outline maintained by Philip Hugenholtz (20) was consulted for phylogenetic classes containing uncultured environmental organisms or unclassified families belonging to named higher taxa. The OTUs comprising each family were clustered into subfamilies by transitive sequence identity according to a previously described method (10). Altogether, 842 subfamilies were found. The taxonomic position of each OTU and the accompanying NCBI accession numbers of the sequences composing each OTU can be viewed at http://greengenes.lbl.gov/Download/Taxonomic_Outlines/G2_chip_SeqDescByOTU_tax_outline.txt.

The objective of the probe selection strategy was to obtain an effective set of probes capable of correctly categorizing mixed amplicons into their proper OTU. For each OTU, a set of 11 or more specific 25-mers (probes) were sought that were prevalent in members of a given OTU but were dissimilar from sequences outside the given OTU. In the first step of probe selection for a particular OTU, each of the sequences in the OTU was separated into overlapping 25-mers, the potential targets. Then each potential target was matched to as many sequences of the OTU as possible. It was not adequate to use a text pattern search to match potential targets and sequences, since partial gene sequences were included in the reference set. Therefore, the multiple-sequence alignment provided by Greengenes was necessary to provide a discrete measurement of group size at each potential probe site. For example, if an OTU containing seven sequences possessed a probe site where one member was missing data, then the site-specific OTU size was only six. In ranking the possible targets, those having data for all members of that OTU were preferred over those found only in a fraction of the OTU members. In the second step, a subset of the prevalent targets was selected and reverse complemented into probe orientation, avoiding those capable of mis-hybridization to an unintended amplicon. Probes presumed to have the capacity to mis-hybridize were those 25-mers that contained a central 17-mer matching sequences in more than one OTU (34, 56). Thus, probes that were unique to an OTU solely due to a distinctive base in one of the outer four bases were avoided. Also, probes with mis-hybridization potential to sequences having a common tree node near the root were favored over those with a common node near the terminal branch. Probes complementary to target sequences that were selected for fabrication are termed PM probes. As each PM probe was chosen, it was paired with a control 25-mer (mismatching [MM] probe), identical in all positions except the 13th base (34). The MM probe did not contain a central 17-mer complementary to sequences in any OTU. The target probe and MM probes constitute a probe pair analyzed together. PM probes for each OTU can be viewed at http://greengenes.lbl.gov/cgi-bin/nph-show_probes_2_otu_alignments.cgi.

The chosen oligonucleotides were synthesized by a photolithographic method, at Affymetrix, Inc. (Santa Clara, CA), directly onto a 1.28-cm by 1.28-cm glass surface at an approximate density of 10,000 probes per μm^2 (6). Each unique probe sequence on the array had a copy number of roughly 3.2×10^6 (Affymetrix, personal communication). The entire array of 506,944 features was arranged as a square grid of 712 rows and columns. Of these features, 297,851 were oligonucleotide PM or MM probes targeting 16S rRNA gene sequences, and the

remaining were used for image orientation, normalization controls, or other unrelated analyses.

16S microarray sample preparation. From each of the 9 pools of 16S rRNA gene amplicons (3 each for Area 2, reduction, and oxidation sampling time points), 2 μg was DNaseI fragmented and biotin labeled, and an aliquot (1.85 μg) was hybridized to custom-made Affymetrix GeneChips (16S PhyloChip) according to the manufacturer's recommended protocol in the same way as cDNA is processed for expression arrays. Hybridization was performed at 60 rpm at 48°C overnight. PhyloChip washing and staining were performed according to standard Affymetrix protocols as described previously (32). In total, 9 microarrays were analyzed. Each PhyloChip was scanned and recorded as a pixel image, and initial data acquisition and intensity determination were performed using standard Affymetrix software (GeneChip microarray analysis suite, version 5.1).

Background subtraction. Background probes were identified as those producing intensities in the lowest 2% of all intensities. The average intensity of the background probes was subtracted from the fluorescence intensity of all probes. The noise value (*N*) was the variation in pixel intensity signals observed by the scanner as it read the array surface. The standard deviation of the pixel intensities within each of the identified background cells was divided by the square root of the number of pixels comprising that cell. The average of the resulting quotients was used for *N* in the calculations described below.

Detection and quantification criteria. Probe pairs scored as positive were those that met two criteria: (i) the intensity of fluorescence from the PM probe was greater than 1.3 times the intensity from the MM control and (ii) the difference in intensity, PM minus MM, was at least 500 times greater than the squared noise value ($>500\ N^2$). These two criteria were chosen empirically to provide stringency while maintaining sensitivity to the amplicons known to be present from sequencing of cloned Area 2 16S rRNA gene amplicons. The positive fraction (PosFrac) was calculated for each probe set as the number of positive probe pairs divided by the total number of probe pairs in a probe set. An OTU was considered present in the sample when over 90% of its assigned probe pairs were positive (PosFrac > 0.90). A hybridization intensity score (HybScore) was calculated in arbitrary units for each probe set as the trimmed average (maximum and minimum values removed before averaging) of the PM minus MM intensity differences across the probe pairs in a given probe set. All HybScores of <1 were shifted to 1 to avoid errors in subsequent logarithmic transformations. Two groupings are used to describe bacteria detected by the 16S microarray, the OTU and the subfamily. An OTU consists of a group of one or more sequences with typically 97 to 100% sequence homology, while a subfamily consists of a group of OTUs with typically no less than 94% sequence homology. Most comparisons are presented in terms of subfamilies as a conservative estimate of array specificity.

Array normalization. To account for variation from array to array, internal standards were added to each experiment. The internal standards were a set of 15 amplicons generated from yeast and bacterial metabolic genes, spiked into each amplicon pool prior to fragmentation. The known concentrations of the amplicons ranged from 4 pM to 605 pM in the final hybridization mix. HybScores resulting from the 15 corresponding probe sets were natural log transformed. Adjustment factors for each array were calculated by fitting a linear model using the least-squares method. The calculated array-specific adjustment factor was then subtracted from each probe set's ln(HybScore) for each array. When summarizing array results to the subfamily, the probe set (OTU) with the highest normalized array intensity (HybScore) was used as a representative.

