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Bacterial Populations Associated with the Oxidation and Reduction of Arsenic in an Unsaturated Soil

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Microbial populations responsible for the oxidation and reduction of As were examined in unsaturated (aerobic) soil columns treated with 75 μM arsenite [As(III)] or 250 μM arsenate [As(V)]. Arsenite [As(III)] was rapidly oxidized to As(V) via microbial activity, whereas no apparent reduction of As(V) was observed in the column experiments. Eight aerobic heterotrophic bacteria with varying As redox phenotypes were isolated from the same columns. Three isolates, identified as *Agrobacterium tumefaciens*-, *Pseudomonas fluorescens*-, and *Variovorax paradoxus*-like organisms (based on 16S sequence), were As(III) oxidizers, and all were detected in community DNA fingerprints generated by PCR coupled with denaturing gradient gel electrophoresis. The five other isolates were identified (16S gene sequence) as *A. tumefaciens, Flavobacterium* sp., *Microbacterium* sp., and two *Arthrobacter* sp.-like organisms and were shown to rapidly reduce As(V) under aerobic conditions. Although the two *A. tumefaciens*-like isolates exhibited opposite As redox activity, their 16S rDNA sequences (~1400 bp) were 100% identical, and both were shown to contain putative *arsC* genes. Our results support the hypothesis that bacteria capable of either oxidizing As(III) or reducing As(V) coexist and are ubiquitous in soil environments, suggesting that the relative abundance and metabolic activity of specific microbial populations plays an important role in the speciation of inorganic As in soil pore waters.

Introduction

The activity of As transforming microorganisms in soils and natural waters has significant implications for the behavior of As because different As species exhibit variation in solubility, mobility, bioavailability, and toxicity (*1*−*4*). Of the dominant inorganic species, arsenite ($H_3AsO_3^0$) is generally considered to be more mobile and more toxic than arsenate ($H_2AsO_4^-$, $HAsO_4^{2-}$) (*1*−*4*). Known As transforming bacteria possess diverse mechanisms for either oxidizing As(III) or reducing As(V), including energy generation and detoxification (e.g., refs *5*−*10*). For example, an As(III)-oxidizing *Agrobacterium*/*Rhizobium*-like bacterium isolated from a gold mine in Australia can grow chemolithoautotrophically, utilizing As(III) as the sole electron donor (*8*). Conversely, *Alcaligenes* sp. and *Agrobacterium albertimagni* strains grow heterotrophically but can rapidly oxidize As(III) using a mechanism consistent with As detoxification rather than energy generation (*7*, *11*). The crystal structure of arsenite oxidase in *Alcaligenes* sp. has recently been elucidated (*12*).

Dissimilatory reduction of As(V) has been shown to occur in at least nine different genera scattered throughout the domain *Bacteria* (*10*, *13*−*15*) and has also been observed in two hyperthermophilic *Archaea* (*16*). These microorganisms are either strict anaerobes, facultative anaerobes, or microaerophiles capable of utilizing arsenate as a terminal electron acceptor. Interestingly, *Thermus* strain HR13 apparently has the capability to both reduce As(V) via respiration under anaerobic conditions and oxidize As(III) via a detoxification mechanism in the presence of oxygen (*13*). Dissimilatory reduction is often considered the primary mechanism responsible for the rapid reduction of As(V) observed in anaerobic environments. However, work also suggests that a variety of soil microorganisms, both anaerobic and aerobic, may reduce As(V) to As(III) via As detoxification (*17*−*19*). Significant prior work has shown that As(V) reduction activity by numerous bacteria is encoded by a variably organized *ars* operon, which may either be plasmid-borne or chromosomal (e.g., refs *20*−*22*). The *ars* genes are inducible by either As(V) or As(III), resulting in the intracellular reduction of As(V) by ArsC, a cytoplasmic reductase, and subsequent excretion of As(III) into the surrounding media (ArsB, A). Homologues of the *ars* operon have been identified in diverse bacteria including *Pseudomonas*, *Bacillus*, *Klebsiella*, *Staphylococcus*, *Salmonella*, *Acidithiobacillus*, *Yersinia*, and *Escherichia* (*21*, *23*−*26*). In addition, putative *ars* homologues have been detected in many of the bacteria and archaea whose genomes have recently been sequenced, suggesting that *ars* genes are relatively common among prokaryotes (*27*).

