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Microbial Populations Associated with the Reduction and Enhanced Mobilization of Arsenic in Mine Tailings

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Microbial reduction of arsenate [As(V)] to arsenite [As(III)] and the subsequent effects on As mobilization in contaminated mine tailings were studied under transport conditions. Molecular analysis of bacterial populations and traditional isolation techniques were used in conjunction with column experiments designed to observe relationships among pH (limed vs unlimed treatments), redox potential (Pt electrode), and mobilization of As. Liming increased pH values from approximately 4 to 8, resulting in a 5-fold increase in total As eluted from sterile columns. Elution of As from limed columns was further enhanced by microbial activity. As(III) was the predominant As species eluted from oxic, nonsterile columns. Conversely, in sterile treatments, As(V) was the predominant valence state in column effluent. Denaturing gradient gel electrophoresis coupled with sequence and phylogenetic analysis of 16S rRNA gene segments revealed that liming of the mine tailings stimulated specific *Caulobacter-, Sphingomonas-,* and *Rhizobium*-like populations. Pure culture isolates of these bacteria demonstrated the ability to rapidly reduce As(V) in aerated serum bottles. An intracellular As detoxification pathway was implicated in the reduction of As(V) by these isolates. These results indicate that microbial reduction of As(V) in As-contaminated soils may occur under aerobic conditions over relatively short time scales resulting in enhanced As mobilization.

Introduction

Microorganisms possess a variety of mechanisms for reducing arsenate [As(V)] and oxidizing arsenite [As(III)] (*1*−*4*). Transformation of As by microorganisms has important environmental implications because As(V) and As(III) have different sorption and toxicological characteristics; As(III) is often considered the more mobile and toxic species and thus more problematic regarding contamination of natural waters (*5*−*7*). Reduction of As(V) to As(III) in anoxic environments is thought to occur primarily by dissimilatory reduction where microorganisms utilize As(V) as a terminal electron acceptor for anaerobic respiration (*8, 9*). To date, dissimilatory reduction has been characterized in at least seven bacteria [*Sulfurospirillum barnesii, Bacillus arsenicoselenatis, B. selenitireducens, S. arsenophilum, Desulfotomaculum auripigmentum, Chrysiogenes arsenatis, and Desulfomicrobium* strain Ben-RB (*1, 10, 11*)], which represent genera scattered throughout the bacterial domain. In addition, dissimilatory reduction of As(V) has been observed in two hyperthermophilic archaea [*Pyrobaculum arsenaticum* and *P. aerophilum* (*12*)]. Microorganisms may also possess reduction mechanisms that are not coupled to respiration but instead are thought to impart As resistance (*2, 13, 14*). Enzymes involved in the detoxification pathway are transcribed by the *ars* operon. Homologues of the *ars* operon have been discovered in the *Pseudomonas, Bacillus, Klebsiella, Enterobacter, Citrobacter, Staphylococcus, Salmonella, Thiobacillus, Yersinia,* and *Escherichia* genera (*13*−*18*), genotypes that also are scattered throughout the bacterial domain. Microorganisms that express As resistance genes are able to withstand higher concentrations of As through the intracellular reduction of As(V) and the subsequent excretion of As(III) into the surrounding media. It is thought that this pathway may function in aerobic as well as anaerobic environments (*10, 11*) and may contribute to apparent nonequilibrium conditions where As(III) has been often observed in oxic surface waters (*19, 20*). Although it is likely that bacteria in soil, especially at As-contaminated sites, possess As detoxification pathways, to our knowledge the effect of aerobic As(V)-reducing bacteria on As behavior in soils has not yet been presented in the literature.

Liming of acidic mine tailings is recognized as an effective method for immobilizing trace metals and promoting plant establishment (*21*). However, as demonstrated by Jones et al. (*22*), liming may also result in enhanced As mobilization due to the pH dependence of As sorption reactions. The work presented here is an extension of the study conducted by Jones et al. (*22*), wherein our primary objective was to examine the effect of microbial processes on As behavior in mine tailings. We hypothesized that microbial reduction of As(V) may enhance As mobilization in mine tailings and that liming may impact the populations and activities of As(V)-reducing microorganisms. These hypotheses were tested by (i) examining the affect of microbial As(V) reduction on mobilization of As in mine tailings under column transport conditions and (ii) using molecular and traditional cultivation techniques to identify microorganisms responsible for As(V) reduction in the mine tailings. Column transport experiments were designed to observe relationships among pH (limed vs unlimed treatments), redox potential (Pt electrode), dissolved As species, and corresponding shifts in microbial populations. Several microbial populations enriched after liming were identified using molecular and phylogenetic analysis of 16S rDNA fragments and through traditional methods of bacterial cultivation. Several isolates matching 16S fragments observed in the environmental samples were shown to reduce As(V) under aerobic conditions, suggesting a potential role of aerobic heterotrophs in As cycling.

Materials and Methods

Sample Collection and Chemical Characterization. Arsenic-contaminated reprocessed mine tailings (RT) were collected near an abandoned copper smelter in Anaconda, MT (EPA Superfund Site MTD093291656). Extensive characterization of this sample by Jones et al. (*22*) included sequential extractions of As fractions, scanning electron microscopy (SEM)/energy dispersive analysis of X-rays (EDAX), and total metal concentrations as determined by X-ray fluorescence. Briefly, Fe represented 15−21% (w/w) of the RT, primarily in the form of iron oxyhydroxides. Total As

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accounted for approximately 0.3% (w/w) of the RT, with about 32% of the As sorbed to the iron oxyhydroxide phases (quantified by the 0.1 M NaOH sequential extraction step, which has been correlated with As surficially bound to iron oxyhydroxide). The largest fraction of As, approximately 60%, was in the "nonlabile" pool, extracted only after a four-acid heat treatment. Extensive analysis of the RT with SEM-EDAX revealed no discrete solid phases concentrated in As.

