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Linking Specific Heterotrophic Bacterial Populations to Bioreduction of Uranium and Nitrate in Contaminated Subsurface Sediments by Using Stable Isotope Probing▽†

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Shifts in terminal electron-accepting processes during biostimulation of uranium-contaminated sediments were linked to the composition of stimulated microbial populations using DNA-based stable isotope probing. Nitrate reduction preceded U(VI) and Fe(III) reduction in [^{13}C]ethanol-amended microcosms. The predominant, active denitrifying microbial groups were identified as members of the *Betaproteobacteria*, whereas *Actinobacteria* dominated under metal-reducing conditions.

Oxidized uranium, U(VI), is highly soluble, toxic, and a widespread contaminant in subsurface environments, especially at nuclear legacy waste sites (29). Nitrate is often a cocontaminant with U(VI) as a result of the use of nitric acid in the processing of uranium and uranium-bearing wastes (5, 28). Immobilization of oxidized uranium can be achieved in contaminated groundwater through the reduction of U(VI) to insoluble U(IV) by indirect (abiotic) and direct (enzymatic) processes catalyzed by microorganisms (23, 41). Current remediation practices for dealing with uranium wastes in contaminated groundwaters favor the reductive immobilization of U(VI) via biostimulation of indigenous microorganisms through pH neutralization and/or the addition of an electron donor (4, 12, 16, 44).

At radionuclide-contaminated sites managed by the U.S. Department of Energy (DOE), microbially mediated uranium reduction is limited under *in situ* conditions often due to low pH and electron donor limitation and the presence of competing and more energetically favorable terminal electron acceptors, e.g., nitrate (2, 9, 10, 13, 19, 25, 45–47). Field manipulation experiments have shown that ethanol is an effective electron donor for the *in situ* biostimulation of terminal electron-accepting processes (TEAPs), resulting in the complete reduction of U(VI) after nitrate reduction (1, 2, 10, 19, 25, 29–31, 38, 47).

Although biostimulation is known to promote the removal of U(IV) and nitrate from solution, the identity of bacterial populations catalyzing these processes in contaminated sediments remains uncertain (2). Previous work was based largely on limited DNA sequence information, and the activity of bacterial populations that mediate the fate of U(VI) in response to electron donor addition was not often confirmed with RNA or stable isotopic tracer approaches. A microcosm-based stable isotope-probing (SIP) experiment was employed to assess whether distinct microbial populations are catalyzing the removal of nitrate and immobilization of U(VI) in subsurface radionuclide-contaminated sediments.

Microbial activity during biostimulation of uranium-contaminated sediments. Sediments from borehole FB097 were sampled from the area 2 experimental plot at the DOE's Oak Ridge Field Research Center (ORFRC) on 1 May 2006, 5 to 7 m below the surface, within the saturated zone, as described previously (2). Microcosms were prepared and sampled as previously described (2) except that the sediment was diluted 1:5 (wt/vol) with sterile, anoxic artificial groundwater (composition per liter: 1.0 g NaCl, 0.4 g $MgCl_2 \cdot 6H_2O$, 0.1 g $CaCl_2 \cdot 2H_2O$, 0.5 g KCl, 1 ml selenite-tungstate solution, 1 ml trace element solution, 2.5 mM NO_3, and 5.0 mM $NaHCO_3$; modified from reference 43). Duplicate microcosms were prepared for treatments N1 (^{13}C-labeled ethanol amended; >99 atomic percent ^{13}C; Cambridge Isotopes) and N2 (unlabeled ethanol amended).

The addition of ^{13}C-labeled or unlabeled ethanol stimulated microbial activity. No difference in the rates of carbon or electron acceptor utilization was observed for treatments N1 and N2, indicating that there was no negative effect of adding a ^{13}C-enriched substrate (data not shown). Nitrate was rapidly consumed in the microcosms from days 2 to 6 (Fig. 1A), corresponding to the initial consumption of supplemental ethanol (Fig. 1B). The concentration of uranium was observed to increase on day 6, whereas U(VI) removal from solution was observed from days 8 to 16 and began prior to the onset of Fe(III) reduction, which occurred from days 11 to 16 (Fig. 1A). Fe(II) ceased to accumulate after day 16, although bioavailable Fe(III) remained (data not shown). Supplemental ethanol was completely consumed at the end of the incubation (day 22) and was incompletely oxidized to acetate (Fig. 1B). Acetate accu-