The reduction of As(V) via detoxification may contribute to apparent nonequilibrium conditions where As(III) has been observed in oxic soils and surface waters (e.g., refs *18*, *28*, and *29*). For example, several As(V)-reducing bacteria have been found to mediate the reduction of As(V) under highly aerobic conditions resulting in enhanced mobilization of As from limed mine tailings (*18*). The characterization of several aerobic heterotrophs isolated from these tailings suggested that the probable mechanism of As(V) reduction was As(V) detoxification. Other investigators have also isolated apparent As detoxifying bacteria from soil, although the relevance of these populations to As redox cycling actually occurring in the environment was not studied (*17*, *19*, *30*). Given the importance of As(V)−As(III) redox cycling in soil−water systems, very little is known regarding the potential role of various As detoxification strategies versus metabolisms capitalizing on As for energy conservation. Consequently, one of the goals of the current study was to improve our understanding of the possible mechanisms and associated microbial diversity responsible for As (III)/(V) cycling in soil systems. Specifically, the objectives of this study were to (i) utilize cultivation-independent 16S rDNA sequence analysis to identify microbial populations associated with observed As redox transformations occurring in a soil environment, (ii) cultivate As(III)-oxidizing and As(V)-reducing aerobic heterotrophic bacteria from the same soil systems, and (iii) determine if isolates with demonstrated As redox activity correspond to those populations detected with molecular methods. These objectives were addressed by conducting

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unsaturated flow column transport experiments where micromolar concentrations of either As(III) or As(V) were used as influent to enrich for microorganisms capable of transforming As under aerobic conditions.

Materials and Methods

Column Experiments. The upper 20 cm of a well-drained, fine loamy, frigid Typic Calciaquoll that contained 2.6 μM soluble As (saturated paste extraction using deionized H_2O equilibrated for 0.5 h; *31*) was collected from an irrigated pasture in the Madison River Valley (Gallatin County, Montana) and used as inocula for column studies. Measurements of soluble As concentrations in Madison River Valley soils typically range from 0.2 to 35 μM (*31*). The fate of As in irrigated Madison River water containing 1−4 μM As originating from Yellowstone National Park (*32*) has been an important regional water quality problem regarding As contamination of soils and shallow groundwaters (*31, 32*). Redox transformations of As within this soil were studied under unsaturated flow conditions using autoclaved polycarbonate columns (length = 100 mm, diameter = 35 mm) packed with a mixture of 5% soil and 95% acid-washed, autoclaved quartz sand (50−70 mesh, Sigma Chemical, St. Louis, MO) for a total mass of 115 g (bulk density ~1.2 g cm^{-3}). The columns received autoclaved influent supplied to the top of the columns with a continuous-flow pump set at 1.8 mL h^{-1} (1.9 pore volume d^{-1}, pore water velocity ~0.77 cm h^{-1}). To ensure thorough aeration and create unsaturated flow within the columns, filter-sterilized air was drawn through the column via a nylon screen at the bottom endcap using a peristaltic pump at a rate of 100 mL h^{-1}. Solution applied to the columns drained through the soil/sand matrix via gravimetric flow and exited the bottom of the columns along with the air stream. The volumetric water content (θ_V) of the soil/sand mixture was maintained at 0.24 cm^3 cm^{-3} (~53% of saturation). The influent was formulated to enrich for aerobic heterotrophs capable of transforming As 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) (*33*) supplemented with micronutrients (*34*), vitamins (*35*), 5 mM glucose (added to enrich for heterotrophic organisms), and either 75 μM NaH_2AsO_3 or 250 μM NaH_2AsO_4. These concentrations of As were significantly higher than concentrations shown to induce genes involved in As detoxification (*21*) but lower than those normally employed for As(III) chemolithotrophic growth or for anaerobic respiration on As(V) (*8, 35*). The pH of the influent was adjusted to 7.0 with NaOH. Column experiments were conducted in duplicate, and sterile controls were performed using autoclaved soil (30-g portions of soil autoclaved for three 1-h cycles).

Arsenic transformations within all columns were monitored periodically during the 14−16-d experiments by collecting 2-mL samples of column effluent, which were then filtered (0.22 μm) and analyzed for As(V) and As(total) using hydride generation−atomic absorption spectrometry (HG-AAS) and $NaBH_4$ speciation, where arsenite is determined by difference (*17*). Upon termination, all columns were dismantled, and subsamples of the soil/sand mixture were used for isolation of As(III)-oxidizing and As(V)-reducing microorganisms and for molecular analysis.

Isolation and Characterization of As-Transforming Aerobic Heterotrophs. Bacteria were isolated from the unsaturated columns by homogenizing the entire soil/sand mixture and adding 1 g to 10 mL of 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 R2A nutrient agar (Difco Laboratories, Detroit, MI), a medium designed to maximize culturability of stressed cells. This plating protocol was used to isolate aerobic heterotrophs consistent with the soil column enrichment conditions and was not intended to cultivate dissimilatory As(V) reducers, which are generally anaerobic or microaerophilic (*10*).