Column Experiments. Mobilization of As under transport conditions was studied using polycarbonate columns (length = 54 mm, diameter = 32 mm) packed with a mixture of 20% RT and 80% acid-purified, autoclaved quartz sand (50−70 mesh, Sigma Chemical, St. Louis, MO) for a total mass of 62 g (bulk density ~1.43 g cm^{-3}). Limed treatments included a mix of 60% $CaCO_3$/40% $Ca(OH)_2$ at a concentration of 25.6 g kg^{-1} (*23*). The column apparatus was either autoclaved or fumigated with chloroform prior to packing. Sterilized treatments used RT that was either autoclaved (12 g of RT portions for 1 h × 2) or exposed to chloroform (~5-mm layer of RT in a vacuum-evacuated desiccator with chloroform for ~250 h). The alternate chloroform sterilization treatment was used to circumvent potential alteration of solid phases during autoclaving; however, our results showed no apparent differences between the two sterilization methods (e.g., steady-state values of pH, Fe and As measured in the effluent of chloroform-treated limed columns bracketed values for the autoclaved columns). Autoclaved influent was supplied to the bottom of the columns with a continuous flow pump set to deliver 0.88 mL h^{-1} (1.1 pore vol d^{-1}, pore water velocity = 0.24 cm h^{-1}). The influent, formulated to simulate a "typical" soil solution (SSE), was modified from Angle et al. (*24*) and contained NH_4NO_3 (1.25 mM), $CaSO_4$ (2 mM), $MgCl_2$ (2 mM), KH_2PO_4 (10 μM), KOH (1.25 mM), and $FeCl_2$ (5 μM) supplemented with 100 μL L^{-1} micronutrient solution (*25*). The pH of the influent was adjusted with HCl to pH 3.6 for unlimed columns and with NaOH to pH 7.0 for limed columns. Effluent pH values in the limed treatments near 7.7 were due to liming amendments as opposed to affects of the unbuffered, pH-adjusted influent solution. Because organic amendments and topsoils rich in organic C are routinely added to mine tailings in reclamation efforts, an additional treatment simulating these high C environments included 0.5 mM glucose and 1.0 mM lactate in the influent. For several specific columns, air was continuously pumped through a port in the bottom endcap at a rate of 10 mL min^{-1} to ensure that oxic conditions were present and to disrupt potential redox gradients.

An effluent collection system was devised to minimize microbial and abiotic transformations of As mobilized from the columns. Solution exiting the top of the columns was plumbed directly into 50-mL glass syringes whose pistons were allowed to freely extend as effluent flowed in. The syringes were housed within a N_2(g)-purged temperature-controlled chamber set at 2 ± 1 °C. To allow for expulsion of air from aerated columns, tubing exiting the top endcap was open to the atmosphere, and effluent flowing from the tube was collected with a fraction collector rather than syringes. During the column transport experiments, samples were removed periodically, filtered (0.22 μm), and analyzed for As(V), As(total), Fe(II), Fe(total), sulfide, and pH. The method used for quantifying As species was modified from Masscheleyn et al. (*26*). Specifically, 5-mL aliquots of effluent or standard were added to each of two 15-mL polyethylene bottles and analyzed separately for total As and As(V), with As(III) determined by difference. Arsenite was liberated from one sample by selectively reducing As(III) to arsine gas and subsequent purging of the arsine gas. This was accomplished by adding 1 mL of 0.25 M NaOH and 0.79 M $NaBH_4$ (over a period of 3 min) to a sample buffered with 1 mL of 2 M Tris (pH 6.5) while sparging with N_2(g). The sample was then sparged for 7 additional min. Total As was analyzed with hydride generation−atomic absorption spectrophotometry as described in Jones et al. (*22*). The phenanthroline method was used to determine Fe(II) and Fe(total) concentrations, and sulfide was measured colorimetrically using methylene blue (*27*). Redox potential within the columns was measured using a Pt wire inserted into the center of the column midway between the top and bottom endcaps and sealed prior to starting the experiments (surface area = 0.5 cm^2) and a reference electrode connected to the top endcap. The Pt electrodes were interfaced to a computer that collected voltage data at prescribed intervals. Prior to the experiments, the Pt electrodes were calibrated in accordance with ASTM Method D1498-76 (*28*) using Fe(II)/Fe(III) reference solutions. All column transport experiments were conducted in triplicate unless otherwise noted. At the conclusion of the experiments, columns were dismantled, and the RT/sand mixture was used for isolation of As(V)-reducing microorganisms and for molecular analysis.