Clone library comparison with microarray analyses. To compare the detection of bacteria using the 16S microarray with a more commonly used clone library approach, aliquots from the same 16S rRNA gene amplicon pools generated from Area 2 soil for array analysis were combined prior to ligation to pCR2.1 vectors. Following ligation, plasmids were transformed into TOP10 cells according to the manufacturer's instructions (Invitrogen, Carlsbad, Calif.). Clones (742) were randomly sequenced at the Department of Energy, Joint Genome Institute. Individual cloned rRNA genes were sequenced from each terminus, assembled using PHRED (14) and PHRAP (17), and required to pass quality tests of Phred 20 (base call error probability $< 10^{-2.0}$) to be included in the comparison. Chimeric sequences were identified using BELLEROPHON (19) and removed. Nonchimeric sequences of >1,300 bp in length were retained for further study and have been submitted to GenBank (see "Nucleotide sequence accession number" below). Representative 16S rRNA genes from each array-detected OTU were aligned with quality-assured sequences from the clone library. For phylogenetic placement, hypervariable regions of the alignment were masked, leaving 1,287 columns for generating a distance matrix. Phylogenetic trees were created from a neighbor-joining distance matrix using the ARB maximum parsimony algorithm (29). Rarefaction analysis of clone library sampling and Chao1 (5) estimates of richness were carried out using the software package DOTUR (46). Coverage was calculated according to the method described by Singleton et al. (49).

Identifying dynamic OTUs and subfamilies. Triplicate PhyloChip arrays were analyzed for each sample type, and normalized hybridization scores of representative OTUs within each subfamily were used to track changes in specific populations. A change in HybScore of 1,000 units is approximate to a 10-fold change in gene copy number (data not shown). To identify the most dynamic 16S rRNA gene amplicons, hierarchical clustering was performed on a prefiltered subfamily list containing only the 100 most variable subfamilies (based on standard deviation). This was performed within the R statistical programming environment (42) using the function "heatmap" within the package "made4" (9). Both samples and subfamilies were clustered with 1 Pearson's correlation as the distance metric and UPGMA (unweighted pair group method with arithmetic mean) as the linkage method. Heatmaps were reordered by dendrogram clustering to relocate covarying subfamilies and samples. The HybScore pattern over time for each cluster was plotted by averaging the intensities of all subfamilies within a cluster.

Real-time quantitative PCR confirmation of *Geothrix fermentans* population dynamics. Previously described primers (7) were used for quantitative real-time PCR (qPCR) (Gx.193F, 5′-GACCTTCGGCTGGGATGCTG-3′; Gx.448R, 5′-AGTCGTGCCACCTTCGT-3′). qPCR was performed using an iCycler iQ real-time detection system with the iQ Sybr Green Supermix kit (Bio-Rad). Reaction mixtures (final volume, 20 μl) contained 1X iQ SYBR green Supermix, 6 pmol of each primer, 10 μg bovine serum albumin, 0.5 μl DNA extract, and DNase/RNase-free water. Following enzyme activation (95°C, 3 min), 40 cycles of 95°C for 20 s, 51°C for 20 s, and 72°C for 30 s were performed. A specific data acquisition step at 87°C for 10 s was set above the melting temperature of potential primer dimers to minimize any nonamplicon SYBR green fluorescence. The copy number of *Geothrix fermentans* 16S rRNA gene molecules was quantified by comparing cycle thresholds to a standard curve (in the range of 10^2 to 10^8 copies), run in parallel, using *Geothrix fermentans* 16S rRNA gene amplicons generated by PCR (using universal bacterial primers 27F and 1492R) (62). Regression coefficients for the standard curves were consistently greater than 0.99, and postamplification melt curve analyses displayed a single peak at 90°C, indicative of specific *Geothrix fermentans* 16S rRNA gene amplification (data not shown).

Nucleotide sequence accession numbers. The nonchimeric sequences of >1,300 bp in length have been submitted to GenBank and assigned accession numbers DQ125500 to DQ125935.

RESULTS

Microbial biomass and activity. Unsurprisingly, column infusion with lactate stimulated microbial biomass, with DNA concentrations in soil samples taken during net U reduction being almost 10 times greater than in the original soil (Fig. 1A). However, biomass decreased between the net reduction and net oxidation phases. Lactate infusion also stimulated microbial activity (Fig. 1B), which increased from barely detectable levels in the original soil (1.2 μg iodonitrotetrazolium formazan [INF] $g^{-1}\ h^{-1}$) to over 690 μg INF $g^{-1}\ h^{-1}$ in samples from the net U reduction phase. Significantly, despite decreased biomass, microbial activity more than doubled to over 1,540 μg INF $g^{-1}\ h^{-1}$ in the net oxidation phase.

T-RFLP analysis of bacterial population shifts. Principal component analysis (PCA) of 16S rRNA gene-based T-RFLP profiles showed bacterial community divergence from the original starting material (Fig. 2), with separation occurring along both axes. The net U reduction phase and net oxidation phase samples also diverged but only along axis 2 (Fig. 2), indicating closer association to each other than to the original Area 2 soil community.

Clone library comparison with microarray analyses. Relatively large clone libraries (742 clones) were sequenced, assembled, and trimmed for quality before being screened for chimeras. After this quality control process, 429 high-quality clones remained, with almost 24% being detected as chimeric,