Isolates obtained from the soil/sand mixture were tested for their ability to oxidize or reduce As during growth in 25-mL bottles containing 5 mL of column influent media (described above) modified to include 5 mM MOPS buffer (pH 7.0), 50 μM NaH_2PO_4, 1 mg L^{-1} yeast extract (added to enhance growth in pure culture), and either 75 μM NaH_2AsO_3 or 250 μM NaH_2AsO_4. Bottles were agitated on a shaker table and were aseptically vented daily to maintain aerobic conditions. The As-transforming isolates were grouped based on comigration of PCR amplified 16S rDNA fragments in denaturing gradient gel electrophoresis (DGGE) (described below) and, finally, by near-full-length sequencing of their 16S rRNA gene (described below). Rates of either As(III) oxidation or As(V) reduction by each isolate were characterized in continuously aerated serum bottles (5 mL min^{-1} filter-sterilized air) containing 50 mL of the same liquid media used for initial testing (discussed above) with the exception that 50 μM NaH_2AsO_3 was used for isolate 3. Prior to inoculation, all isolates were grown in the same liquid media without As. Serum bottles were inoculated to attain an initial cell density of 10^6 cells mL^{-1} based on optical density (OD) measurements (A_{500}) of cell suspensions. At each sampling interval, 3.0 mL of suspension was removed for determination of OD and concentrations of As(V) and As(III) as described above. Arsenic oxidation and reduction rates were determined from maximum slopes of As concentration curves versus time and normalized to cell number (OD correlating to position of maximum slope) using an empirically developed relationship between cell enumeration with phase contrast microscopy and OD measurements (A_{500}) of cell suspensions.

To confirm that As(III) oxidation was dependent on the presence of microbial cells, culture filtrates from As(III)-oxidizing isolates were tested for oxidization of As(III) (*5*) by spiking 3 mL of filtrate (0.22 μm) from actively oxidizing cell suspensions (inoculated similarly and grown in the same media described for the oxidation rate experiments) with 200 μM As(III) and measuring oxidation after 3 h. The ability of the As(III)-oxidizing isolates to grow chemolithoautotrophically with As(III) as the sole electron donor and CO_2 as the primary C source was tested using the serum bottle method described above with 25 mL of column influent media (described above) supplemented with 50 μM NaH_2PO_4, 30 mM $NaHCO_3$ (added to replace glucose as the C source), and 5 mM As(III) (modified from ref *8*). The serum bottles were sealed to maintain the partial pressure of CO_2. The potential for the As(V)-reducing isolates to respire on As(V) was tested in N_2(gas)-purged serum bottles containing column influent media modified to contain 5 mM MOPS buffer (pH 7.0), 50 μM NaH_2PO_4, 1 mg L^{-1} yeast extract, 4 mM As(V), and 1 mM cysteine; NH_4NO_3 and glucose were replaced with 2.5 mM NH_4Cl and 20 mM lactate, respectively, based on past reports of media used to culture As(V) dissimilatory reducers (*35*).

DNA Extraction, PCR, and DGGE Analysis. Total DNA was extracted from the homogenized soil/sand mixture using the FastDNA SPIN Kit for Soil (Bio 101, Vista, CA). DNA extracts were used as template for polymerase chain reaction (PCR) which targeted a specific 322 bp segment within the 16S rRNA gene. The 1070 forward primer 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; *36*). The reverse primer was modified to contain a 40 bp GC-rich clamp to facilitate analysis by DGGE (*37*). PCR mixtures (50 μL) contained 1−5 μL of template DNA (2−20 ng), 10 mM Tris-HCl (pH 8), 50 mM KCl, 0.1% Triton X-100, 4.0 mM $MgCl_2$, 800 μM dNTPs, 0.5 μM of each primer, and 1.25 U *Taq* DNA polymerase (Promega, Madison, WI).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.

PCR products were separated by DGGE as described by Ferris et al. (*37*) with the following modifications. A DCode System (Bio-Rad, Hercules, CA) was used to resolve the PCR products in gels consisting of 8% acrylamide and a 40−70% gradient of urea/formamide. Electrophoresis was performed at 60 V at 60 °C for 17 h. DGGE gels were stained with SYBR Green II (Molecular Probes, Eugene, OR) for 30 min and photographed using UV transillumination. DGGE bands of interest were stabbed with a sterile pipet tip and used as template for PCR amplification, purification (repeated PCR amplification and DGGE until a pure band was obtained), and subsequent sequencing reactions. The templates were amplified using primers 1070 forward and 1392 reverse (without the GC clamp) 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 (Perkin-Elmer, Foster City, CA). The samples were processed on an ABI Prism 310 capillary sequencer (Perkin-Elmer), and the resultant sequences were aligned and edited using Sequencher 3.1.1 software (Gene Codes Corporation, Ann Arbor, MI). The sequences were then compared with those found in the GenBank database using BLAST (*38*).

Full-Length 16S rDNA Amplification and Sequencing of Isolates. Total DNA from each of the isolates was used as template to amplify nearly the entire 16S rRNA gene. Template for PCR 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 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 PCR 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. (*36*). Sequencing reactions and analysis were conducted as described above. The near-full-length 16S rDNA sequences for the isolates obtained in this study have been submitted to GenBank and have been assigned the accession numbers AF388027, AF388028, AF388029, AF388030, AF388031, AF388032, AF388033, and AF388034.