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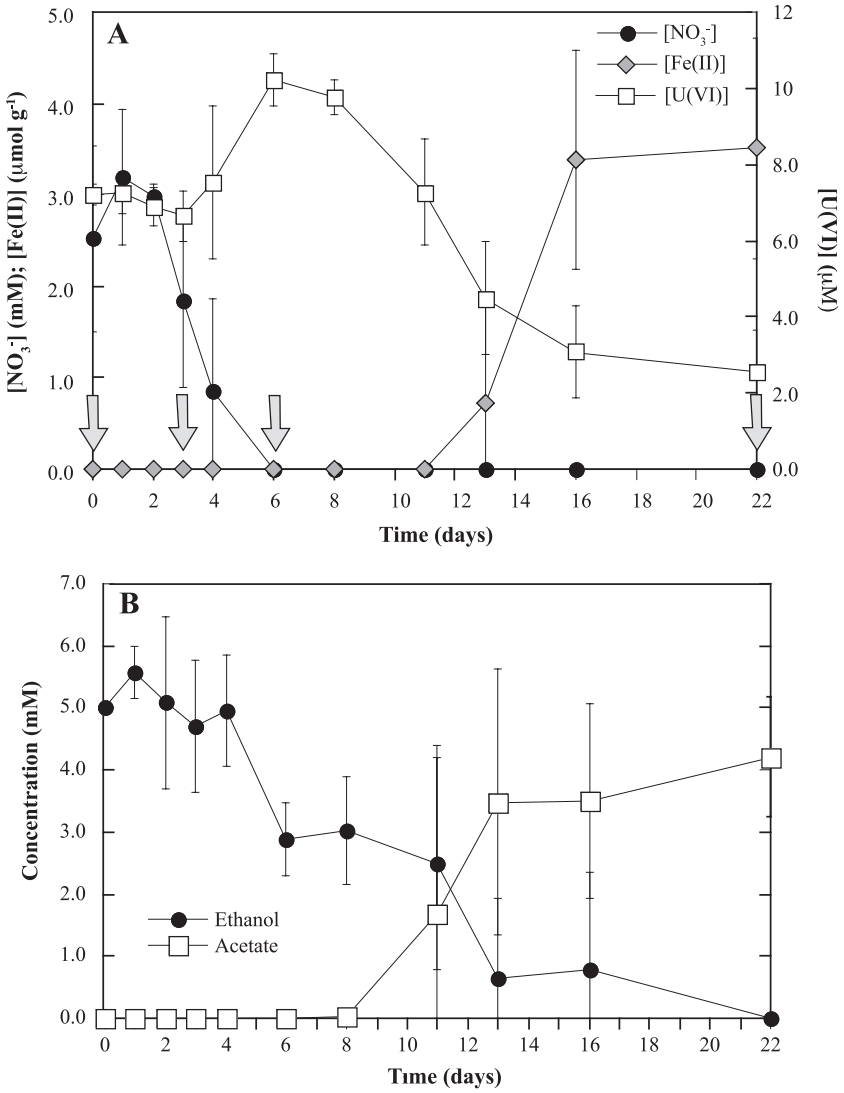

FIG. 1. Electron acceptor (A) and electron donor (B) utilization in ethanol-amended microcosms. Arrows indicate sampling points for TRFLP community fingerprinting. Values are averages ± standard deviations of the results for the four microcosms established for treatments N1 [^{13}C]ethanol) and N2 (unlabeled ethanol).

mulation was observed on day 8, and the accumulated acetate was not utilized as a secondary carbon substrate during the course of the incubation.

