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Uranium (VI) Reduction by Denitrifying Biomass

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ABSTRACT Groundwater near the S3 ponds at the US Department of Energy's Y-12 site in Oak Ridge, Tennessee, is contaminated by high levels of nitrate (up to 160 mM) and U(VI) (~0.3 mM). To minimize nitrate inhibition, the authors proposed extraction of contaminated groundwater, nitrate removal in a denitrifying fluidized bed bioreactor (FBR), and return of nitrate-free effluent to the aquifer to stimulate in situ microbial reduction of U(VI). In the presence of carbonate, U(VI) sorption to biomass was negligible, but in its absence, sorption was significant. Biomass reduced U(VI) to U(IV), exhibiting slow first-order removal with respect to U(VI). Addition of electron donor increased rates. Addition of an inhibitor of sulfate reduction (molybdate) slowed the rate and inhibited sulfate reduction. Denitrifying *β-Proteobacteria* dominated clone libraries of SSU rRNA and *dsr*A gene sequences. Approximately 10% were low-G+C microorganisms that had 90% to 92% sequence identity with *Sporomusa, Acetonema*, and *Propionispora*. The *dsr*A sequences were dominated by a single clone with ~80% nucleotide identity to *dsr*A of *Desulfovibrio vulgaris* sub sp. *oxamicus*. The authors conclude that some members of this denitrifyng community reduce uranium, and that sulfate-reducing bacteria likely contribute to this capability.

KEYWORDS denitrification, fluidized bed reactor, microorganisms, uranium reduction

INTRODUCTION

Bioremediation is a promising technology for the immobilization of U(VI) (Abdelouas et al., 1998, 1999; Bender et al., 2000; Lutze et al., 2001). Under anaerobic conditions, a diverse set of microorganisms can reduce uranyl U(VI) to U(IV), precipitating it as the highly insoluble mineral uraninite (UO_2) (Lovley et al., 1991; Ganesh et al., 1997). Microorganisms with this capability include Fe (III)-reducing bacteria (FeRB), such as *Shewanella* spp. and *Geobacter* spp. and gram-positive bacteria (Holmes et al., 2002; Lovley et al., 1991, 1993c; Fredrickson et al., 2000); sulfate-reducing bacteria (SRB), such as *Desulfovibrio* spp. (Lovley and Phillips, 1992a, 1992b; Lovley et al., 1993a, 1993b; Tucker et al., 1996; Ganesh et al., 1999; Payne et al., 2002), *Desulfosporosinus* spp. (Suzuki, et al., 2003), and *Desulfotomaculum* spp. (Tebo and Obraztsova, 1998); *Clostridium* spp. (Francis et al., 1994), *Salmonella*

(Shebolina et al., 2004) and *Cellulomonas* (Sani et al., 2002) also reduce U(VI).

Groundwater near the S-3 waste disposal ponds at the US Department of Energy Y-12 site in Oak Ridge, Tennessee, has U(VI) (up to ~60 mg/L), high levels of nitrate (to ~160 mM), sulfate (2 to 10 mM), and a pH of ~3.4 (Gu et al., 2002, 2003). A potential strategy for remediation is pH adjustment followed by subsurface delivery of electron donors and nutrients to stimulate biological reduction of U(VI). The high nitrate levels pose a serious obstacle to this approach, however, because nitrate inhibits U(VI) reduction (Senko et al., 2002), nitrate and nitrite can oxidize reduced U(IV) (Senko et al., 2002), and the stimulation of in situ denitrification would result in the copious production of biomass and gas, and loss of hydraulic control. Accordingly, nitrate removal is best accomplished above ground where nitrogen gas and biomass can be efficiently removed. Above-ground denitrification can potentially generate water with acceptably low levels of nitrate and high alkalinity, which can then be injected back into the aquifer, increasing pH and inoculating the aquifer. To test the feasibility of this approach, we operated a pilot-scale denitrifying fluidized bed reactor (FBR) inoculated with a denitrifying enrichment obtained from the Y-12 site, and we characterized properties of the biomass for U(VI) reduction. Mature biofilms developed on the granular activated carbon (GAC) carrier used within the FBR, and these biofilms mediated efficient nitrate removal. Organisms in the FBR effluent were capable of U(VI) reduction.

MATERIALS AND METHODS

Medium and Inoculum

All medium and stock solutions were prepared anaerobically under a helium atmosphere. The chemicals used were obtained from Aldrich (Milwaukee, WI), J. T. Baker Chemical (Phillipsburg, NJ) or from Fisher Scientific (Fair Lawn, NJ) except for ethanol, which was obtained from Gallade Chemical (Santa Ana, CA). The basal medium used for the enrichment of denitrifying culture contained (per liter distilled water): $NaNO_3$, 1.0 g; $NaHCO_3$, 0.16 g; KH_2PO_4, 2.77 g; K_2HPO_4, 0.99 g; Na_2SO_4, 0.21 g; trace nutrient solution, 5 ml; mineral solution 100 ml. The trace element solution contained (per liter): HCl, 6.4 ml; $FeCl{\cdot}4H_2O$, 0.3 g; $ZnSO_4{\cdot}7H_2O$, 0.1 g; $MnSO_4{\cdot}H_2O$, 0.085 g; HBO_3, 0.06 g; $CoCl_2{\cdot}6H_2O$, 0.019 g; $CuSO_4$, 0.004 g; $NiSO_4{\cdot}6H_2O$, 0.028 g; $Na_2MoO_4{\cdot}2H_2O$, 0.04 g. The mineral solution contained (per liter): $MnSO4{\cdot}H_2O$, 0.2 g; $MgCl_2{\cdot}6H_2O$, 1.0 g; and $CaCl_2{\cdot}2H_2O$, 2.57 g. The medium was distributed into 158-ml serum bottles (50 ml per bottle) and sealed with a butyl rubber stopper and aluminum cap. The bottles were supplemented with 0.5 ml of either ethanol (1 M) or sodium lactate solution (1 M) as an electron donor. The pH of the medium was 6.52. Groundwater (5 ml) from monitoring well TPB-16 at the Y-12 site was added as inoculum. TPB-16 is located downstream from the source zone, in a region of less contamination. The groundwater in this well contained (mg per liter): U(VI), 1.2; NO_3^-, 15.9; and SO_4^{2-}, 86. The pH was 6.38. Bottles were incubated at ambient temperature (20°C to 22°C). After 11 days, turbidity increased significantly in all cultures, N_2 headspace gas concentrations increased, and nitrate concentrations decreased, confirming denitrification. Enrichments were transferred twice, with ethanol and lactate as added substrates (5% inoculum).