Amplification, Hybridization, and Phylogeny of *arsC* Genes. Four sets of primers for *arsC* genes were designed based on different groupings of *arsC* sequences (*23*). Sequences of 17 characterized and putative *arsC* genes were obtained from GenBank and aligned using the ArbEdit Fast Aligner function in the ARB software package (W. Ludwig and O. Strunk, Technical University of Munich). Primer set 1 was derived from the *arsC* genes of enteric bacteria (5′-ATGAGCAACATYACCAT-3′ forward and 5′-TTATTTCAGYCGTTTACC-3′ reverse; corresponding to positions 1−426 of *E. coli arsC*). Primer set 2 was derived from the *arsC* genes of Gram-positive bacteria (5′-ATTTAYTTTATATGYACAG-3′ forward and 5′-GATCATCAAAACCCCAAT-3′ reverse; corresponding to positions 16−317 of the *Bacillus subtilis arsC*). Primer set 3 was derived from the *arsC* genes of *Pseudomonas aeruginosa* and *P. putida* (5′-AGTCCTGTTCATGTGYAC-3′ forward and 5′-TGGCGTSGAAYGCCG-3′ reverse; corresponding to positions 6−365 of the *P. aeruginosa arsC*). The fourth primer set was designed from the annotated *arsC* in the *Agrobacterium tumefaciens* genome sequence (GenBank Accession No. AE008073) (5′-ATGTCCGATTTTTCACAATCCG-3′ forward and 5′-TTTCCTTCATTGTCGAGGACCTGC-3′ reverse; corresponding to positions 11−416 of the *A. tumefaciens arsC*). The *arsC* genes of *E. coli* (strain K-12, *21*), a *Geobacillus* sp. (isolate from a Yellowstone National Park geothermal soil, GenBank Accession No. AF391973 for 16S rDNA), *P. aeruginosa* (strain PAO1; *24*), and *A. tumefaciens* (strain C58, gift from D. Wood, University of Washington) were used as controls in amplification reactions using each set of primers. PCR conditions were as described for 16S rRNA gene amplification except for annealing temperature (which was reduced to 37 °C for primer sets 1 and 2, to 42 °C for primer set 3, and to 40 °C for primer set 4) and cycle number (which was increased to 35). Attempts were made to amplify *arsC* genes from DNA extracted from each isolate with each primer set. PCR products of positive controls were confirmed as *arsC* genes by sequencing. *E. coli*, *P. aeruginosa*, and *A. tumefaciens arsC* genes corresponded to previously reported *arsC* sequences in GenBank (Accession Nos. X80057, AF010234, and AE008073, respectively). The putative *Geobacillus* sp. *arsC* gene was novel and was submitted to GenBank (AF393651). PCR products of all four genes were used as probes for dot blot hybridizations. Probes were labeled with ^{32}P using the Megaprime DNA Labeling System (RPN 1606, Amersham Pharmacia Biotech, Piscataway, NJ). Total DNA from each of the isolates was applied to nylon membranes (GeneScreen Plus, NEN Life Sciences, Boston, MA) using the Bio-Dot microfiltration apparatus (BioRad), and hybridizations with each probe were performed according to the manufacturer's instructions. Probed membranes were visualized using autoradiography with Kodak BioMax MS film (Rochester, NY).

An unrooted *arsC* phylogenetic tree was constructed using sequences of 14 characterized and putative *arsC* genes obtained from GenBank and from sequences described in this study. Sequences were aligned using the neighbor joining procedure as described above; only positions that were unambiguous for all sequences were used, and gaps were ignored.

Results and Discussion

Column Experiments. Arsenate was the predominant species of As eluted from nonsterile unsaturated columns after 3 d, regardless of whether they received As(III) or As(V) (Figure 1). The value of the column-derived first-order rate constant (k) for As(III) oxidation in treatments receiving 75 μM As(III) was determined to be >0.60 h^{-1} ($t_{1/2} < 1.2$ h) using an analytical solution to the advection−dispersion equation (eq 9 in ref *41*), assuming that all As(III) was converted to As(V) (detection limit = 0.05 μM). Although the oxidation of As(III) to As(V) is thermodynamically favored under oxic conditions (*42*), sterilized treatments did not result in detectable conversion of As(III) to As(V). Consequently, the rapid oxidation of As(III) observed in the nonsterile columns was mediated by microbial processes.