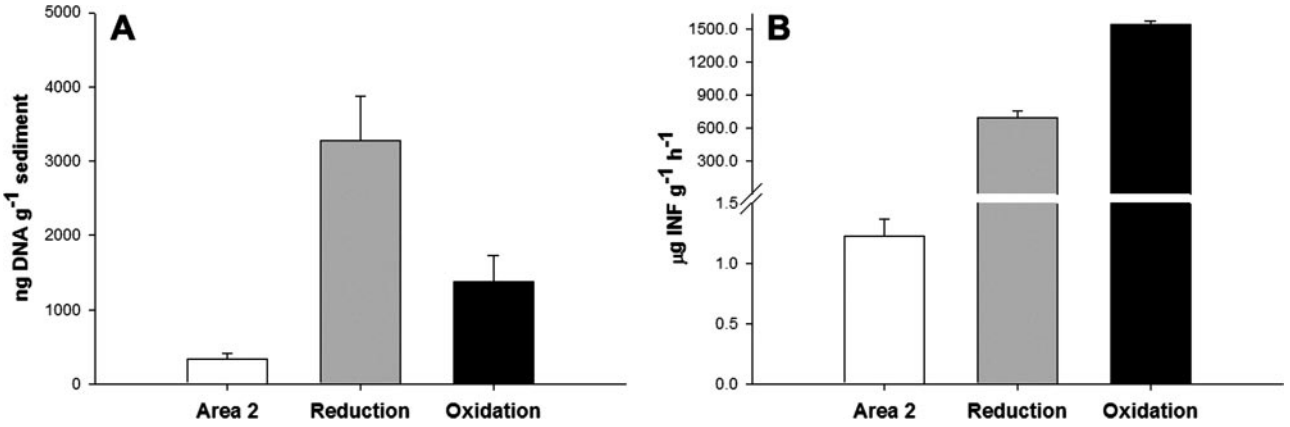

FIG. 1. Plots show DNA concentration (A) and microbial activity (dehydrogenase) (B) in soil samples taken from Area 2 soil before column packing and during net U reduction and oxidation phases. Bars show means ± standard errors ($n = 3$).

possibly due to the harsh nature of bead beating. Figure 3 shows a rarefaction curve with a sampling efficiency determined at 100% sequence homology (i.e., unique clones) and 99% sequence homology (i.e., the array defined OTU level). Neither curve reached an asymptote, indicating incomplete sampling of the Area 2 soil bacterial community after 429 high-quality clone sequences were analyzed. Coverage rates were 0.49 at 100% homology (unique), 0.61 at 99% homology (OTU), and 0.84 at 94% homology (subfamily).

Phylogenetic analysis of the composition of the Area 2 clone library (Fig. 4) shows an amplicon pool dominated by the *Actinobacteria* phylum accounting for almost 60% of the clones sampled. The families *Micrococcaceae* and *Microbacteriaceae* dominated the *Actinobacteria* clones, with the dominant species having their closest cultured homologs in *Arthrobacter* spp. and *Microbacterium* spp., respectively. The *Firmicutes* and the *Proteobacteria* represented the next most abundant phyla at 16% of clones each, with the latter being dominated by *Alphaproteobacteria*. Only one clone within the *Deltaproteobacteria* was detected.

In total, all clones detected could be assigned to 63 groups, most at the family or subfamily level. Of the 63 clone groups detected, the 16S microarray accurately identified a member of the same group in 59 cases, the other four cases consisted of two novel singleton clones (AKAU3608 and AKAU3516) not placed in any recognized bacterial grouping, a group of 11 clones within the family *Nocardiaceae* (subfamily 3) and a group of 13 novel *Chloroflexi* clones (which we have termed "uranium-contaminated soil clones") with low homology to previously sequenced *Chloroflexi* (3). In addition to the 59 groups of sequences in common between the clone and array approach, the array also detected 194 additional groups at the subfamily level. A phylogenetic tree comparing array and clone library detection of bacterial groups is available (see Fig. S1 in the supplemental material). Among the groups neglected by the cloning approach but detected by the array were organisms of significance to uranium bioremediation such as the

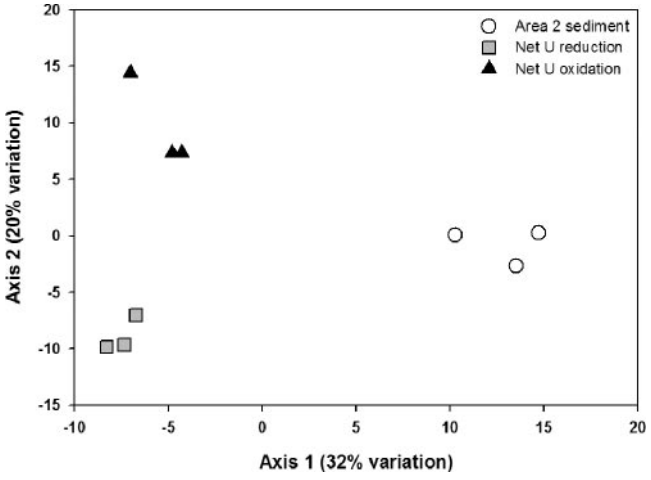

FIG. 2. PCA of 16S T-RFLP profiles showing bacterial community divergence from original FRC Area 2 soil prior to column packing and through net uranium reduction and reoxidation phases.

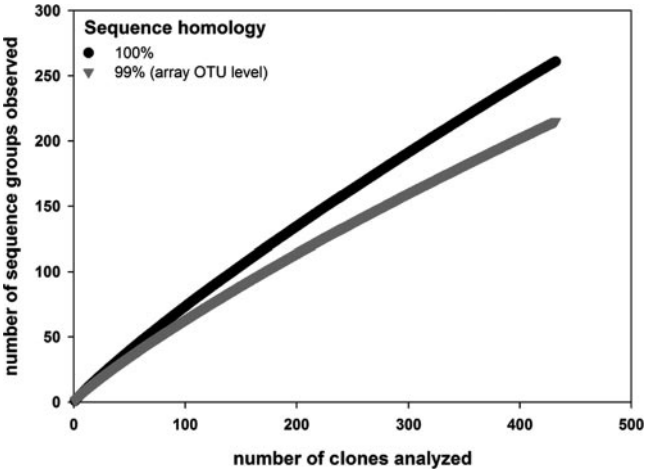

FIG. 3. Rarefaction analysis of clone library coverage of microbial diversity in uranium-contaminated Area 2 soil samples. Two sequence homology levels are shown: 100% represents the coverage estimate when clones are placed in groups using this homology cutoff and 99% represents the coverage estimate based on clones grouped using a 99% sequence homology cutoff, which corresponds to the typical divergence of sequences within array-defined OTUs.