Three-liter well mixed reactors were inoculated with 75 ml of ethanol-grown enrichment plus 75 ml of the lactate-grown enrichment, then fed a mixture of ethanol and lactate (1:1 as mole/mole). A pilot-scale FBR was then inoculated with a 6-L mixture of the three culture reactors. Kinetic characteristics of the inoculum were evaluated at pH 7.0 and 22°C where the electron donor was a mixture of ethanol and lactate at a ratio of 1:1 (g/g) as Chemical Oxygen Demand (COD).

Denitrifying Fluidized Bed Reactor

A pilot-scale FBR inoculated as described in the previous section was used to denitrify synthetic groundwater at ambient temperatures. Reactor dimensions were as follows: height, 2.05 m; diameter of fluidization region, 5 cm; diameter of settling region, 7.62 cm; fluidization volume, 6.7 L; and total system volume, 15.3 L. GAC (2.7 L, Calgon type MRX, and 10 × 30 mesh) served as media for attachment of biomass. The fluidization flow rate was 1.48 m^3/m^2-min. Synthetic groundwater was prepared with tap water at Stanford University (Stanford, CA) supplemented with (per liter): HNO_3, 35.5 mmoles; NaOH, 35.5 mmoles; $MgCl_2$, 0.1 mmoles, Na_2SO_4, 0.12 mmoles; $Na_3P_3O_4$, 0.06 mmoles; and trace element solution (as described previously), 1 ml. Electron donor solution contained ethanol (64.9 mM) and lactic acid (62 mM), giving a COD concentration of 12 g/L. The synthetic groundwater and

electron donor solution was fed separately to the FBR with a COD to NO_3^--N ratio ranging from 4.2:1 to 4.8:1. The ratio was adjusted by slightly changing the feed rate to obtain low effluent concentrations of soluble COD (<70 mg/l) or NO_3^--N (<1 mg/l) at the desired loading rate or hydraulic retention time (HRT). After inoculation, the reactor was continuously operated at ambient temperature conditions (20° C to 25° C) for more than 20 months.

Batch U(VI) Reduction Assays

U(VI) reduction assays were performed in 75-ml serum bottles with helium gas headspace at ambient temperature (20° C to 21° C). The effluent from the FBR (pH 7.5) contained ~35 mM bicarbonate. Effluent was collected and distributed into serum bottles as 35-ml aliquots. Biomass (~100 ml) was collected after gentle shearing of the GAC using a brush and transferred to a 158-ml serum bottle. All the bottles were crimp sealed with a butyl rubber stopper and an aluminum cap, and the headspace flushed with He containing 5% CO_2. The biomass was dispersed into a homogenous suspension by passing it through a 5-ml syringe equipped with a 24-gauge needle prior to inoculation. Twenty milliliters of sample were withdrawn from the bottle to determine biomass concentration as volatile suspended solids (VSS). Different volumes of suspension were injected into the serum bottles to obtain the desired biomass levels. The biomass concentration of FBR effluent was measured as COD and assumed equal to the difference of the concentrations between soluble and total COD. To convert into volatile suspended solids, a COD/biomass ratio of 1.42 g COD/g VSS was assumed (Rittman and McCarty, 2001). After inoculation, electron donor solutions (ethanol and/or lactate) were added. Abiotic controls consisted of autoclaved FBR effluent or autoclaved effluent plus added biomass (30 min, 121° C). Uranyl nitrate was added from a stock solution (20 mM) to achieve the desired initial U(VI) concentration of around 60 mg/L. A mixture of sodium lactate and ethanol (1:1 as COD) served as electron donor, with an initial COD concentration of approximately 500 mg COD/L. For some selected experiments, Na_2MoO_4 (1 M) and Na_2SO_4 solutions were added. Test bottles were incubated under ambient temperature (22°C to 24°C). Samples were periodically withdrawn for U(VI) analysis.

U(VI) Adsorption

To determine the potential for sorption of U(VI) on the FBR biomass, we obtained adsorption isotherms of U(VI) in the presence of either bicarbonate or nitrate (10 mM). FBR biomass suspension was mixed with U(VI) as $UO_2(NO_3)_2$ at concentrations ranging from 0 to 60 mg/L. The final volume was adjusted to 15 ml, with a final biomass concentration of 3 g/L (dry weight). Samples were shaken overnight (~18 h) and filtered using a syringe filter (0.2 μm) to remove biomass and sorbed U(VI). The mass of sorbed U(VI) was estimated as the difference between the mass of U(VI) in the solution in the absence of biomass and the mass in the filtered supernatant solution.

DNA Extraction and Purification

Biomass was harvested by centrifugation (10,000 × *g* force, 4° C for 30 min), and the pellets were stored at −80° C prior to DNA extraction. To recover DNA, pellets were resuspended in a lysis buffer and the cells disrupted by grinding with sterile sand in liquid-nitrogen (Zhou et al., 1996). DNA was extracted as described previously (Zhou et al., 1996, 1997), and the precipitated DNA purified by gel electrophoresis and a mini-column preparation (Wizard DNA Clean-Up system; Promega, Madison, WI).

Polymerase Chain Reaction (PCR) Amplification and Cloning

The partial SSU rRNA gene, intergenic region, and partial 23S rRNA gene sequence were amplified in a 9700 Thermal Cycler (Perkin-Elmer) with the primer pair 925 F (5′ AAA ACT YAA AKG AAT TGA CGG 3′) and 23SR (5′ GGG TTB CCC CAT TCR 3′), and the primer 23SR was previously described (Fisher and Triplett, 1999). The PCR parameters were as follows: 80°C for 30 s, 94°C for 2 min; 94°C for 30 s, 55°C for 1 min, 72°C for 1 min, 28 cycles; 72°C for 7 min. The dissimilatory (bi-) sulfite reductase (*dsr*) primers used in this study were those of Karkhoff-Schweizer et al. (1995). The PCR reactions (20 μl) contained 2 μl 10× buffer (500 mM KCl, 100 mM Tris HCl pH 9.0 and 1% Triton X-100), 1.5 μl 25 mM/L $MgCl_2$, 0.2 μl 400 ng/μl bovine serum albumin (Boehringer Mannheim, Indianapolis, IN), 0.2 μl 25 mM 4× dNTPs (USB Corporation, Cleveland, OH), 10 pmol each primer, 2.5 U Taq polymerase, and 1 μl purified DNA

(5 to 10 ng). The thermal cycling protocol used included initial denaturation at 94°C for 2 min, followed by 25 cycles of 94°C for 30 s, 58°C for 1 min, and 72°C for 1 min. To minimize PCR-induced artifacts, the optimal number of cycles was determined and five PCR reactions were combined prior to cloning as described previously (Qiu et al., 2001; Yan et al., 2003). PCR products were analyzed on 1.5% (*w/v*) TAE solution (40 mM TRIS acetate; 2 mM EDTA) agarose gels. The PCR products were separated by electrophoresis in a low-melting-point agarose gel (0.8%), the appropriate band excised, and the DNA extracted with a Wizard Prep Kit (Promega, Madison, WI) according to manufacture's instructions. Recovered DNA was resuspended in 6 μl double-distilled H_2O (ddH_2O), 2 μl was ligated with pCR2.1 vector from a TA-cloning kit, and competent *Escherichia coli* cells were transformed according to the provided protocol (Invitrogen, San Diego, CA).