Molecular Analyses. DGGE was used to obtain DNA fingerprints of microbial populations present in the original soil inoculum and in each of the column enrichments. Replicate DGGE profiles of the untreated soil appeared identical, exhibited numerous bands, and suggested that numerous microorganisms were present in the original soil inoculum (Figure 2). The As(III) and As(V) treatments produced somewhat different banding patterns, indicating that enrichment conditions, including As speciation and/or concentration, influenced the selection of soil microbial populations. Replicate columns of each As treatment yielded similar DGGE banding patterns; however, differences in band intensity between the replicates were observed, indicating potential variation in inoculum and/or subsequent column conditions. As with all defined enrichment environments, the chemical and physical attributes of the media select for organisms that are competitive or highly adapted to such conditions. Consequently, the use of glucose as a potential C and energy source and the relatively high As concentrations used in these experiments do not necessarily enrich for organ-

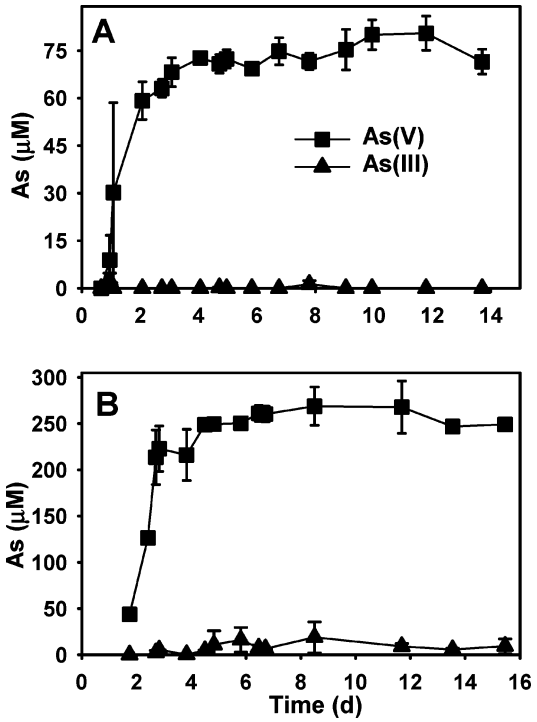

FIGURE 1. Concentrations of As(V) and As(III) in the effluent from unsaturated soil columns receiving either (A) 75 μM As(III) or (B) 250 μM As(V). Error bars are standard errors of duplicate column experiments. [Preliminary results from these experiments were presented in a review chapter (*39*) based on results of Macur et al. (*40*)].

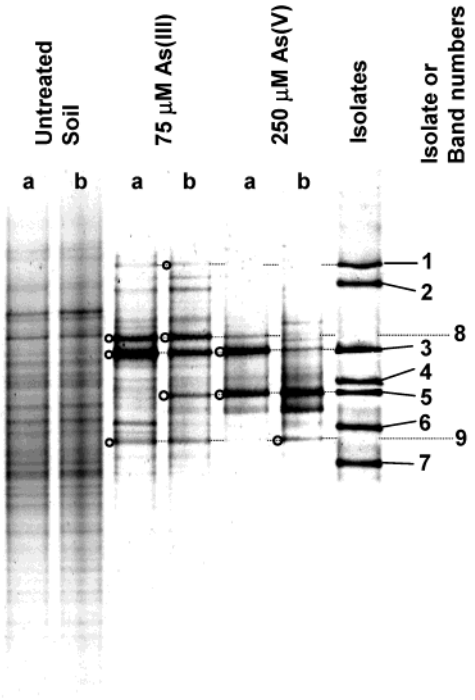

FIGURE 2. Separation of PCR-amplified 16S rDNA fragments using denaturing gradient gel electrophoresis (40−70%). DNA was derived from either untreated soil, soil columns (replicates a and b) that had received either 75 μM As(III) (14 d treatment) or 250 μM As(V) (16 d treatment), and As-transforming isolates obtained from the columns. Band numbers labeled on the right side of gel correspond to (1) *Variovorax paradoxus*, (2) *Flavobacterium heparinum*, (3) *Pseudomonas fluorescens*, (4) *Microbacterium* sp., (5) *Agrobacterium tumefaciens*, (6) *Arthrobacter aurescens*, and (7) *Arthrobacter* sp.-like organisms. Bands observed in the soil columns that did not correspond to any isolates represent (8) *Alcaligenes* sp. and (9) *Agrobacterium vitis*-like populations based on comparison of band sequences to entries in the GenBank Database. Dashed lines show comigrating bands, and open circles indicate bands that were purified and sequenced. [A preliminary gel from these experiments was presented in a review chapter (*39*) based on results of Macur et al. (*40*)].

isms that may predominate in natural soil environments containing other C substrates and lower As concentrations.

Purification and sequencing of selected bands in the DGGE gels and comparison with sequences in GenBank revealed that the two most prominent bands in the columns supplemented with As(III) represented populations that were 99.4% similar to *Pseudomonas fluorescens* (band 3) and 99.6% similar to *Alcaligenes* sp. (band 8, GenBank Accession No. AF536820) (Figure 2). Less conspicuous bands in this treatment that were successfully purified and sequenced most closely matched *Variovorax paradoxus* (99.5% identity, band 1), *A. tumefaciens* (100% identity, band 5), and *A. vitis* (100% identity, band 9, GenBank Accession No. AF536821). Major bands in columns supplemented with As(V) represented *P. fluorescens* (band 3) and *A. tumefaciens* (band 5), while minor bands represented *Alcaligenes* sp. (band 8) and *A. vitis* (band 9)-like populations. Thus, all of the sequenced bands were found in each of the two treatments with the exception of *V. paradoxus*, which was only observed in the As(III)-supplemented columns. The most apparent differences between treatments were decreased intensity of the *Alcaligenes* sp. band (no. 8) and increased intensity of the *A. tumefaciens* band (no. 5) in the As(V)-treated columns relative to the As(III)-treated columns. These differences in band intensity suggested that the *Alcaligenes* sp.-like population may have been favored in the presence of As(III), and conversely, the *A. tumefaciens*-like population was favored in the presence of As(V).