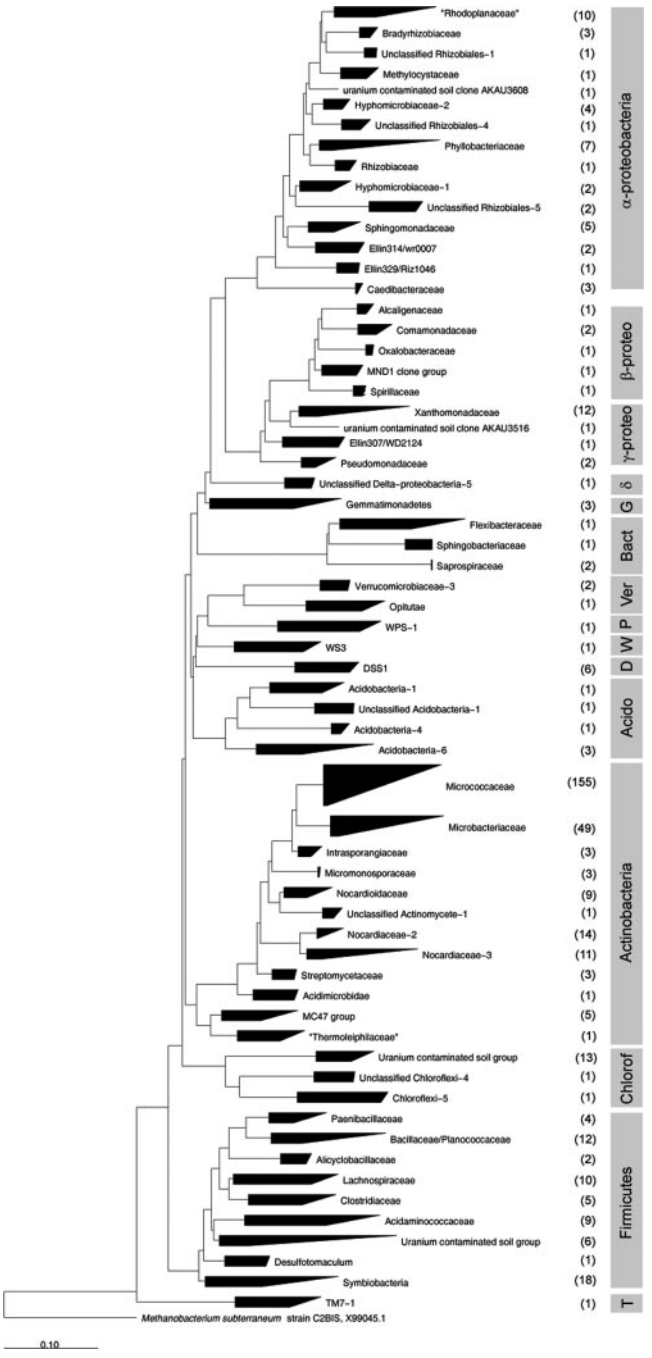

FIG. 4. Neighbor-joining tree showing phylogenetic position of 16S rRNA gene clones generated from Area 2 soil prior to column packing. Sequences were inserted by parsimony into a tree containing all bacteria and archaea in the "greengenes" database (as of 3 May 2005). Abbreviations are as follows: β-proteo, *Betaproteobacteria*; γ-proteo, *Gammaproteobacteria*; δ, *Deltaproteobacteria*; G, *Gemmatimonadetes*; Bact, *Bacteroidetes*; Ver, *Verrucomicrobia*; P, *Planctomycetes*; W, candidate division WS3; D, candidate division DSS1; Acido, *Acidobacteria*; Chlorof, *Chloroflexi*; T, candidate division TM7. Values in parentheses indicate numbers of clones in each group. *Methanobacterium subterraneum* strain C2BIS was used to root the tree. The scale bar represents 10 inferred nucleotide changes per 100 nucleotides analyzed.

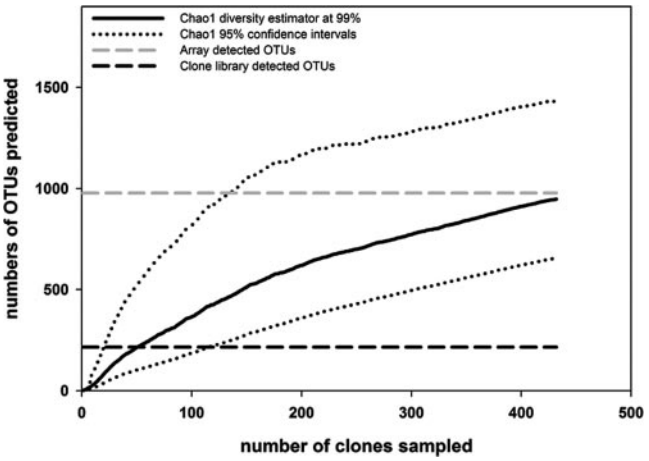

FIG. 5. Chao1 richness estimates of Area 2 soil bacterial communities based on clone library sampling. Richness estimates were made using a 99% sequence homology group classification equivalent to array-defined OTUs. The dashed gray line denotes the mean number of OTUs detected by array in Area 2 samples. The dashed black line denotes the number of OTUs detected by clone sequence analysis in Area 2 samples.

Geobacteraceae, which we have previously shown by primer-specific PCR to be present in this Area 2 sediment genomic DNA sample (60).

As rarefaction curves did not reach an asymptote, a nonparametric richness estimator, Chao1 (5), was used to predict richness (Fig. 5). The predicted richness of 948 OTUs corresponds well with the array-detected richness of 978 OTUs. While the curve has not yet leveled off, it is also possible that richness is being underestimated.

Identifying dynamic OTUs and subfamilies. Dynamic OTUs and subfamilies were detected by confining the analysis to the 100 subfamilies exhibiting the greatest standard deviation between the phases sampled. Hierarchical cluster analysis allowed detection of correlations between samples and also between subfamilies (Fig. 6). Array analysis indicated that Area 2 soil sample communities formed a separate cluster from those sampled during the net uranium reduction or oxidation phases, with the latter two arising from a common node. Overall, five groups of dynamic subfamilies were detected by cluster analysis (Fig. 6; see Table S2 in the supplemental material).