Sequence and Phylogenetic Analysis

White colonies were picked after blue-white screening, and PCR products (100 μl) amplified with vector-specific primers were purified with the Millipore membrane filters according to manufacturer instructions with the primer 925F. DNA sequences were determined with a BigDye Terminator kit (Applied Biosystem, Foster City, CA) using a 3700 DNA analyzer (Perkin-Elmer, Wellesley, MA) according to the manufacture instructions. DNA sequences were assembled and edited using the Sequencher program (v. 4.0; Gene Codes Corporation, Ann Arbor, MI). The edited sequences (approximately 400 nucleotide [nt]) were checked with ChimeraCheck (Ribosomal Database), were aligned with ClustalW (Thompson et al., 1994), and alignments compared with reference sequences from the database. Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 2.1 (Kumar et al., 2001), and phylogenetic trees were constructed with distance matrices and the neighbor-joining method within MEGA as previously described (Yan et al., 2003). Trees constructed with maximum-parsimony and neighbor-joining methods were not significantly different.

Analytical Methods

U(VI) was measured using the steady-state phosphorescence technique. This method is specific for U(VI) (Brina and Miller, 1992; Gu and Chen, 2003). It involved the addition of 0.1 ml of sample solution into 4 ml of deoxygenated phosphoric acid (10%) in a quartz vial. Phosphoric acid was used to complex U(VI) and enhance its phosphorescence. Samples were filtered through a 0.25-μm filter to determine soluble U(VI), and unfiltered samples were used for total U(VI). Fluorescence intensity is directly proportional to the concentration of U(VI), and the detection limit is less than 0.1 mg U(VI)/L. All measurements were performed with a Fluorolog-3 fluorescence spectrometer equipped with both excitation and emission monochromators (Johin-Yvon-SPEX Instruments, NJ). A 450-W Xenon arc lamp was used as the excitation source, and the emission spectra were collected from 482 to 555 nm with an excitation wavelength of 280 nm. The peak emission at 515.4 nm was used for the calculation of U(VI) phosphorescence intensity or U(VI) concentration in solution. The concentrations of sulfate and nitrate were determined using an ion chromatograph equipped with an IonPac AS-14 analytical column and an AG-14 guard column (Dionex, Sunnyvale, CA). Nitrogen gas concentration was measured using a Series 580 TCD GC (GOW-MAC Instrument Co., Bridgewater, NJ). COD was measured by the colorimeteric method using Hach COD tubes (Loveland, CO).

RESULTS AND DISCUSSION

Denitrifying Cultures and Performance of the Denitrifying FBR

For 18 h after inoculation, the FBR was operated in a continuous recirculation mode without feed. Thereafter it received a continuous feed of synthetic groundwater together with an electron donor stock solution containing 1:1 ethanol:lactate (as COD). Mature biofilms developed within 45 days. The initial nitrate loading was 0.87 g NO_3-N/L-day, and the initial COD loading rate was 4.3 g COD/L-day. Over a 4-month period, these loading rates were gradually increased to 3.1 g NO_3-N/L, and 12.8 g COD/L-day. Nitrate removal consistently exceeded 99% at a hydraulic residence time of ~11 h. The reactor was then operated to steady state at different constant loading rates. After 7 months' operation, biomass was removed for U(VI) reduction assays and microbial community analysis. Table 1 summarizes the operational performance of the FBR at a steady-state condition (30-day period) during the period of U(VI) reduction assays. In addition to nitrate and COD removal, the influent concentration of sulfate (0.12 mM) decreased by more than 90%.

TABLE 1 Operational Parameters and Performance of the FBR at Steady State After 7 Months of Operation

Temperature (°C)	22–23
Effluent pH	7.35–7.50
Hydraulic retention time (h)	10.83 ± 1.48
COD loading rate (g/L-day)	3.90 ± 0.72
Effluent COD (mg/L)	64 ± 20
NO_3-N loading rate (g/L-day)	0.93 ± 0.14
Effluent NO_3-N (mg/L)	0.50 ± 0.29
COD/NO_3-N consumption ratio (g/g)	4.15 ± 0.58

Note. The data are averages over a 30-day period. Hydraulic retention time and loading rates were calculated based on the volume of the reaction region in the FBR (6.4 L).

U(VI) Reduction Kinetics

U(VI) reduction kinetics were determined in two different assays using initial biomass concentrations ranging from 0.27 to 1.07 g VSS/L. The time courses of U(VI) removal for an assay performed at 0.27 to 1.07 g/L are shown in Figure 1. The U(VI) concentrations shown are for unfiltered samples and therefore represent total U(VI) concentration, including both soluble U(VI) and U(VI) sorbed on/to biomass. The reduced U(IV) does not give fluorescence or interfere with the analysis of U(VI), as indicated earlier. The same trends were observed at both cell concentrations. No U(VI) reduction was observed in sterile filtered FBR effluent or in an autoclaved cell suspension (data not shown), indicating that the reduction of U(VI) was due to biological activity.

The kinetics of U(VI) reduction were a function of biomass concentration and the addition of ethanol and lactate. The more biomass added, the faster the U(VI) removal rate. Addition of electron donor also enhanced U(VI) removal, especially in the bottles with lower biomass concentrations.

To ensure that U(VI) was not simply sorbed or precipitated, adsorption experiments were performed in background electrolyte solutions of either bicarbonate or nitrate. U(VI) sorption on FBR biomass was negligible in the presence of carbonate or bicarbonate (Figure 2), but significant in its absence (i.e. in the presence of nitrate). These observations can be explained by the formation of soluble uranyl carbonate species such as $UO_2(CO_3)_2^{2-}$ or $UO_2(CO_3)_3^{4-}$ in media containing significant carbonate concentrations. Negatively-charged U(VI) species are presumably repelled by ionized carboxyl and hydroxyl functional groups on the cell surfaces. In the absence of carbonates, the uranyl cation (UO_2^{2+}) complexes with carboxyl, hydroxyl, and