Isolates. Traditional cultivation methods were used to isolate microorganisms from the As-treated columns. Serial dilutions of the soil/sand mixture obtained after column enrichment were plated on R2A media and after 72 h, 24 colonies representing 10 different colony morphologies were picked from the most dilute plates (10^{-5}−10^{-4} dilutions) and tested for their ability to either oxidize As(III) or reduce As(V). Because all of these isolates were obtained from plates inoculated with dilute serial suspensions, they represented the cultivatable organisms that were enriched as a result of the As(III) or As(V) treatments. As with any cultivation method, the microorganisms selected under specific enrichment conditions, in this case on R2A media, do not necessarily represent all the populations that were important in the soil columns. Of the 24 bacteria isolated from plates, 10 were capable of oxidizing As(III) and 10 were capable of reducing As(V). Four others grew poorly in the solution media and were therefore dropped from the experiment. Both the As(III)-oxidizing and the As(V)-reducing isolates were obtained from columns that received either As(III) or As(V), indicating that As oxidizers or reducers were not limited to a specific treatment (Table 1). The fact that both oxidizers and reducers were obtained from the same treatments suggests that As oxidation and reduction may have been occurring simultaneously. These organisms were identified by near-full-length sequencing of their 16S rRNA genes, revealing that a total of three different As(III)-oxidizing populations and five different As(V)-reducing populations were represented by these isolates (Table 1). BLAST searches showed that the closest matches to the three As(III) oxidizers were *P. fluorescens*, *V. paradoxus*, and *A. tumefaciens*. The closest GenBank matches to the five As(V)-reducing isolates were *Flavobacterium heparinum*, *A. tumefaciens*, *Microbacterium* sp., *Arthrobacter aurescens*, and *Arthrobacter* sp. The

TABLE 1. Closest GenBank Neighbors, Sequence Similarities, and As-Transforming Characteristics of Isolates Cultivated from Unsaturated Soil Columns[a]

isolate or DGGE band no.	isolate accession no.	closest GenBank neighbor (% similarity)[b]	DNA sequences detected in soil columns[a,c]	column treatment from which isolates were obtained[a]		As(III) oxidation/ As(V) reduction parameters	
				As(III)	As(V)	rate[d] (μmol d^{-1} 10^{-9} cell)	half-life[e] (d)
		As(III)-Oxidizing Isolates					
1	AF388028	*Variovorax paradoxus* (99.3)	yes	√		1.7	0.5
3	AF388027	*Pseudomonas fluorescens* (99.1)	yes	√	√	1.4	0.2
5A	AF388033	*Agrobacterium tumefaciens* (99.9)	yes		√	0.7	0.3
		As(V)-Reducing Isolates					
2	AF388029	*Flavobacterium heparinum* (94.9)	no	√		0.5	0.6
4	AF388031	*Microbacterium* sp. (98.1)	no		√	2.4	0.2
5B	AF388030	*Agrobacterium tumefaciens* (99.9)	yes	√	√	3.3	0.1
6	AF388032	*Arthrobacter aurescens* (99.6)	no	√	√	2.9	0.1
7	AF388034	*Arthrobacter* sp. (97.8)	no		√	1.6	0.2

[a] Isolates were obtained from treatments indicated with checkmark, and detection of their 16S rDNA sequences in soil columns is indicated. [b] Closest GenBank neighbors and similarities of near full-length 16S rDNA sequences of isolates were determined using BLAST (*38*). [c] 16S rDNA sequences in columns were analyzed by purifying and sequencing DGGE bands derived from PCR amplification of DNA extracts. [d] Rates of As(III) oxidation and As(V) reduction demonstrated by isolates were measured during logarithmic growth under aerated serum bottle conditions. Rates normalized to cell number using an empirically developed relationship between cell enumeration with phase contrast microscopy and optical density (OD) measurements (A_{500}) of cell suspensions. [e] Apparent half-lives ($t_{1/2}$) are dependent on experimental conditions and are estimated based on rates assuming psuedo-first-order reaction dependent on As(III) (oxidizing isolates) or As(V) (reducing isolates).

Pseudomonas and *Agrobacterium* genera contain members that were previously shown to have As-transforming capabilities (*11*, *21*). Interestingly, the two *A. tumefaciens* isolates that exhibited opposite As redox phenotype have identical 16S rDNA sequences (across ~1400 bp). Further discussion regarding these two strains follows below. The similarity in colony morphology between the *A. tumefaciens* isolates as well as among other isolates prevented attempts to enumerate specific populations from either column based on colony forming units (cfu).