Cluster group 1 sequences increased following lactate amendment to reach a peak during the reduction phase and subsequently decline during the oxidation phase. This group contained mostly *Proteobacteria* with some *Actinobacteria*, *Firmicutes*, and members of the *Deinococcus-Thermus* and *Planctomyces* phyla. The *Proteobacteria* which responded in this manner were mostly orders within the α (*Azospirillales*, *Caulobacterales*, *Sphingomonadales*) or β (*Burkholderiales*, *Neisseriales*, *Rhodocyclales*) subphyla and are typically capable of nitrate reduction. Sequences within the *Cellulomonadaceae* family (order *Actinomycetales*) also increased following lactate addition and subsequently declined. Two orders of *Deltaproteobacteria*, *Bdellovibrionales* and *Desulfobacterales*, and a *Firmicute* of the homoacetogenic genus *Sporomusa* (44) also responded in a similar way.

Cluster group 2 sequences increased following lactate stim-

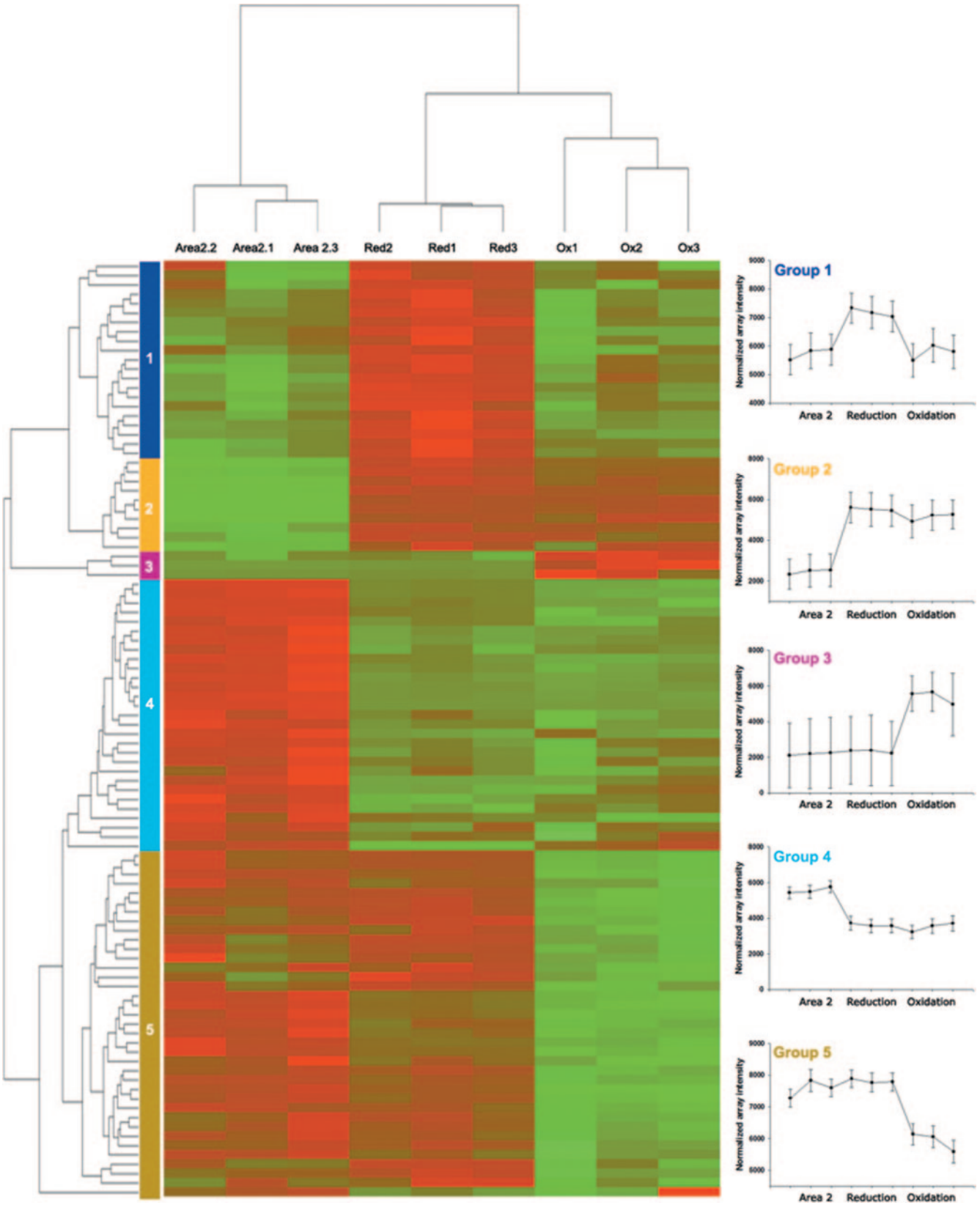

FIG. 6. Heatmap and dendrograms showing the response of 100 bacterial subfamilies (shown on *y* axis) exhibiting the highest standard deviation between samples (shown on *x* axis) taken pre-lactate stimulation (Area2.1, Area2.2, and Area2.3), during net uranium reduction (Red1, Red2, and Red3), and during net uranium reoxidation (Ox1, Ox2, and Ox3). The color gradient from green to red represents increasing array hybridization intensity. Five main response groups were detected, and the average intensity (HybScore) of the cluster group response is presented in line plots to the right of the heatmap. Error bars represent standard errors in HybScore differences between all subfamilies in a cluster.

ulation and remained at elevated concentrations in the amplicon pool throughout the net uranium reduction and oxidation phases. This group contained three families of *Deltaproteobacteria*, the *Nitrospinaceae*, *Geobacteraceae*, and *Anaeromyxobacter*. In addition to *Deltaproteobacteria*, members of the *Acidobacteriaceae* were also detected, specifically the species *Geothrix fermentans*. This cluster also contained *Rhodocyclaceae*, *Comamonadaceae* (*Malikia spinosa*), and an *Actinomycete* of the genus *Intrasporangium*.

Cluster group 3 sequences comprised three subfamilies which were of lower abundance in the original Area 2 soil and reduction-phase soil amplicon pools but increased during the net uranium oxidation phase. Two of the subfamilies were from the *Acidobacteria*, with both representative clones coming from halogenated organic carbon-degrading consortia (47, 59). The other member of cluster group 3 was a bacterium within the *Desulfovibrionaceae* family.