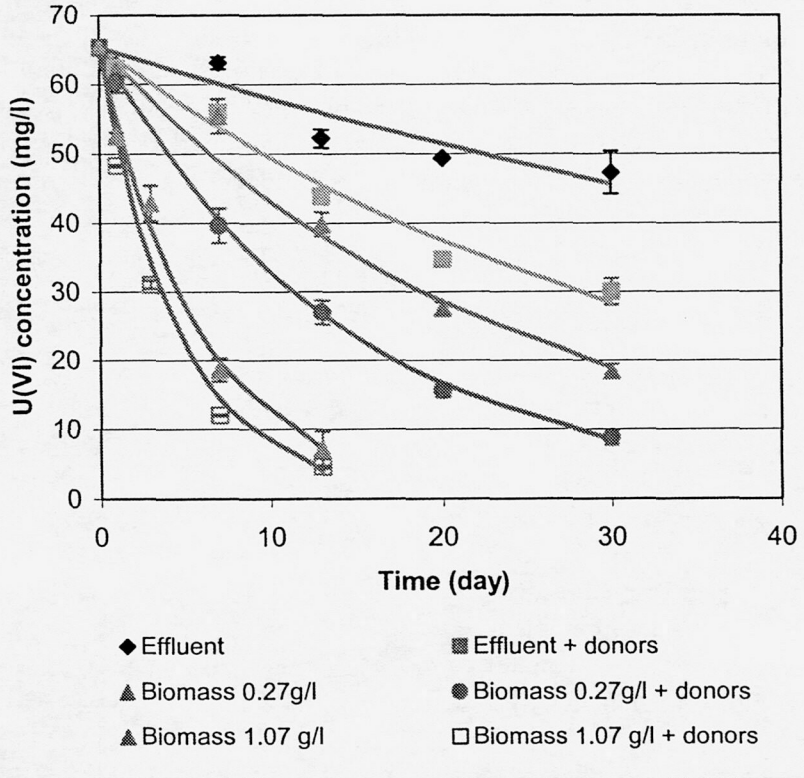

FIGURE 1 Time course of U(VI) reduction by FBR biomass in the presence and absence of added electron donors (ethanol and lactate). The biomass of the FBR effluent was estimated to be 0.07 g VSS/L. Sterile filtered FBR effluent was the abiotic control. Error bars give the ranges for duplicates.

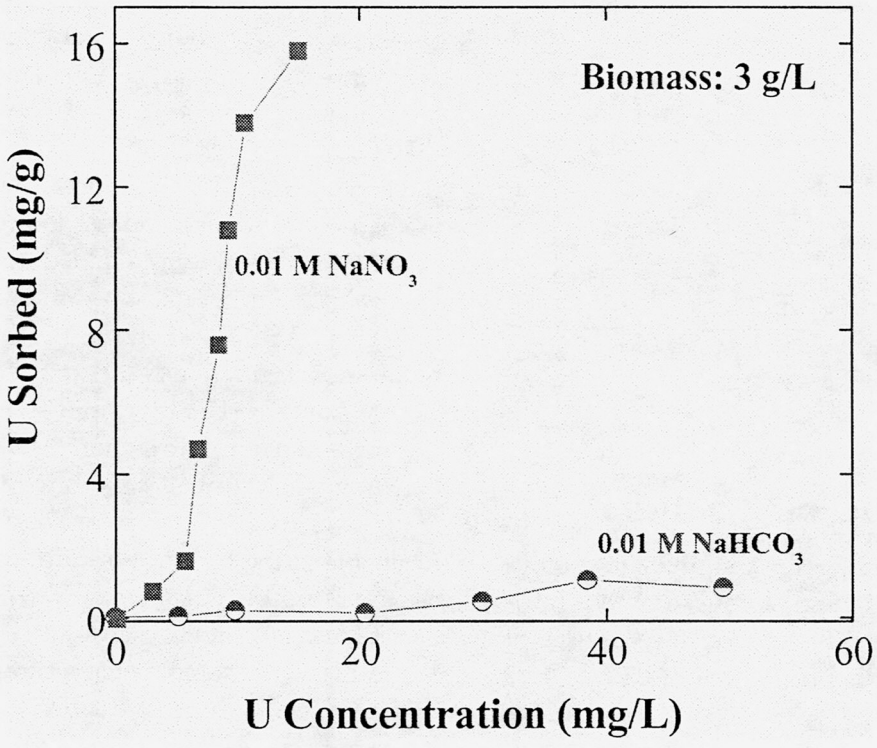

FIGURE 2 U(VI) sorption or precipitation on FBR biomass in 0.01 M $NaHCO_3$ or $NaNO_3$ background electrolyte solution.

neutrally-charged phosphoryl functional groups on the cell wall (Choppin, 1992; Lenhart et al., 2000; Fowle et al., 2000; Kelly et al., 2002; Francis et al., 2004). This likely explains why U(VI) was strongly retained by FBR biomass in a low nitrate solution. Because U(VI) reduction experiments were conducted in the presence of elevated carbonate, the observed removal of U(VI) cannot be attributed to its sorption to biomass.

Kinetics data for microbial reduction of U(VI) were quantified. Others have used saturation kinetics to quantify U(VI) reduction by pure cultures of SRB and FeRB (Truex et al., 1997; Spear et al. 1999, 2000; Lui et al., 2002). Reported half saturation coefficients (K) were high, ranging from 0.13 to 0.88 mM (or 31 to 209 mg/L). These values indicate that U(VI) reduction kinetics can be generally described as first order with respect to the concentration of U(VI). This view is supported by Liu et al. (2002) who reported that the saturation kinetic expression are over parameterized for metal reduction, and that more precise coefficients can be obtained using a simpler first order approximation. Accordingly, we adopted a pseudo-second-order rate expression to describe U(VI) reduction kinetics by the FBR biomass. The differential equation is $-\mathrm{d}S/\mathrm{d}t = k_1XS$, and, when biomass concentration is constant over time, its integrated form is $S = S_0\exp(-k_1Xt)$, where S = U(VI) concentration (mg/L) at time t (day), S_0 = initial U(VI) concentration, k_1 = pseudo-second-order rate coefficient (L/g VSS-day), and X = biomass concentration (g VSS/L). Because biomass concentration did not change during these assays, the slope of $\ln(S/S_0)$ versus Xt represents a first order coefficient k ($= k_1X$). The pseudo-second-order rate coefficient k_1 was obtained by dividing k by X.

Table 2 summarizes k_1 values in the presence and absence of added electron donors (lactate/ethanol) and

TABLE 2 The U(VI) Reduction Rate Coefficient (k_1) of the Pseudo-Second-Order Reaction Versus Different Biomass Concentrations in the Absence and Presence of Added Electron Donors

	Biomass concentration (g/L)	k_1 (L/g VSS-day)	Regression coefficient, R^2
No electron donors	0.07	0.175	0.892
	0.27	0.154	0.975
	0.32	0.142	0.983
	1.07	0.159	0.989
With added	0.07	0.390	0.985
electron donor	0.05	0.304	0.985
(ethanol/lactate	0.27	0.254	0.998
mixture)	0.32	0.201	0.972
	1.07	0.201	0.985

Note. All assays were performed twice. Duplicates were used for each test condition. VSS was calculated from insoluble COD data, assuming 1.42 g COD/g VSS.

at different biomass concentrations. Higher k_1 values were observed in the presence of lactate/ethanol (Table 2). This supports the view that soluble substrate furnishes electrons for U(VI) reduction at a faster rate than biomass decay.