As(V)-Reducing Isolates. Bacteria were isolated by adding 1 g of post-experimental RT/sand mixture to 10 mM NaCl and shaking at 100 cycles min^{-1} for 5 min. The slurry was serially diluted, and 0.1-mL aliquots of each dilution were plated onto various media designed to culture aerobic and anaerobic bacteria and, specifically, bacteria capable of anaerobic As(V) respiration. Bacteria were isolated using yeast−extract peptone−glucose (YEPG) agar media and SSE agar media supplemented with 1 mM glucose and 2 mM lactate (SSE+C). Both media also contained 13 μM As(V). These plates were incubated under both aerobic and anaerobic conditions. To specifically isolate As(V)-respiring bacteria, SSE agar media was prepared with 500 μM As(V) and 250 μM cysteine; NH_4NO_3 was replaced with 2.5 mM NH_4Cl. Anaerobic plates were degassed for several days, inoculated, and then incubated in a chamber containing a GasPak Plus generator (Becton Dickinson, Sparks, MD). Isolated colonies were restreaked several times to obtain pure cultures.

Isolates obtained from the post-experimental RT/sand were screened for their ability to reduce As(V) in serum bottles containing 50 mL of SSE+C media and 13 μM As(V). Aerobic treatments were maintained by continuously purging the solution with filter-sterilized air (>5 mL min^{-1}). Anaerobic treatments were conducted using N_2(g)-purged serum bottles containing 13 μM As(V). The potential for isolates to respire on As(V) was tested in N_2(g)-purged serum bottles containing SSE media supplemented with 500 μM As(V) and 250 μM cysteine; NH_4NO_3 was replaced with 2.5 mM NH_4Cl. Aerobic isolates that were capable of reducing As(V) were further characterized using serum bottles containing SSE media plus 5 mM MOPS buffer, 20 mM glucose, 50 μM NaH_2PO_4, and 200 μM Na_2HAsO_4. Serum bottle experiments with isolates obtained from the C-supplemented columns utilized the same media with exception of 10 μM P and 1.4 or 156 μM As(V). Serum bottles were inoculated to attain an initial cell density of 10^6 cells mL^{-1}, as determined using an empirically developed relationship between cell enumeration with epifluorescence microscopy of DAPI stained cells and OD measurements (A_{500}) of cell suspensions. At each sampling interval, 3.5 mL of suspension was removed for determination of OD (A_{500}), As(V), and As(total) concentrations as described above.

DNA Extraction and Purification. Total sample DNA was extracted using the FastDNA SPIN Kit for Soil (Bio 101, Vista, CA) following the manufacturer's instructions. The extracted DNA was electrophoresed in a 1% SeaKem GTG agarose gel (FMC BioProducts, Rockland, ME) and stained with ethidium bromide.

Partial 16S rDNA Amplification and Denaturing Gradient Gel Electrophoresis (DGGE). DNA extracts were used as

TABLE 1. Mean Steady-State pH Values and Fe and As Concentrations in Effluent from Reprocessed Tailing (RT) Columns[a]

treatments	pH	E_H (mV)	Fe(II) (μM)	Fe(total) (μM)	As(III) (nM)	As(total) (nM)
unlimed sterile[b]	3.9 (0.1)[c]	473.2 (12.3)	158.6 (9.9)	162.5 (12.4)	8.3 (5.0)	16.7 (8.4)
unlimed nonsterile	3.8 (0.03)	522.5 (19.6)	78.3 (42.5)	76.4 (43.7)	16.3 (7.9)	24.5 (5.9)
limed sterile	7.8 (0.07)	418.3 (38.6)	0.0 (0.0)	0.0 (0.0)	3.0 (3.0)	99.3 (14.3)
limed nonsterile	7.7 (0.06)	412.9 (17.4)	0.0 (0.0)	0.0 (0.0)	252.1 (52.8)	300.3 (61.4)
limed nonsterile aerated	7.8	393	0.0	0.0	299.5	340.0

[a] Influent for these treatments was not supplemented with C. [b] Values for sterilized treatments are means of three replicate columns experiments; two of the experiments used RT pretreated with chloroform, and the third used RT that was autoclaved. [c] Standard errors of three replicate column experiments in parentheses.

template for polymerase chain reaction (PCR) that targeted a 322-bp region within the 16S rRNA gene. The 1070 forward primer (Integrated DNA Technologies, Coralville, IA) targeted the domain Bacteria (*Escherichia coli* positions 1055−1070), and the 1392 reverse-GC primer targeted a universally conserved region (*E. coli* positions 1392−1406). The reverse primer contained a 40-bp GC-rich clamp used in DGGE (5′-CGC CCG CCG CGC CCC GCG CCC GGC CCG CCG CCC CCG CCC CAC GGG CGG TGT GTA C-3′; *29*). PCR reaction mixtures (50 μL) contained 1−5 μL of template DNA (2−20 ng), 2 mM Tris-HCl (pH 8), 10 mM KCl, 10 μM EDTA, 2.5 mM $MgCl_2$, 800 μM dNTPs, 0.5 μM of each primer, and 1.25 U of *Taq* DNA polymerase (Promega, Madison, WI). PCR reactions were run on a 9700 GeneAmp PCR System (Perkin-Elmer, Foster City, CA). The protocol was 94 °C for 4 min; 30 cycles of 94, 55, and 72 °C each for 45 s; and a final 7-min extension period at 72 °C. DNA was quantified by electrophoresis on a 3% SeaKem GTG agarose gel (FMC BioProducts, Rockland, ME) run with a Low DNA Mass Ladder (Gibco BRL, Grand Island, NY) and stained with ethidium bromide.