Cluster group 4 was a large cluster containing sequences from 28 bacterial subfamilies whose amplicons declined in abundance or disappeared following lactate addition. The groups consisted of four families of *Actinomycetes*, including *Microbacteriaceae*, one of the dominant families in the original Area 2 soil amplicon pool. Bacteria within the *Chloroflexi*, *Cyanobacteria*, and *Acidobacteria* also followed this pattern. Five families within the order *Bacillales* also declined following lactate addition, as did 8 families within the *Alphaproteobacteria*. The *Xanthomonadaceae*, which initially was the most abundant *Gammaproteobacteria* family in the Area 2 soil clone library, also declined.

The final cluster group observed, group 5, consisted mainly of *Actinobacteria*, *Firmicutes*, *Cyanobacteria*, and *Alphaproteobacteria* sequences, representing most of the initially dominant clones present in the original Area 2 soil, including the family *Micrococcaceae*. Following lactate addition, these amplicons appeared to maintain similar abundance through the uranium reduction phase but showed a sharp decline during the uranium reoxidation phase.

Analysis of the dynamics of all subfamilies detected indicated that, between the net U reduction and net U oxidation phases, the amplicon signatures of many (206 subfamilies) did not change, while a large number (139 subfamilies) declined, with only a small proportion (13 subfamilies) increasing in quantity (see Table S1 in the supplemental material). However, compared with other bacterial groups, a disproportionate amount of *Actinobacteria* and α-, β-, and γ-proteobacteria subfamilies showed a decline in amplicon concentration between the reduction and oxidation phases.

Real-time quantitative PCR confirmation of *Geothrix fermentans* population dynamics. We have previously demonstrated via 16S microarray analysis and confirmed by qPCR that bacteria within the family *Geobacteraceae* increased in abundance following lactate addition and maintained an increased abundance through both uranium reduction and reoxidation phases (60). To confirm array observations that the *Acidobacteria* species *Geothrix fermentans* showed this pattern of increased abundance, as detected by the 16S microarray, we used primers specific for this species together with a SYBR green-based quantitative PCR approach. This showed that initial populations of *G. fermentans* in Area 2 soil were below detection limits (10^3 copies g soil^{-1} for qPCR) (Table 1); however, following lactate stimulation, *G. fermentans* abundance increased to over 10^6 copies g soil^{-1} during the uranium reduction phase. Samples from the uranium reoxidation phase also showed increased *G. fermentans* populations over 10^6 copies g^{-1}, correlating well with 16S microarray observations (Table 1).

TABLE 1. Real-time quantitative PCR analysis of changes in *Geothrix fermentans* gene copy number compared with array intensity

Sample source[a]	Replicate no.	Normalized array intensity[d]	No. of 16S rRNA gene copies g^{-1}
Area 2	1	133[b]	ND[c]
	2	119[b]	ND
	3	135[b]	ND
Reduction	1	6,309	1.7×10^6
	2	6,295	3.3×10^6
	3	6,122	4.7×10^6
Oxidation	1	5,497	2.7×10^6
	2	6,054	4.0×10^6
	3	6,433	9.5×10^6

[a] Area 2 indicates the original sediment prior to column packing, reduction indicates samples taken during the net uranium reduction phase, and oxidation indicates samples taken during the net uranium oxidation phase.
[b] The *Geothrix fermentans* probe set was below the positive fraction threshold of 0.90 (90% of probe pairs must pass) in these samples and therefore is considered not detected by the array.
[c] ND, none detected.
[d] Array intensities are expressed in arbitrary fluorescence units.

DISCUSSION

The observed reoxidation of bioreduced U(IV) reported by Wan et al. (60) is of significant concern, considering the limited options available for uranium remediation. From this work, it now appears that even under highly reducing (methanogenic) conditions, the stability of bioreduced U(IV) can be transient. A by-product of microbial metabolism, dissolved inorganic carbon, coupled with high Ca^{2+} concentrations was shown by Wan et al. (60) to drive the redox potential of the U(VI):U(IV) oxidation couple more negative, thus accounting for the thermodynamic feasibility of U(IV) oxidation under reducing conditions. The terminal electron acceptor(s) for U(IV) oxidation have not yet been identified for this process, but it has been hypothesized that either Fe(III) or Mn(IV) is the likely candidate (60). What remains unclear is the role of alterations in microbial community composition coincident to the changing process dynamics. Is there an active microbially catalyzed oxidation process? Or is the microbial role indirect, simply through the production of CO_2? If U(VI) reduction and U(IV) reoxidation are thermodynamically favorable under similar redox conditions, then the possibility remains that a balance exists between the two processes and that during the net reoxidation phase, reoxidation rates exceed those of reduction. In terms of microbial community composition, a possible explanation for this would be the loss of microbial species or functional groups capable of U(VI) reduction. It is conceivable, for example, that *Geobacter* species, having reduced all available iron and uranium within their immediate vicinity or range of motility, are faced with no terminal electron acceptor with which to couple the oxidation of organic carbon, resulting in population decline as other organisms, possibly sulfate re-

ducers, fermenters, or methanogens, are able to out-compete the metal reducer. To test for functionally significant alterations in bacterial community composition, we analyzed samples from both the net U reduction and net U reoxidation phases of a column incubation using historically contaminated soil from Oak Ridge, Tenn.