In the absence of added electron donor, k_1 values were independent of biomass concentration. Under these conditions, the source of electrons for U(VI) reduction was the biomass itself (presumably from stored polymers, lysis of dead cells, etc.). In the presence of added electron donor, k_1 values declined with increasing biomass concentration. The reasons for this pattern are not clear, and require further investigation. It is possible that the ratio of soluble electron donor mass to biomass influences rates of reduction.

Spear et al. (2000) observed first order U(VI) reduction kinetics for a mixed culture of *D. vugaris* and *Clostridium* sp. and pure culture of *D. desulfuricans*. Their values for k_1 are 100 times higher than those observed in this study. This is probably because the majority of organisms in the FBR biomass were denitrifiers rather than U(VI) reducers, as discussed in the section on microbial community analysis.

Respike Experiments

The available literature suggests that the U(VI) reduction may support growth of U(VI)-reducing organisms. Lovley et al. (1993b) reported that *Dsulfovibrio vulgaris* reduces U(VI) but does not use it as a terminal electron acceptor. On the other hand, the spore-forming SRB *Desulfotomaculum reducens*, which is different from most *Desulfotomaculum* species (Tebo and Obraztsova, 1998), and FeRB, *Geobacter metallireducens*, and *Shewanella putrefaciens* can grow anaerobically with U(VI) as the terminal electron acceptor (Lovley et al., 1991). Such organisms can utilize lactate and ethanol as electron donors for growth, so it seemed possible that the FBR biomass might include representatives of these organism types. To determine whether U(VI) reduction supported growth of U(VI)-reducing microorganisms in the FBR biomass, we performed respike experiments. If growth accompanies U(VI) reduction, respiking with U(VI) should result in an increase in k_1 values. Accordingly, U(VI) respikes were added, and the rates of U(VI) removal monitored (Figure 3). The results do not support the hypothesis of U(VI)-supported growth: in the absence of electron donor, k_1 for the first spike was 0.159 L/g VSS-day, and k_1 for the second spike was 0.136 L/g VSS-day (R^2 = 0.989 versus 0.988, respectively); in the presence of added electron donors, k_1 for the first spike was 0.201, and k_1 for the second spike was 0.145 L/g VSS-day (R^2 = 0.976 versus 0.985, respectively).

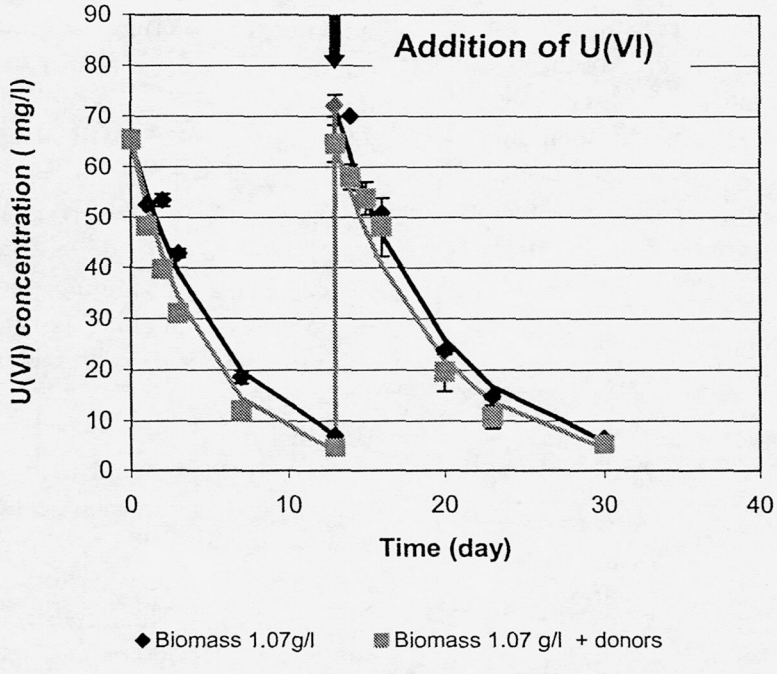

FIGURE 3 Time course of U(VI) reduction by sequential spiking U(VI). The initial biomass concentration was 1.07 g VSS/L. Uranyl nitrate was added to test bottles on day 13.

Effect of Sulfate and Molybdate on U(VI) Reduction

Some SRB reduce U(VI), but not all. Lovley et al. (1993a) reported that *Desulfovibrio* spp. was capable of reducing U(VI) but *Desulfobacter curvatus, Desulfobacterium autotrophicum, Desulfolobus propionicus, Desulfomonile tiedjei*, and *Desulfotomaculum acctoxidans* did not. *Desulfovibrio* grows on hydrogen, ethanol, and lactate, and is the most abundant SRB in soils, aquifers, and wastewater treatment plants. Species of SRB which reduce U(VI) include spore-forming *Desulfotomaculum reducens* (Tebo and Obraztsova, 1998) and *Desulfosporosinus* spp. (Suzuki et al., 2002). These SRB grow on lactate and/or ethanol, so it seemed possible that the observed reduction of U(VI) by FBR biomass might be due to the presence of such populations as minority members within the FBR biomass. To assess this possibility, we tested the effects of sulfate and molybdate addition.

The effect of sulfate was tested by addition of 0, 2, 5, and 10 mM of Na_2SO_4 plus the mixture of ethanol and lactate. Sulfate was partially removed (Table 3), but sulfate addition had little or no effect on U(VI) removal (Figure 4). No U(VI) reduction was observed in abiotic controls (autoclaved cell suspension).

Molybdate is an effective and relatively selective inhibitor of SRB (Taylor and Oremland, 1979). The effect of molybdate (10 mM) in the presence of various sulfate concentrations (2, 5, and 10 mM) is presented in Figure 5A–C. The addded molybdate could cause complete inhibition of SRB grown on both ethanol and lactate with sulfate as electron acceptor (Yadav and Archer, 1988; Wu et al., 1991). The results indicated that the addition of the molybdate slightly decreased the U(VI) reduction rate or caused a lag in its reduction. Inhibition was more pronounced in bottles containing higher levels of added sulfate. A comparison of sulfate concentrations before and after 48 days of incubation confirmed that sulfate reduction was severely inhibited in the presence of molybdate (Table 3). The results suggest that SRB contribute to the observed U(VI) reduction by FBR biomass, and that the presence of sulfate may stimulate the growth of such organisms.