PCR products were separated and visualized using DGGE as described by Muyzer et al. (*30*) with the following modifications. A DCode Universal Mutation Detection System (Bio-Rad, Hercules, CA) was used to resolve the PCR products. The gel consisted of 8% acrylamide and a 35−70% gradient of urea/formamide increasing in the direction of electrophoresis (running buffer = 1X TAE [40 mM Tris, 20 mM acetic acid, and 2 mM EDTA at pH 8.5]; 60 V at 60 °C for 17 h). DGGE gels were stained with SYBR Green II (Molecular Probes, Eugene, OR) in 1X TAE for 30 min and photographed using UV transillumination. DGGE bands of interest were stabbed with a sterile pipet tip, rinsed in sterile molecular biology grade water, and used as a template for PCR amplification and subsequent sequencing reactions.

PCR Amplification and DNA Sequencing of DGGE Bands. Template for sequencing of DGGE bands was amplified using primers 1114 forward and 1392 reverse (without the GC clamp; *31*) as described above. The product was purified with a QIAquick PCR Purification Kit (Qiagen Inc., Valencia, CA), and the sequencing reaction was carried out using an ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit as described by the manufacturer (Perkin-Elmer). The samples were processed on an ABI Prism 310 capillary sequencer (Perkin-Elmer), and the resultant sequences were aligned using Sequencher 3.1.1 software (Gene Codes Corporation, Ann Arbor, MI). Phylogenetic information was obtained by using BLAST to compare the sequences with sequences found in the GenBank database (*32*).

Full-Length 16S rDNA Amplification and Sequencing. DNA from each of the isolates was amplified using primers that amplify nearly the entire 16S rRNA gene. Template for the reactions was obtained by scraping several colonies with a sterile pipet tip and swirling the tip in 50 μL of DNase-free water. The suspension was heated at 98 °C for 10 min, and 1.0 or 5.0 μL was used as template for PCR. Primers for the initial PCR consisted of the *Bacteria*-specific primer Bac8 forward (5′-AGAGTTTGATCCTGGCTCAG-3′) and the universal primer Univ1492 reverse (5′-GGTTACCTTGTTACGACTT-3′). The products were purified with a QIAquick PCR Purification Kit. All primers for the full-length sequence reactions were derived from the probes described by Amann et al. (*33*). Sequencing conditions and sequence analysis were conducted as described above.

Results and Discussion

Column Experiments. The mean steady-state effluent pH increased from 3.85 in unlimed treatments to 7.75 after liming (Table 1), consistent with lime requirement calculations for this acid mine soil (*23*). Iron eluted from unlimed columns was predominantly Fe(II), which is consistent with thermodynamic predictions of Fe speciation at pH < 4 and E_H < 530 mV (Table 1; *34*). As expected, the pH increase after liming resulted in undetectable concentrations of Fe in the effluent (<0.8 μM) and thus confirmed the use of liming as an effective method for immobilizing Fe in RT. No detectable sulfide (>3.0 μM) was released from either unlimed or limed columns. Estimates of redox potential using Pt electrodes suggested that limed columns not supplemented with C were too oxidized to support significant concentrations of As(III) (Figure 1; Table 1). At pH 7.75, thermodynamic predictions suggest that redox potential values must be less than −23 mV to support significant concentrations of As(III) (>1% of total; *35*); observed Pt E_H values at steady state were never less than 380 mV (Table 1). In limed columns that received influent containing 0.5 mM glucose and 1.0 mM lactate, redox potential values that theoretically favored the presence of As(III) were attained after 5 d (Figure 2).

In unlimed columns, both the sterilized and the nonsterilized treatments released an initial pulse of As, which ranged from 100 to 250 nM and then gradually declined to approximately 20 nM As after 1−5 d (Figure 1A,B). After liming, steady-state concentrations of total As mobilized from sterile columns increased significantly to about 100 nM (Figure 1C; Table 1). In nonsterile treatments, mean steady-state As concentrations increased further to approximately 300 nM (Figure 1D, Table 1). In the absence of microbial activity, As(V) was the predominant species mobilized after liming. Conversely, in the limed, nonsterile treatments, As(III) was the predominant species mobilized after 3 d, and the increased mobilization of As was associated with the reduction of As(V) to As(III). To verify that As(V) reduction and enhanced As mobilization occurred under oxic conditions, an additional experiment was conducted during which air was continuously pumped through a limed nonsterile column. Steady-state effluent As(III) and total As concentrations for the aerated treatment were essentially identical to concentrations in the nonaerated limed treatments (Table 1), supporting the conclusion that microbial As(V) reduction within these limed columns occurred under redox conditions considered oxic as determined using Pt electrode measurements and Fe^{2+} concentrations (*34*). Limed columns that received influent supplemented with C released about 450 nM total As after 3 d, nearly all as As(III) (Figure 2).