Shifts in TEAPs were consistent with thermodynamic predictions and previous studies in which the addition of ethanol stimulated microbial populations to rapidly reduce nitrate, allowing for a shift to metal reduction once nitrate was depleted (5, 8, 10, 21, 25, 28, 29, 31). The concentration of soluble U(VI) was observed to increase during nitrate reduction, likely due to abiotic oxidation of U(IV) by nitrate reduction intermediates (13, 36–38). The onset of U(VI) reduction preceded Fe(III) reduction in the experiment and was hypothesized to occur via direct enzymatic reduction. However, rapid U(VI) reduction can occur via abiotic reduction with Fe(II) in the presence of Fe(III) (oxyhydr)oxides, and U(IV) can rapidly reduce Fe(III) (20, 22, 34). This rapid redox cycling of Fe(III)-Fe(II) could mask the onset of metal reduction and be mistakenly attributed to direct enzymatic processes. At the end of the experiment, dissolved U(VI) concentrations decreased to <3 μM. Although U(VI) was not completely removed, the final concentration was below the Environmental Protection Agency (EPA) guidelines for drinking water, indicating successful bioremediation. The incomplete removal of U(VI) may have been influenced by the carbon substrate specificity of the microbial flora; however, previous work indicated that incomplete U(VI) reduction in area 2 sediments was likely due to geochemical and not microbiological factors (27).

Characterization of metabolically active microbial populations. For microbial community analyses, samples from each replicate microcosm were collected at days 3, 6, and 22. DNA was extracted from 0.25 g of a solid-phase microcosm sample by using a modified phenol-chloroform procedure, as described previously (2). Density gradient centrifugation and separation of ^{13}C- and ^{12}C-DNA bands were performed according to the method of Gallagher et al., with ^{13}C-labeled carrier DNA added to assist visualization of the ^{13}C-DNA band in the density gradient (14).

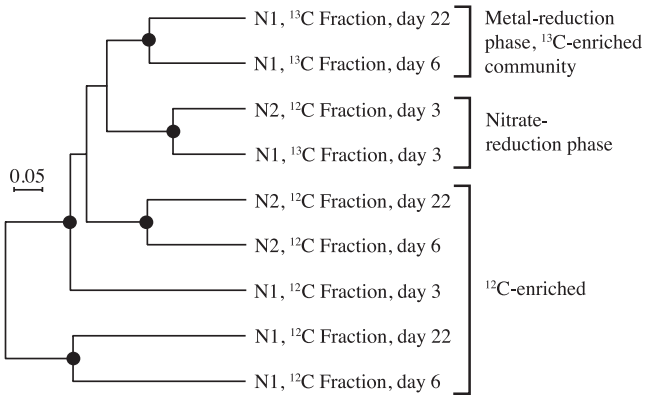

FIG. 2. Dendrogram showing the similarity of MnlI TRFLP profiles for ^{13}C and ^{12}C fractions obtained from microcosm samples of treatments N1 ([^{13}C]ethanol) and N2 (unlabeled ethanol). Clustering analysis based on Bray-Curtis distance, incorporating the relative abundance for each peak, and the unweighted-pair group method using average linkages (UPGMA). One thousand bootstrap analyses were conducted, and circles represent bootstrap values greater than 50%.

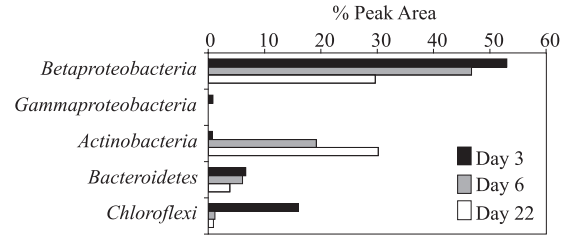

FIG. 3. Relative abundance of phylogenetic groups detected in ^{13}C-DNA fractions of treatment N1 ([^{13}C]ethanol-amended microcosms). Phylogenetic affiliation of TRFLP peaks was determined by matching peak sizes from MnlI profiles against the ORFRC sequence database.

Small-subunit (SSU) rRNA genes from ^{13}C- and ^{12}C-DNA bands were PCR amplified, purified, screened using terminal restriction fragment length polymorphism (TRFLP) analysis, and analyzed as described previously (2).