TABLE 3 Sulfate Removal in the Absence and Presence of Molybdate After 48 Days of Incubation

Sulfate (mM)	Molybdate (mM)	Initial SO_4^{2-} (mg/L)	Final SO_4^{2-} (mg/L)	SO_4^{2-} removal (%)
2	0	198	113	42
	10	206	189	8
5	0	487	393	19
	10	538	502	6.7
10	0	1038	902	13
	10	1090	1049	3.8

Note. Results were the average values of duplicates.

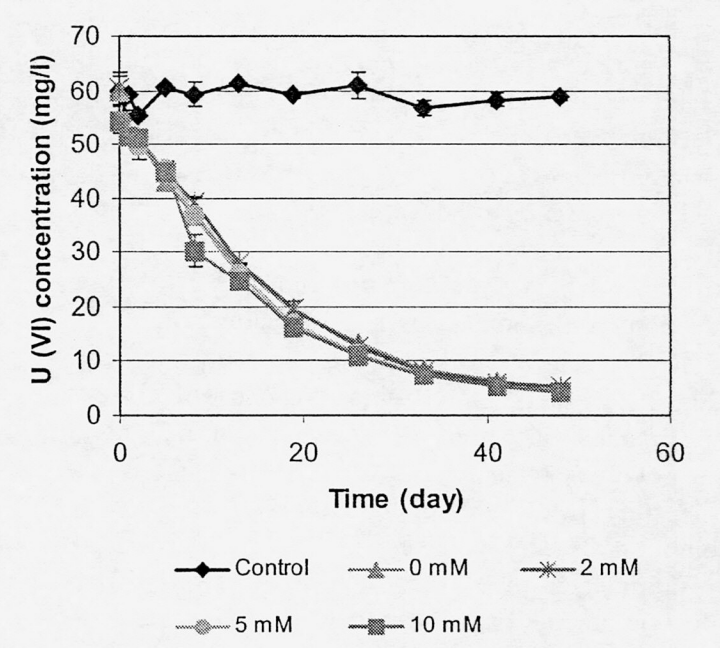

FIGURE 4 Time course of U(VI) reduction by FBR biomass in the presence of various initial sulfate concentrations (0, 2, 5, and 10 mM). An autoclaved cell suspension was the abiotic control.

In previous studies, molybdate had an insignificant effect on U(VI) reduction. Column test using immobilized *D. desulfuricans* in polyarcylamide showed no inhibition of molybdate concentration as high as 2000 mg/L (or 20 mM) with an influent U(VI) concentration of 5 mg/L (Tucker et al., 1998). This SRB species did not use U(VI) as a terminal electron acceptor (Lovley et al., 1993b). In an experiment conducted over a 250 minute period, molybdate up to 10 mM did not inhibit Fe(III) reduction by *D. desulfuricans* (Lovley et al., 1993a). However, molybdate does inhibit the growth of SRB on sulfate. This study suggests that molybdate adversely affects U(VI)-reduction capabilities over the long term when SRB are present as minority community members. Some decrease in U(VI) removal in the presence of added molybdate might also be attributed to the formation of uranyl molybdate complexes. These species have limited solubility and hence could potentially reduce the bioavailability of U(VI) for microbial reduction (Prasad and Barros, 1998; Kiran and Apblett, 2004).

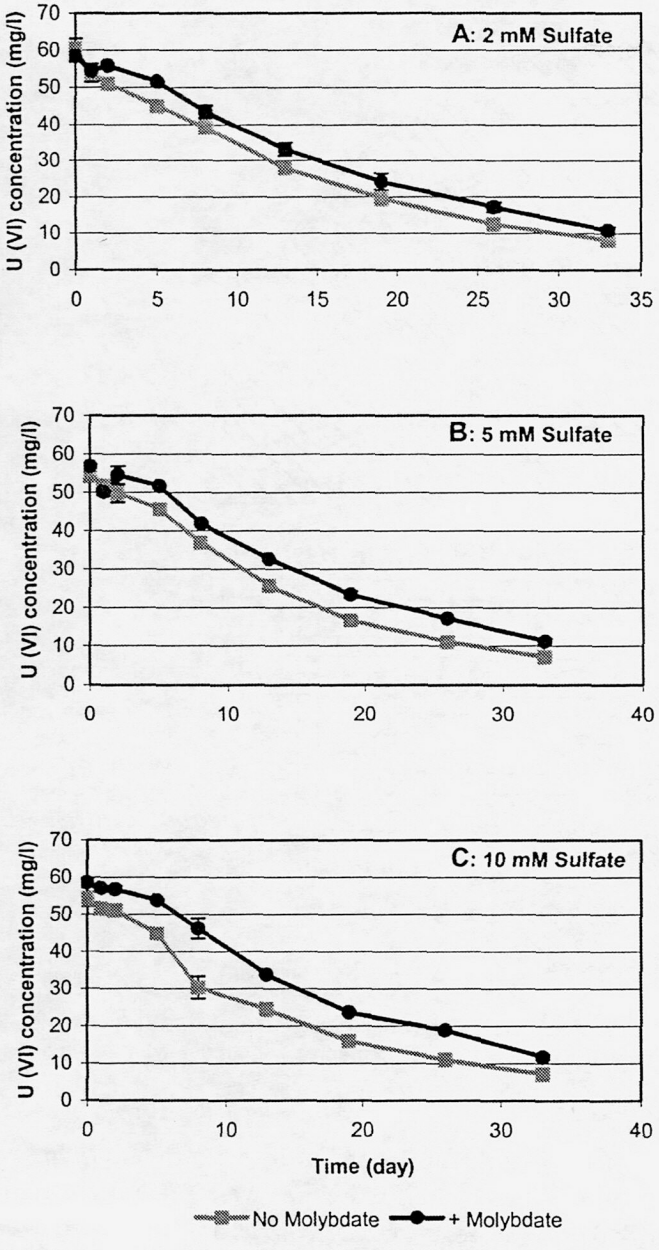

FIGURE 5 Effect of addition of Na_2MoO_4 (10 mM) on U(VI) reduction in the presence of various initial sulfate concentrations (2, 5, and 10 mM).