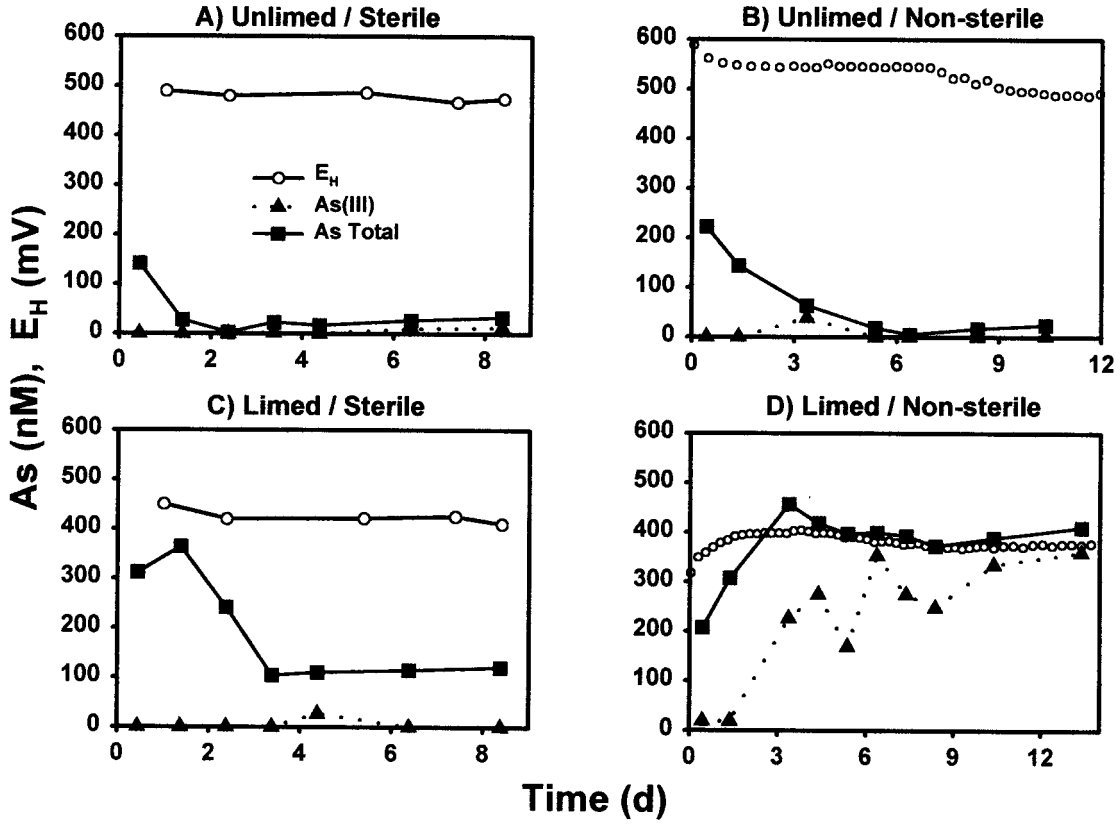

FIGURE 1. Total As and As(III) concentrations in the effluent from unlimed and limed, sterile (autoclaved) and nonsterile RT columns. Influent was not supplemented with C. The As(V) concentration is the difference between total As and As(III) concentrations. E_H was measured with a Pt electrode positioned in the center of the columns.

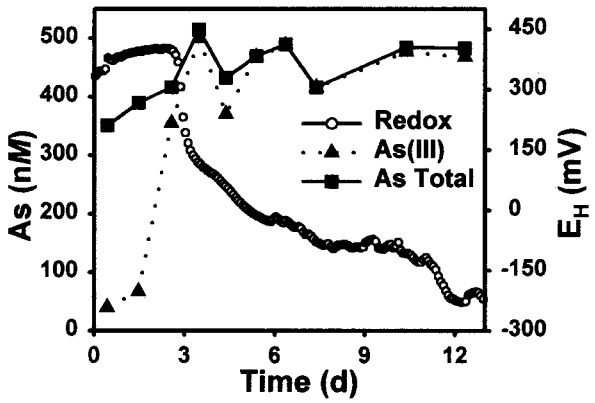

FIGURE 2. Total As and As(III) concentrations in the effluent from limed nonsterile RT column that received influent containing 1.0 mM lactate and 0.5 mM glucose. The As(V) concentration is the difference between total As and As(III) concentrations. E_H was measured with a Pt electrode positioned in the center of the columns.

Molecular Analysis. DNA fingerprints of the microbial populations present in untreated (preexperimental) RT and in two of the three unlimed and limed column replicates were obtained using DGGE. Distinct banding patterns, representing different microbial assemblages, were associated with the different treatments (Figure 3). The presence of many bands in the untreated RT suggested that a relatively diverse microbial community resided in the RT prior to treatment. Significant differences in the banding patterns of unlimed versus limed treatments demonstrated that pH and its consequent impacts on solution chemistry are strong determinants of microbial selection. Comparisons between the banding patterns from column replicates revealed the comigration of many bands and suggested that similar microbial populations were stimulated in the replicate experiments (compare unlimed lane A vs lane B and limed lane A vs lane B); however, some differences between the replicates were observed, indicating potential variation in inoculum or subsequent column conditions or during DNA extraction and amplification of 16S rDNA segments.

Sequence analysis of prominent bands in the denaturing gradient gels and comparison with sequences in the GenBank database revealed that diverse bacterial populations were selected in the column environments. Prominent bands in unlimed column lane B represent populations that were 100% identical to a *Delftia* species and 100% identical to a *Frateuria* species within the region of the rRNA gene analyzed (~322 bp; Figure 3). Prominent bands in the limed column lanes represent *Caulobacter leidyi, Sphingomonas yanoikuyae, Rhizobium* species, and *Leptothrix discophora* (99.1% identical) like populations based on the ~322-bp region analyzed (Figure 3). The prominent upper band in limed column B represented at least two populations that were not easily resolved and therefore were not sequenced.

Isolates. We cultivated and characterized several bacterial populations that may have been responsible for As(V) reduction in the limed RT columns. Bacteria were isolated from two of the three limed (no C supplement) RT column replicates and screened for their ability to reduce As(V). No microorganisms capable of anaerobic growth, including anaerobic respiration using As(V), were isolated during this study. However, three microorganisms capable of aerobic As(V) reduction were isolated from each of these replicate limed columns. Near full-length sequence analysis of the 16S rRNA genes of these three bacteria and comparison with sequences in the GenBank database revealed that their closest phylogenetic neighbors were *C. leidyi, S. yanoikuyae,* and *R. loti* (Table 2).