Using DNA as a proxy of microbial biomass, it was clear that lactate infusion into contaminated soils resulted in significant biomass stimulation, which reached a peak during the net U reduction phase. However, biomass was observed to decline from the net U reduction to the net U oxidation phase despite a nonlimiting supply (60) of OC. A decrease in suitable terminal electron acceptors (TEAs), as indicated by methane production, low effluent concentrations of iron, uranium, and manganese, and low redox potential (60), may have resulted in decreased biomass as microorganisms become unable to fuel cellular metabolism. This effectively would result in a decline in organisms solely reliant on oxygen, nitrate, iron, uranium, manganese, or sulfate as TEAs for oxidation of OC. Despite the significant reduction in bacterial biomass, microbial activity (using dehydrogenase activity as a proxy) more than doubled between net reducing and oxidizing phases. However, the INT reduction assay used to estimate dehydrogenase activity may also be considered an assay of total reducing potential. Nonviable cells may possess residual reduction capacity, and nonbiological reduction of INT may be possible in environments with redox potentials below that of INT (−90 mV) (50), as found in the soil columns during this study (−250 to −150 mV) (60). Nevertheless, as the system redox potential was below that of INT in columns during both U reduction and oxidation phases, the increase in INT reduction during the oxidation phase cannot be solely due to abiotic effects. Studies using *Escherichia coli* have demonstrated that dehydrogenase activity, measured by INT reduction, is greater in cells growing fermentatively than in cells exhibiting anaerobic respiration (50). Such a shift to fermentation as the dominant process following TEA exhaustion may explain the observed increase in INT reduction in the net U oxidation phase despite reduced biomass.

To investigate shifts in the composition and dynamics of bacterial populations during the remediation process, we employed a high-density DNA microarray approach together with a traditional clone library as validation. Both methods sample a gene amplicon pool following PCR amplification; however, clone libraries are known to underestimate microbial diversity either due to insufficient number of clones sequenced (46) or cloning bias (58). DNA microarray approaches, on the other hand, are not subject to cloning bias and have the capacity to sample a far greater number of molecules, which represents a significant advantage, particularly when highly abundant sequences mask clone sampling efforts. However, it must be noted that, in its current form, the DNA microarray approach described here is subject to end-point PCR bias in the same way as the accepted 16S rRNA gene clone libraries. This bias is primarily due to primer degeneracies resulting in preferential amplification of specific sequences and potential misrepresentation of sequence abundance within genomic DNA extracts (40); however, misrepresentations based on these studies of noncomplex mixtures appear to be on the order of two- to fourfold (36). This bias is thought to be magnified by increasing the number of PCR cycles and has only recently been tested in complex environmental mixtures (1, 30). Acinas et al. (1) observed no effect of cycle number on PCR bias, where clone distribution was generally in good agreement with previously observed distributions for bacterioplankton. In this study, we used a conservative approach by confining analyses to the 100 most dynamic subfamilies detected, all of which exhibited changes 10-fold or greater. PCR bias is also thought to be most significant in the early cycles where random priming effects may result in misrepresentation of phylotype abundances. To minimize this effect, we combined triplicate PCRs for each sample (40), and although PCR bias due to template reannealing is thought to be minimal in complex environmental samples (51, 52), we attempted to reduce any bias by using the fastest possible temperature ramp times during PCR cycling (25). Like all PCR-based clone library analyses, we cannot rule out bias in altering the relative abundance of phylotypes or OTUs; however, we have taken several steps to minimize its influence.

The design of the probes contained in the 16S microarray was based on approximately 30,000 sequences of 600 bp or greater which were available in public databases in March 2002. Therefore, it was possible that recently published novel sequences would not be detectable using the 16S microarray. To confirm the ability of the 16S microarray to accurately classify bacterial sequences in the uranium-contaminated soil from Area 2 at the Oak Ridge Field Research Center (60), 16S amplicon pools from three independent soil extractions were processed and hybridized to separate 16S microarrays. For clone libraries, an aliquot of the three amplicon pools was combined and used in the ligation reaction. Assembled clone sequences were obtained for 742 clones, which after quality screening, yielded 429 high quality sequences.

Amplicons from the initial Area 2 soil bacterial community were dominated by high-G+C gram-positive bacteria, particularly the families *Micrococcaceae* (*Arthrobacter* spp.) and *Microbacteriaceae* (*Microbacterium* spp.). *Arthrobacter* species have been shown to be resistant to high concentrations of uranium while also capable of intracellular heavy metal accumulation (53) and uranium adsorption (55); as such, they may play a role in natural uranium immobilization at the Oak Ridge site. Although *Microbacterium* spp. are known to be capable of heavy metal (chromate) reduction (21) and have been reported to accumulate actinides (plutonium) intracellularly (23), their potential role in uranium immobilization is not clear. Other initially abundant 16S sequence types included α-proteobacteria within the *Rhizobiales* (*Hyphomicrobiaceae*, "*Candidatus* Rhodoplanaceae," *Phyllobacteriaceae*) and the *Sphingomonadales*, organisms typically considered oligotrophic. *Hyphomicrobium* has previously been detected at the Oak Ridge site as the dominant *nirK* type denitrifier (63).

Comparison of phylogenetic composition of the Area 2 soil by clone library and 16S microarray analysis showed that most clone groups (59 of 63) were detected by the array. As might be expected, two of the clone groups not identified by the array were singletons which could not be placed in any recognized bacterial phylum. The other two groups consisted of 11 clones within the *Nocardiaceae* (subfamily 3) and 13 clones representing novel *Chloroflexi*. While the 16S microarray was capable of accurately detecting most bacteria sampled by the clone library analysis, the 16S microarray detected many more (253 groups)

bacteria than the clone library (63 groups). One explanation for this observation may be inadequate clone library sampling, resulting in an underestimation of bacterial richness. Rarefaction analysis of clone library sampling demonstrated that the number of clones sequenced was indeed insufficient to adequately determine diversity within the Area 2 soil sample. As is typical in analysis of soil microbial communities, the predicted richness (948 OTUs), by Chao1 estimates in this case, was far greater than that observed by cloning (215 OTUs). Array analysis, however, detected a number (978 OTUs) similar to the Chao1 diversity estimate; therefore, it appears the array approach is capable of detecting substantially more sequence types than a relatively large sample clone library.

Another possible explanation for the discrepancy between array and clone library observed bacterial richness is the influence of nonspecific hybridization leading to false positives in array analysis. In designing this 16S microarray,we specifically chose the Affymetrix platform due to its probe capacity and the added confidence provided by the probe-pair strategy. Here, each perfectly matching probe is paired with a corresponding mismatch probe containing a single-base mismatch at the central nucleotide position, providing a nonspecific hybridization control for each probe on the array. In addition, we have previously confirmed by primer-specific PCR that families of bacteria that are detected by the 16S microarray and overlooked by cloning are indeed present (60).