Microbial Community Characterization

To characterize the microbial community of the FBR biomass, partial SSU rRNA gene sequences from the liquid ($n = 260$) and biofilm ($n = 260$) fractions of the FBR were PCR-amplified, cloned, and partial sequences determined. The biofilm community was dominated by *β-Proteobacteria* (I, II, III), and the partial sequences suggested that the clones were *Zoogloea*, *Dechlorosoma*, and *Dechloromonas*, respectively. These microorganisms constituted greater than 75% of the clonal library, and the most closely related sequence matches have been previously shown to be denitrifiers (Figure 6). The biofilm did not appear to contain two populations that were observed in the liquid fraction (*Pseudomonas* and *Stenotrophomonas*), and these two populations constituted approximately 30% of the liquid fraction community (data not shown). Both the liquid fraction of the FBR and the biofilm community contained low-G+C microorganisms, and approximately 10% of the respective libraries could be classified in the *Acidaminococcaceae* subgroup of the *Clostridiales* (Figures 6 and 7). Group I sequences (liquid fraction) were more closely related to *Propionispora vibrioides* (98% sequence identity), but two sequences amplified from the biofilm (group II) were more closely related to *Sporomusa aerovorans* (96% sequence identity) (Figure 7). *P. vibrioides* is a gram-negative, spore-forming bacterium isolated from compost that can ferment sugar alcohols with the production of propionate, acetate, and hydrogen (Biebl et al., 2000). *S. aerovorans* is an oxygen-reducing, homoacetogenic bacterium isolated from the termite gut. However, sulfate and/or iron reduction have not been reported for these microorganisms.

The group III sequences were more closely related to uncultivated clones, and had approximately 91% with *S. aerovorans, Propionispora hippei*, or *Acetonema longum*. *P. hippei* and *A. longum* are also gram-negative, spore-forming acetogens, and *A. longum* was isolated from the gut of a termite (Kane and Breznak, 1991). Group IV contained sequences from both the liquid and biofilm fractions, and the biofilm clones had 93% sequence identity with *P. hippei*. One sequence from the biofilm (GAC2-H08) did not group with the other FBR clones, and had 92% sequence identity with *P. hippei* or *Anaeroarcus burkinensis*. *A. burkinensis* is a non-spore-forming anaerobe that produces acetate, propionate, and succinate, and can reduce ferric iron but not sulfate or nitrate (Strompl et al., 1999).

Seven genera were identified from the biofilm fraction, and the predominant populations were denitrifying *β-* and *γ-Proteobacteria* that are not known to be dissimilatory iron- or sulfate-reducing microorganisms. *Sporomusa* species have been isolated from flooded rice field soil with lactate and ethanol, and multiple, phylogenetically distinct strains were observed (Rosencratz et al., 1999). Recently, enrichments from uranium-contaminated sediments also contained low-G+C microorganisms, including *Desulfosporosinus* and *Clostridium* (Suzuki et al., 2003). In a previous study, low-G+C

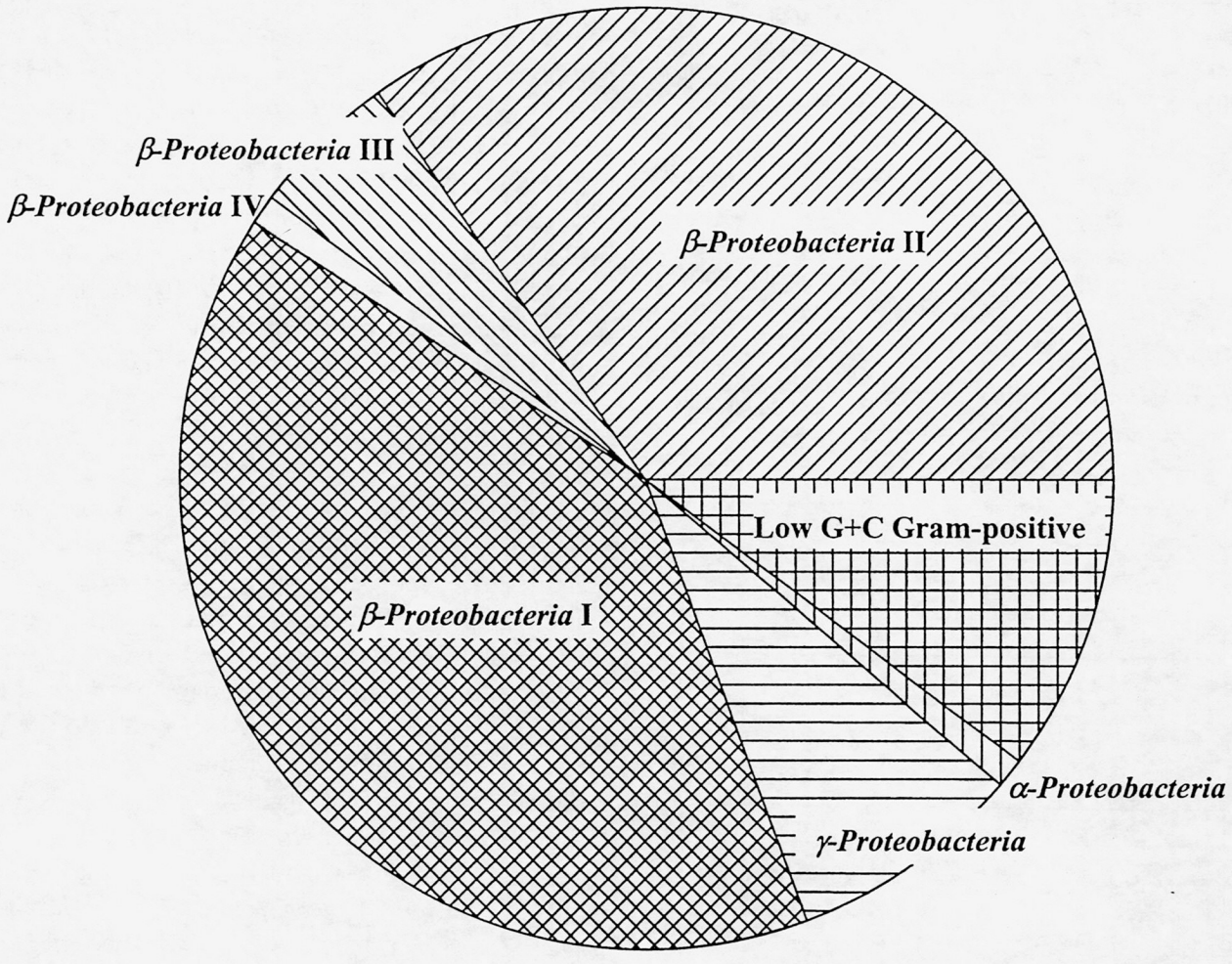

FIGURE 6 Major bacterial groups identified in the FBR biofilm based upon predominance in clonal library of partial SSU rRNA gene sequences (approximately 400 nt of 3′ end). Unique operational taxonomic units (OTUs) were designated as sequences with less than 97% sequence similarity.

microorganisms were isolated from sulfate-reducing enrichments that were inoculated from freshwater lake sediments, and some of the bacteria had approximately 95% sequence identity (SSU rDNA) with *Sporomusa termitida* (Sass et al., 1998).