TABLE 2. Phylogenetic Affiliation and As(V) Reduction Rate Parameters of Isolates Cultivated from Limed Columns Containing Reprocessed Tailings (RT)[a]

		nearest GenBank relative[b]			As(V) reduction parameters[c]			
isolate	GenBank Accession No.	species	phylogenetic group	% similarity	k^d (AU^{-1} d^{-1})	r^2	max rate (mM d^{-1})	half-life[d] (d)
1	AF331660	*Caulobacter leidyi*	α-proteobacteria	100	0.037	0.93	0.054	2.6
2	AF331661	*Sphingomonas yanoikuyae*	α-proteobacteria	100	0.073	0.88	0.401	0.4
3	AF331662	*Rhizobium loti*	α-proteobacteria	98.3	0.033	0.88	0.293	0.5
4	AF331663	*Pseudomonas aeruginosa*	γ-proteobacteria	99.9	0.939	0.97	0.118	0.9
5	AF331665	*Pseudomonas fluorescens*	γ-proteobacteria	99.0	0.224	0.98	0.066	1.7
6	AF331664	*Sphingomonas echinoides*	α-proteobacteria	99.4	0.469	0.82	0.0004	2.4

[a] Measured optical density (OD) values and As(V) concentrations were used to fit a second-order rate expression (eq 1) to obtain values for an apparent second-order rate constant (k). Maximum rates (max rate) of As(V) reduction observed under serum bottle conditions are also reported. [b] Phylogenetic affiliations were determined by comparing near full-length 16S rDNA sequences of these isolates to sequences in the GenBank database. [c] Initial As(V) concentrations were 160−200 μM for isolates 1−5 and only 1.4 μM for isolate 6. [d] Apparent rate constants and half-lives ($t_{1/2}$) dependent on experimental conditions and limited to OD values achieved in current experiment; half-lives estimated based on maximum rates assuming psuedo-first-order reaction dependent on As(V).

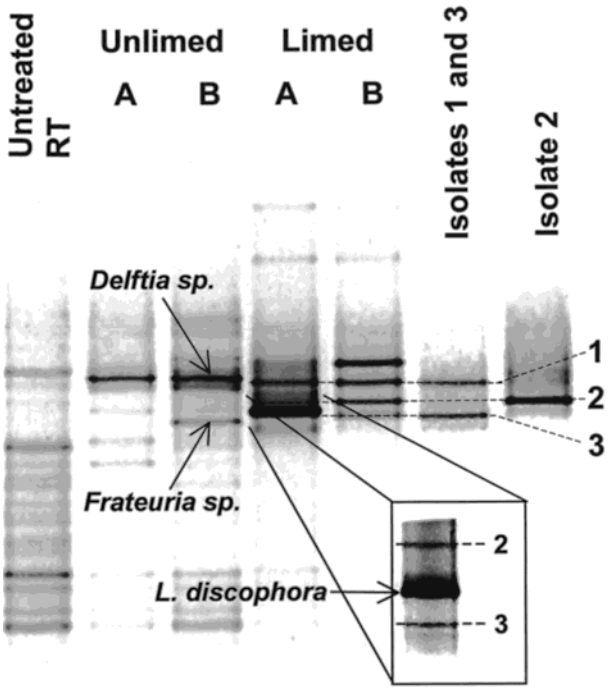

FIGURE 3. Denaturing gradient gel (35−70%) of PCR-amplified 16S rDNA fragments from untreated RT, unlimed RT columns (replicates A and B), limed RT columns (replicates A and B), and *C. leidyi* (1), *S. yanoikuyae* (2), and *R. loti* (3) -like isolates. To resolve the *S. yanoikuyae* and *R. loti* bands in the limed RT column (replication A), the sample was run with a narrower (40−60%) denaturing gradient (inset). Comigration of isolate bands with bands from limed RT columns is indicated with dashed lines.

The relevance of these cultivated organisms in the column environment was examined by comparing DGGE band migration and sequences of bands detected in the column environment to those of each isolate. Bands representing the *C. leidyi*-, *S. yanoikuyae*-, and *R. loti*-like populations in RT had the same mobility in the DGGE gels as isolate bands, and sequence analysis verified that they were 100% identical (Figure 3). These observations suggest that our isolates represented at least three of the bacterial populations that were stimulated by the liming treatment.

Three additional microorganisms capable of reducing As(V) in aerated serum bottles were isolated from replicate limed RT columns that received supplemental C in the influent. This treatment was specifically designed to enrich for microorganisms that may be important under the elevated C levels resulting from remediation treatments that incorporate C amendments. Although the conditions within these columns may have been conducive to the growth of anaerobic bacteria, no bacteria were isolated under anaerobic conditions; the three bacteria obtained from this treatment were all isolated under aerobic conditions. Near full-length sequences of the 16S rRNA genes of these isolates were compared with sequences in the GenBank database; the nearest phylogenetic neighbors were *Pseudomonas fluorescens*, *P. aeruginosa*, and *Sphingomonas echinoides* (Table 2). All six of the As(V)-reducing bacteria isolated from the mine tailings were members of either the α- or γ-proteobacteria subdivisions (Table 2). The 16S rDNA sequences of these isolates were deposited in GenBank under the accession numbers AF331660−AF331665 (Table 2).