Correlating Isolates with Column Populations. Sequences of 16S rDNA fragments amplified from nucleic acid extracts of column samples were compared to sequences of As-transforming isolates described above. These comparisons showed that three DGGE bands present in the column treatments represented 16S rDNA sequences that were 100% identical to the *V. paradoxus* (band 1), *P. fluorescens* (band 3), and *A. tumefaciens* (band 5)-like isolates (Figure 2), all of which were As(III) oxidizers. However, with exception of the *A. tumefaciens* band (no. 5), no DGGE bands corresponding to other As(V)-reducing isolates were observed in DGGE profiles. The direct identification of As(III)-oxidizing populations in the column environments using molecular methods was consistent with the microbial oxidation of As(III) observed during solute transport. However, the fact that two strains of *A. tumefaciens* [an As(III) oxidizer and an As(V) reducer] could not be differentiated by DGGE and that other As(V) reducers were isolated from these columns preclude a conclusion that only As(III)-oxidizing microorganisms were relevant to the net As redox output observed in these columns. Template bias (*43*) may have reduced the sensitivity of the molecular approach and may explain the lack of detection of As(V)-reducing populations using PCR-DGGE. Furthermore, detection of the As(V)-reducing populations using DGGE was potentially limited by the addition of ~100 ng of total DNA per lane; it is possible that higher quantities of DNA may have allowed detection of these bands, although overall band resolution may have suffered. Regardless of the actual population size of As(V)-reducing organisms in the soil columns, the combined metabolic activity of the As(V) reducers failed to dominate the net As redox activity in the column communities.

Rates of As Transformation by Bacteria. Rates of As(III) oxidation or As(V) reduction by each of the eight isolates cultivated from the column environments were measured in continuously aerated serum bottles (Figure 3). Rates of As(III) oxidation during logarithmic growth of the three As(III)-oxidizing isolates varied from 0.7 to 1.7 μmol d^{-1} per 10^9 cell with corresponding As(III) half-lives ranging from 0.2 to 0.5 d (Table 1). In comparison, rates of As(V) reduction by the five As(V)-reducing isolates ranged from 0.5 to 3.3 μmol d^{-1} per 10^9 cell (half-lives of 0.1−0.6 d; Table 1). Assuming that As(III) oxidation was the only transformation process in the soil column experiments, the number of As(III)-oxidizing organisms required to support the column-derived As(III) oxidation rates was estimated to range from 1 to 3 × 10^8 cells/g of soil, based on the As(III) oxidation rates obtained for each of the As(III)-oxidizing isolates (Table 1). Although this estimate assumes that the physiological status of the isolates was similar in pure culture to that under column conditions, it suggests that either of the three oxidizing isolates would be capable of supporting the oxidation of As(III) observed in the soil columns, either acting individually or in concert.

Mechanisms of As Oxidation/Reduction. No oxidation of As(III) was observed in sterile controls or in experiments using cell-free filtrate taken from isolate suspensions that were actively oxidizing As(III), indicating that As(III) oxidation required the presence of microbial cells. In addition, none of the As(III)-oxidizing isolates grew in media designed for chemolithotrophic metabolism using As(III) as the sole electron donor [same medium as used during column enrichment except glucose was replaced by CO_2 and As(III) was increased to provide adequate energy source]. This result taken together with the fact that As(III) oxidation profiles for each isolate corresponded with microbial growth (Figure 3) suggests that the mechanism of As(III) oxidation by these isolates was related to As detoxification rather than energy generation. Likewise, none of the As(V)-reducing isolates could grow in media designed for dissimilatory As(V) reduction, where lactate served as the primary C and energy source and As(V) was present as the primary electron acceptor. This result was not surprising considering that the isolated organisms were enriched under aerobic conditions. Although these findings must be tempered with the possibility