Conserved PCR primers for the dissimilatory sulfite reductase gene (*dsr*A) were used to amplify possible products from the FBR biomass, and partial sequences of clones (n = 60) were determined. Both the liquid and biofilm fractions were predominated by two different Operational taxonomic units (OTUs) that had <97% nucleotide sequence identity and these results indicated that the *dsr*A functional diversity was extremely low (data not shown). The FBR *dsr* sequences had between 78% and 79% nucleotide identity with the *dsr*A of *Desulfovibrio vulgaris* subspecies *oxamicus* and the *dsr* of an uncultivated sulfate-reducing bacterium from a uranium mill tailings site (Chang et al., 2001). These results indicated that microorganisms with the biochemical capacity for sulfate-reduction were present in the FBR, but the *dsr* sequences were novel and unique compared to previously observed sequences. When the FBR biomass was used as an inoculum for sulfate-reducing enrichments, the resulting cultures could reduce uranium (data not shown).

Although acetogens are generally considered obligate anaerobes, they can colonize habitats that are not strictly anoxic. Recently, Boga and Brune (2003) hypothesized that homoacetogens (e.g., like those observed from termite guts) could reestablish anoxic conditions because of tolerance to temporarily low pO_2 and the capacity to reduce O_2. *Desulfosporosinus* spp. are homoacetogens and can reduce U(VI) in the absence of chloride and bicarbonate (Suzuki et al. 2003). Some of the clones from the FBR biofilm were most closely related to sequences from acetogenic bacteria

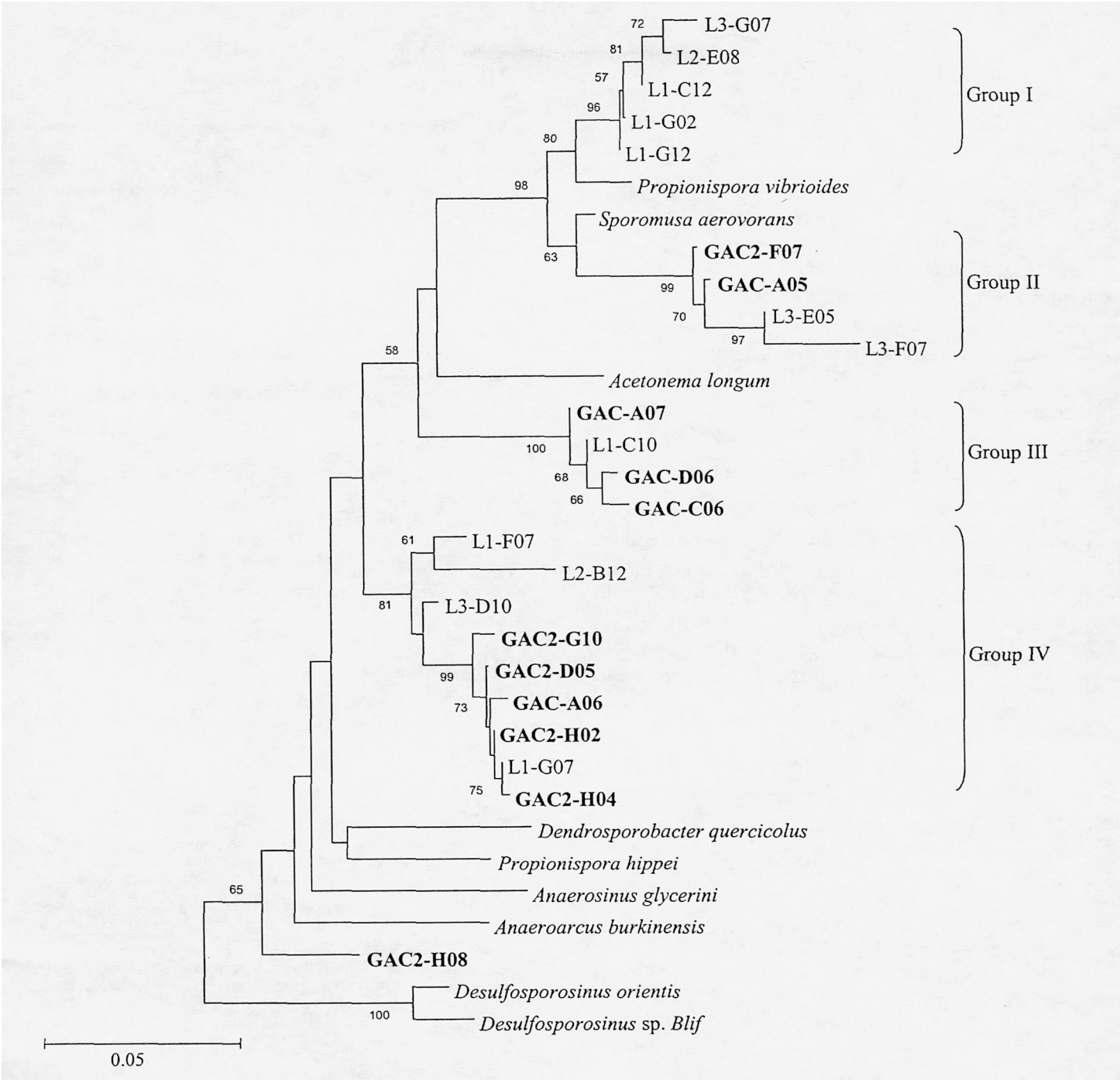

FIGURE 7 Phylogenetic analysis of partial SSU rRNA sequences with neighbor-joining method. Bootstrap values below 50 are not shown. Numbered sequences are from liquid (L) or biofilm (GAC) fractions of the FBR. GenBank accession numbers for reference sequences are as follows: P.v. (AJ279799), S.a. (AJ506191), A.l. (AJ010964), D.q. (M59110), P.h. (AJ508928), A.g. (AJ010960), A.b. (ABAJ0961), D.o. (Y11570), and D. Blif (AF159121).

isolated from the termite gut. These results suggested the possibility that acetogenic and sulfate-reducing microorganisms in the FBR biofilm could contribute to the reduction of uranium and alleviate O_2 related toxicity. The presence of microorganisms that can tolerate low pO_2, reduce O_2, and reduce uranium would be advantageous for the establishment and maintenance of environments conducive for subsurface uranium reduction. Further work is needed to understand the exact role of the uranium-reducing microorganisms in the FBR, and the effects of these organisms on U(VI) reduction upon introduction into the subsurface environment.

CONCLUSIONS

A pilot-scale FBR inoculated with a denitrifying enrichment derived from the Y-12 site efficiently removed high levels of nitrate. The biomass that developed was able to reduce U(VI), and the rate was adequately described by a simple pseudo-second-order rate expression. Rates declined when U(VI) was repeatedly added, suggesting a cometabolic process. The denitrifying community included some sulfate-reducing bacteria, and these organisms likely contributed to U(VI) reduction. We conclude that the strategy evaluated in this study is promising for field application, and so have proceeded with field-testing of this strategy at the Y-12 site.

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