As(V) Reduction Kinetics of Isolates. The kinetics of As(V) reduction were measured in continuously aerated serum bottles for each of the six isolates cultivated from limed tailings (Figure 4). The dependence of As(V) reduction rate on As(V) concentration and OD during the growth phase of the isolates was modeled using the following second-order rate equation (*36*):

$$-d[\mathrm{As(V)}]/\mathrm{d}t = k[\mathrm{As(V)}]X \qquad (1)$$

where X represents OD in absorbance units (AU) and k is the apparent As(V) reduction rate constant (in units of $AU^{-1}\,h^{-1}$). Measured values of OD and As(V) as a function of time were used as inputs to eq 1 to obtain fitted values of k for each isolate (Table 2). Predicted concentrations of As(V) obtained using the fitted k values and measured OD values correlated well with measured data for all isolates ($r^2 > 0.8$; Table 2; Figure 4). The apparent rate constants (k) for isolates 1−3 were significantly lower as compared to isolates 4−6, due in part to the different growth characteristics of the isolates and the composition of the media used in these experiments. For example, the P nutritional status and the P:As ratio may be very important to the growth rates and As(V) reduction kinetics observed in these experiments (*14*). Isolates 1−3 received 50 μM P and achieved much higher OD values than isolates 4−6, which received only 10 μM P. Because the fitted second-order rate constants (k) depend on both As(V) concentration and OD, variation in growth kinetics among isolates under the culture conditions employed influences the magnitude of k. Although we are interested in the affect of P:As ratio on the reduction kinetics of As(V), the current objectives were focused on establishing that these isolates may be responsible for As(V) reduction observed in the limed columns under aerobic conditions. Toward this end, the serum bottle experiments clearly showed that the reduction of As(V) was coincident with microbial growth under aerobic