Microbial communities clearly assimilated supplemental ^{13}C-labeled ethanol, and shifts in phylogenetic structure were observed to coincide with changes in electron-accepting processes. ^{13}C-labeled substrate incorporation into microbial biomass was detected by day 3, corresponding to the onset of nitrate reduction and ethanol depletion. DNA extract concentrations from day 0 of the experiments were below the minimum concentration required for PCR amplification. ^{13}C-DNA was not present in the unlabeled ethanol treatment (N2), and profiles generated from this treatment are representative of the total microbial community present in the microcosms. Peaks detected in the ^{12}C fractions from the ^{13}C treatments represent those groups not utilizing supplemental carbon substrates, such as dead, senescent, autotrophic, or spore-forming bacteria. Similar TRFLP profiles were obtained from the ^{13}C- and ^{12}C-DNA bands from the two treatments, indicating that the majority of the microbial community assimilated ^{13}C from the addition of ^{13}C-ethanol (see Fig. S1 in the supplemental material). Clustering analysis, based on Bray-Curtis distance of the percent peak area of TRF peaks, revealed significant changes in community structure with incubation time, treatment, and incorporation of the ^{13}C label (Fig. 2). TRFLP profiles obtained from treatments N1 and N2 during nitrate reduction were most similar and clustered separately from the active community profiles obtained from days 6 and 22 (Fig. 2). The ^{13}C-enriched community incorporating ^{13}C during metal reduction was significantly different from the inactive (treatment N1, ^{12}C-enriched DNA) and total (N2) microbial communities analyzed on the same day.

Active members of the microbial community were identified by matching peak sizes to predicted terminal restriction fragment (TRF) sizes of sequences in the ORFRC sequence database. The ORFRC sequence database consists of >2,000 SSU rRNA gene sequences, and >850 of the sequences were of the appropriate size to generate TRF sizes using *in silico* digestion. Peaks in ^{13}C-DNA TRFLP profiles matched to members of the *Betaproteobacteria*, *Gammaproteobacteria*, *Actinobacteria*, *Bacteroidetes*, and *Chloroflexi* (Fig. 3). The *Chloroflexi* phylum represented 16.2% of the total peak area at day 3 of the experiment, when active nitrate reduction was observed, and decreased in abundance during the metal reduction phase (Fig. 3). This suggests that these organisms mediated nitrate reduction and were not actively involved in metal reduction in the microcosms. Members of the *Chloroflexi* phylum contain genes in the denitrification pathway (*nirK*, *nosZ*) (15), the group is often detected in sediments under nitrate-reducing conditions (7, 18), and recent evidence from bacterial populations in wastewater treatment plants indicates that the *Chloroflexi* are capable of denitrification (24). Further, the *Chloroflexi* were shown to assimilate [^{13}C]acetate under nitrate-reducing conditions in activated sludge (32).

Once nitrate was removed as a competing electron acceptor, U(VI) and Fe(III) reduction could proceed. During the metal reduction phase of the incubation, we observed an increase in peaks matching the TRF size of the *Actinobacteria* phylum, e.g., a peak at 245 bp matching to *Arthrobacter* (Fig. 3; see also Fig. S1 in the supplemental material). Although *Arthrobacter* species are not known to reduce Fe(III) or U(VI), other members of the *Actinobacteria*, such as *Cellulomonas*, can reduce U(VI) and Fe(III) in pure culture (35). Therefore, our observation of *Actinobacteria* during U removal suggests that these organisms are important at this site during bioremediation. Further work is needed to identify the exact role of these organisms and to determine if *Arthrobacter* species can reductively immobilize U.

Members of the *Betaproteobacteria*, e.g., *Dechloromonas*, *Diaphorobacter*, *Azoarcus*, *Ralstonia*, and *Alcaligenes*, dominated microcosm communities under all treatment conditions, in corroboration with previous studies of nitrate-rich uranium-contaminated subsurface sediments (2, 3, 11, 26, 30, 40). As the genera to which these organisms belong all contain known denitrifiers (17, 39), it is likely that they are actively catalyzing nitrate removal in this experiment. Surprisingly, in addition to being linked to nitrate reduction, *Betaproteobacteria* taxa predominated in the active communities under metal-reducing conditions when nitrate was depleted. Thus, we hypothesize that members of the *Betaproteobacteria* were coupling oxidation of supplemental carbon to Fe(III) and/or U(VI) reduction or were detected in high abundance due to cross-feeding of supplemental ^{13}C. The prolonged dominance of *Betaproteobacteria* members during the entire incubation and the short incubation time suggests that these organisms are active at later time points and not an artifact of the SIP

method. In corroboration, previous studies by our group and others indicate that organisms belonging to the *Betaproteobacteria* catalyze metal reduction in sediments (2, 33, 42) and in uranium-contaminated soils without nitrate as a cocontaminant (6). In addition, our findings confirm the importance of this group to the removal of nitrate and maintenance of reducing conditions during bioremediation in contaminated ORFRC subsurface sediments.

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