A major strength of using high-density arrays is the ability to perform replicated analyses in a routine and reproducible manner. While clone libraries may also be replicated, the cost is generally prohibitive, and additional bias may occur (52). In this study, we required an approach that allowed statistical analysis of the changes in bacterial communities and their members over time to determine if the observed uranium reoxidation was a result of shifts in microbial groups of interest. T-RFLP analysis indicated that the bacterial community composition had changed following lactate addition but that the communities during the U reduction and U oxidation phases were only separated along one PCA axis. This is a useful approach, but 16S rRNA gene-based T-RFLP of diverse communities with universal primers does not provide reliable information regarding community composition. To mine the large quantities of data produced by this phylogenetic array and identify groups of covarying bacterial sequences, we used clustering approaches in a similar manner to those used for more standard gene expression arrays (9). Hierarchical clustering of the 100 most variable subfamilies detected by the 16S microarray demonstrated that variable sequences fell into 5 primary response groups from which it is possible to suggest common population dynamics. Cluster group 4 exhibited a distinct profile, declining rapidly following lactate addition through the U reduction and U reoxidation phases. This cluster consisted mostly of sequences from the actinobacteria, bacilli, and α-proteobacteria, which presumably declined as bacteria with more efficient lactate utilization flourished. Cluster group 1 sequences increased initially following the lactate amendment but declined during the U reoxidation phase. This group was primarily composed of α- and β-proteobacteria typically capable of nitrate reduction in addition to some bacteria recognized as potential uranium reducers, such as the family *Cellulomonadaceae* (45). While some *Deltaproteobacteria* sequences were also in this cluster, neither group has been reported to reduce U(VI). Interestingly *Bdellovibrio* spp. are bacterial parasites, and their increase may simply be a response to the increased biomass of their prey. The significant increase of this cluster of sequences followed by a significant decline may indicate a rapid oxidation of added carbon coupled with reduction of available TEAs and a subsequent decline as suitable TEAs are exhausted. Cluster group 5 sequences were initially at a higher density in the original Area 2 sediment amplicon pool prior to lactate amendment, but unlike cluster group 4, they did not immediately decline following carbon addition. Again, like cluster group 4, this sequence group contained many families of *Actinobacteria*, including the family *Micrococcaceae*, the dominant 16S rRNA clone type. This may be significant in explaining some of the observed mobilization of uranium in these column studies, as any intracellular uranium accumulated by these organisms would be released upon cell lysis. Cluster group 4 also contained many *Alpha-* and *Gammaproteobacteria* capable of nitrate reduction, which may have retained competitiveness until the in situ nitrate (only 1.1 mg kg^{-1}) was depleted.

Cluster group 2 sequences increased following lactate addition but showed no decline during the observed reoxidation phase, and significantly, this group contained many typical U(VI) reducing bacteria such as the family *Geobacteraceae* and the genera *Geothrix* and *Anaeromyxobacter*. These bacteria have been detected before in either iron- or uranium-reducing enrichments using other NABIR FRC sediments (36, 38, 48). We confirmed this observation for *Geothrix fermentans* using qPCR in this study and for *Geobacteraceae* in a previous study (60). The fact that these bacterial sequences did not decline during the oxidation phase strongly suggests that a shift in community composition away from metal reducers was not a factor in reoxidation of U(IV).

However, the questions remain, how would these bacterial sequences remain at elevated densities despite low concentrations of U(VI) and other suitable TEAs in highly reducing (methanogenic) conditions, and why did uranium reduction rates decline? One possible strategy for survival in the absence of a suitable abiotic TEA would be a syntrophic association with an organism acting as a biological electron acceptor for excess electrons generated during fermentation. The significant increase observed in dehydrogenase activity between the U reduction and U reoxidation phase may support such a shift to fermentative pathways. A closer look at other bacterial sequences in this cluster also indicates bacteria capable of survival under highly reducing conditions (i.e., reductive dechlorination, TCE-contaminated site clone FTLM5 [accession no. AF529125]). Cluster group 3 contained bacteria whose amplicons increased in abundance between the U reduction and U oxidation phases; significantly, two of the organisms detected (*Acidobacteria*) matched clones previously detected in reductive dechlorinating enrichments (47, 59), while the third organism was a *Desulfovibrio*-related isolate. This covariation of a sulfate reducer and reductive dechlorinating organisms may indicate an interaction such as interspecies hydrogen transfer (13). Drzyzga et al. (12) demonstrated the maintenance of a *Desulfovibrio* sp. in a chemostat in coculture with a dehalorespiring bacterium, which occurred despite the absence of sulfate. The authors proposed that the two organisms were

in syntrophy with *Desulfovibrio*, fermenting lactate and using the dehalogenating bacterium as a "biological electron acceptor." Another possibility may be a syntrophic association between the *Desulfovibrio*-like organism and methanogens (41), as we observed methane production (60) during both the U reduction and oxidation phases. Significantly, *Geobacter* spp. may also benefit from syntrophic relationships with biological electron acceptors. *Geobacter sulfurreducens* was shown to be capable of sustaining growth by electron transfer, following acetate oxidation, to an anaerobic partner bacterium in the absence of Fe(III) or other TEAs (8).

While the observed increase in a *Desulfovibrio* species deserves further investigation, the observation that other metal reducing bacteria, *Geothrix* and *Geobacter* spp., showed no decline indicates that a community shift away from more-efficient U(VI) reducers was not a primary factor in the net oxidation of U(IV), as was perhaps the case in the Colorado field study of Anderson et al. (2). The reoxidation of bioreduced U(IV) has been shown to be thermodynamically possible under the reducing conditions in these column studies (60), with Fe(III) and Mn(IV) being identified as possible electron acceptors. While the bacterial dynamics documented here by the array data do not support direct microbial involvement in this process, neither do the data eliminate this possibility. This is a focus of future studies. The high-density oligonucleotide-based microarray approach used here constitutes a powerful tool which holds significant promise for application in microbial ecology. The ability to detect and monitor a substantial portion of the microbial population in a routine, accurate, and reproducible manner will aid our understanding of the functioning of microbial communities, ranging from natural ecosystems to managed microbial consortia.

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