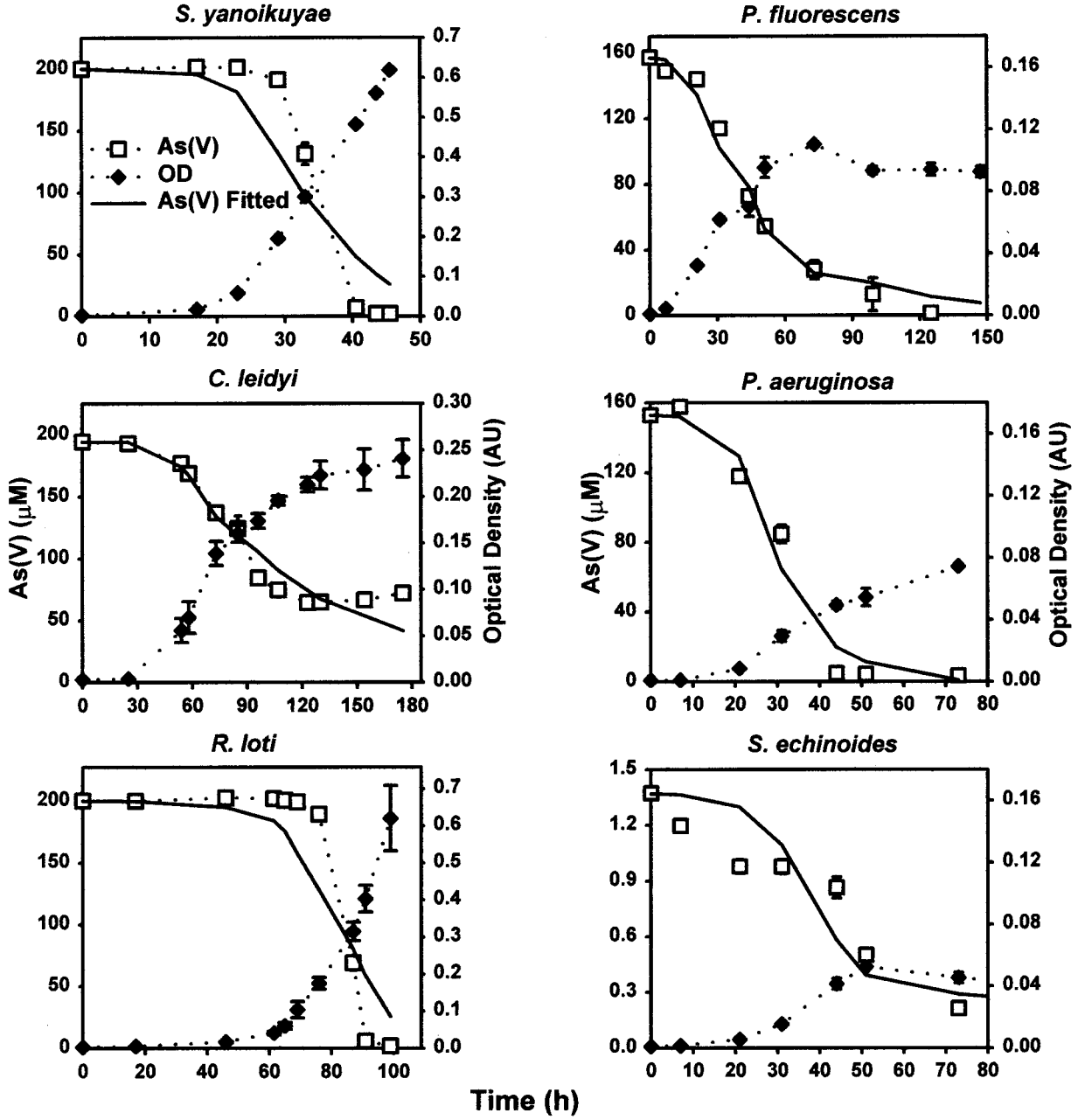

FIGURE 4. Microbial biomass (as optical density, OD) and As(V) concentrations as a function of time for six As(V)-reducing isolates incubated under aerated conditions. Isolates are named after their nearest phylogenetic neighbor found in the GenBank database. Serum bottles inoculated with *S. yanoikuyae*, *C. leidyi*, and *R. loti* contained 50 μM P and 200 μM As(V). Serum bottles inoculated with *P. fluorescens* and *P. aeruginosa* contained 10 μM P and 157 μM As(V). Because *S. echinoides* did not reduce any As(V) at an initial As(V) concentration of 157 μM, reduction experiments with this isolate were conducted using 1.4 μM As(V) and 10 μM P. Error bars represent standard errors of three replicate serum bottle experiments.

conditions. Moreover, maximum OD values were not significantly different in the absence of As(V) (data not shown), suggesting that growth was not coupled to reduction of As(V) and that growth was not inhibited by the presence of As(V) at these levels. Maximum rates of As(V) reduction by these isolates ranged from about 0.4 mM d^{-1} for *S. yanoikuyae* to about 0.4 μM d^{-1} for *S. echinoides* (Table 2). Since the maximum concentration of As(III) eluted from the limed columns was about 0.4 μM (at a flow rate of 21 mL d^{-1}), it is reasonable that these isolates could have been responsible for the reduction of As(V) that was observed during transport. Nevertheless, the As(V) reduction rates demonstrated by several of these isolates are comparable to rates measured for other bacteria. For example, researchers have reported dissimilatory As(V) reduction rates as high as 7 mM d^{-1} (*37*) while As(V) reduction rates attributed to detoxification have been observed to be as high as 1 mM d^{-1} (*11*, *36*).

Mechanisms of As Reduction and Implications for As Cycling. In the RT used in the current study, liming enhanced the mobilization of As due to both chemical (pH-dependent sorption; *38*) and microbiological processes (Table 1). A combination of cultivation and cultivation-independent methods were used to evaluate specific bacterial populations responsible for the reduction of As(V) in mine tailings after liming. Six different microbial populations representing *Sphingomonas*, *Caulobacter*, *Rhizobium*, and *Pseudomonas* genera were isolated from limed reprocessed tailings, and all were capable of rapidly reducing As(V) in aerated serum bottles. The appearance of 16S rDNA bands representing these bacteria in denaturing gradient gels revealed that several

of these populations were present in the limed columns and in some cases appeared to be dominant members of the microbial community. The reduction of As(V) to As(III) by these microorganisms was associated with the enhanced mobility of As in column transport experiments. However, reductive dissolution of the iron oxide phase was not a significant factor in mobilizing As as judged by the absence of detectable Fe(II) or Fe(III) in column effluent. Conversely, the pH change from 4 to 7.7 resulting from lime amendment would be expected to cause significant precipitation of iron oxyhydroxides and measured dissolved Fe concentrations before and after liming were consistent with this expectation.

Several lines of evidence suggest that the microbial reduction of As(V) in these column transport experiments occurred via a process other than As(V) respiration. The fact that aeration had little effect on As(V) reduction rates (Table 1) suggests that reduction of As(V) was not coupled to anaerobic respiration. Furthermore, the bacterial isolates shown to be relevant members of the microbial community did not demonstrate growth under anaerobic conditions with As(V) as an electron acceptor but, rather, were able to grow and rapidly reduce As(V) under oxic conditions. These data suggest that As(V) reduction in the mine tailings occurred via a detoxification process that is functional under oxic conditions. Cai et al. (*17*) demonstrated that strains of *P. aeruginosa* and *P. fluorescens*, which are closely related to two of our isolates, carry *ars* operon homologues that confer increased resistance to As. This pathway results in intracellular reduction of As(V) and the subsequent efflux of As(III) via a transmembrane pump. DNA sequence homologues of As detoxification genes have also been detected in *Thiobacillus ferrooxidans*, an obligately chemolithotrophic bacterium common in pyritic mine tailings (*13*). Although we recognize that anaerobic microsites within soil aggregates may have contributed to dissimilatory As(V) reduction, the molecular analysis and cultivation techniques employed in the current study did not reveal any potential As(V)-respiring bacteria. It is certainly possible that these microorganisms may have been present in our columns, but they were not detected due to limitations of the techniques including (i) the inability to extract or amplify DNA from these bacteria, (ii) the inability to identify and/or sequence less prominent DGGE bands representing organisms capable of dissimilatory As(V) reduction, and (iii) the inability to successfully simulate the column environment during attempts to cultivate these microorganisms. However, while dissimilatory reduction of As(V) may be an important process in strict anaerobic environments, the results from this study suggest that rapid reduction of As(V) in limed mine tailings, and potentially in other soils and natural waters, may be facilitated by microorganisms under aerobic conditions. Consequently, the prediction of As valence and, thus, the behavior of As based solely on redox status may be problematic. Because the ability to reduce As(V) via detoxification pathways may be a widely distributed trait in soil and aquatic microorganisms, these processes need to be taken into account in order to fully understand As cycling in natural systems.

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