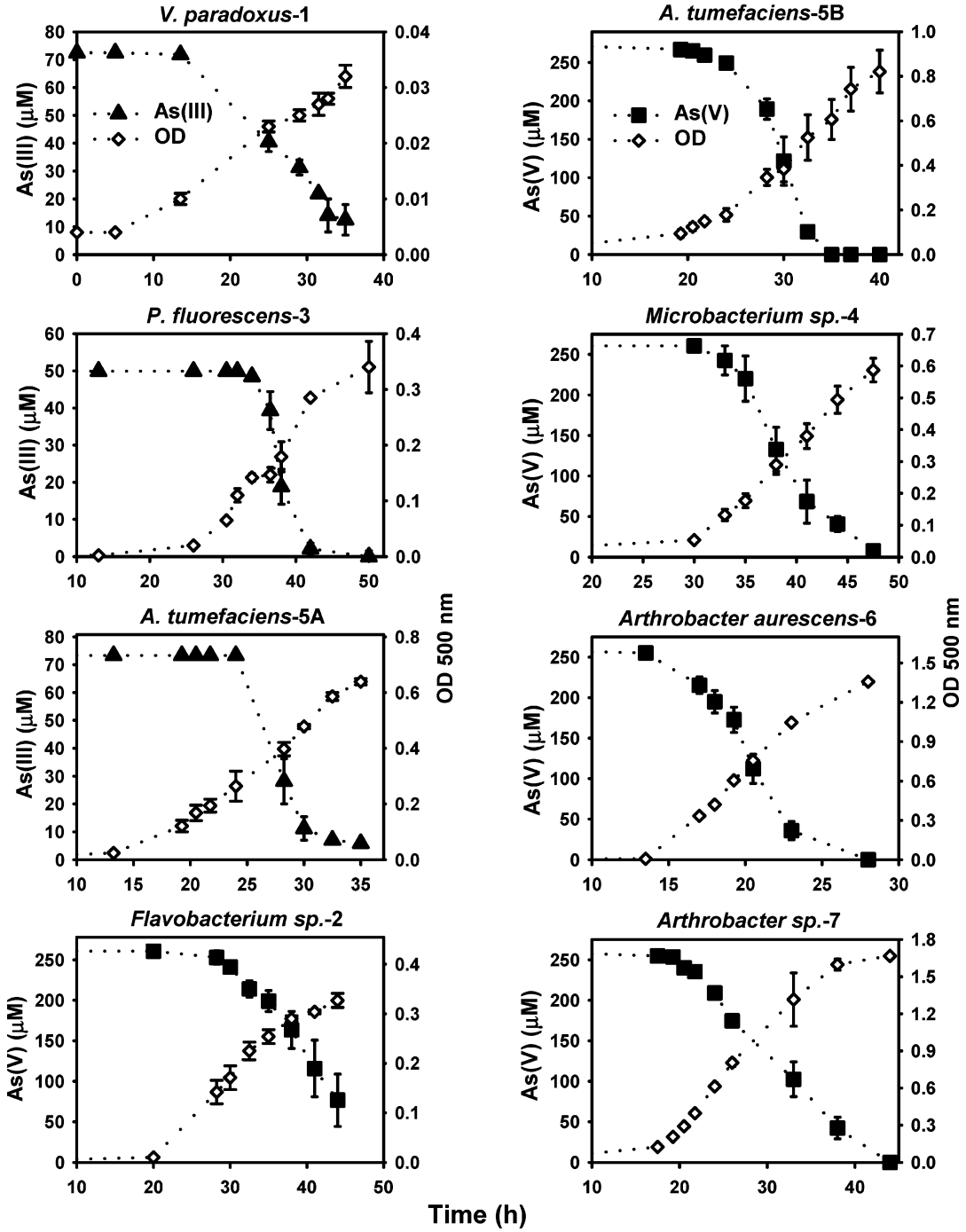

FIGURE 3. Microbial biomass (as OD 500 nm, ◇) and As(III) (▲) or As(V) (■) concentrations as a function of time for three As(III)-oxidizing isolates and five As(V)-reducing isolates incubated under aerated serum bottle conditions. Total concentrations of As in solution ([total As] = [As(III)] + [As(V)]) remained nearly constant during each experiment. Isolates are named after their nearest match found in the GenBank Database. Error bars represent standard errors of three replicate experiments for *P. fluorescens* and two replicate experiments for all other isolates. [Preliminary results for *P. fluorescens* were presented in a review chapter (*39*) based on results of Macur et al. (*40*)].

that some or even all of these bacteria may utilize As for energy metabolism in different media, under the conditions tested here, none of these organisms demonstrated energy-conserving reactions with As. Thus, all of the bacteria isolated from the As-supplemented columns demonstrated the capacity to either oxidize As(III) or reduce As(V), apparently for detoxification purposes.

Hybridization experiments using *arsC* probes derived from *P. aeruginosa*, *Geobacillus*, *E. coli*, and *A. tumefaciens* for dot blots of DNA obtained from the soil isolates revealed significant homology between only the *A. tumefaciens arsC* probe and the DNA from the *A. tumefaciens* isolates (Figure 4A). To characterize the apparent *arsC* homologues in these *A. tumefaciens* isolates, the homologues were PCR amplified and sequenced (GenBank Accession Nos.: AY286230 for isolate 5B, AY286231 for isolate 5A). The nucleotide sequences of these putative *arsC* genes (375 nucleotides) were 99.2% identical to each other, and their inferred amino acid sequences were 80% identical and 88% similar to the annotated *arsC* in *A. tumefaciens* (across 124 amino acids). Amino acid alignments (Figure 4B) showed that the cloned *A. tumefaciens arsC* genes shared extensive homology with the well-characterized *arsC* from *E. coli*, including the highly conserved amino acids Cys12, Ser15, Arg60, Arg94, and Arg107

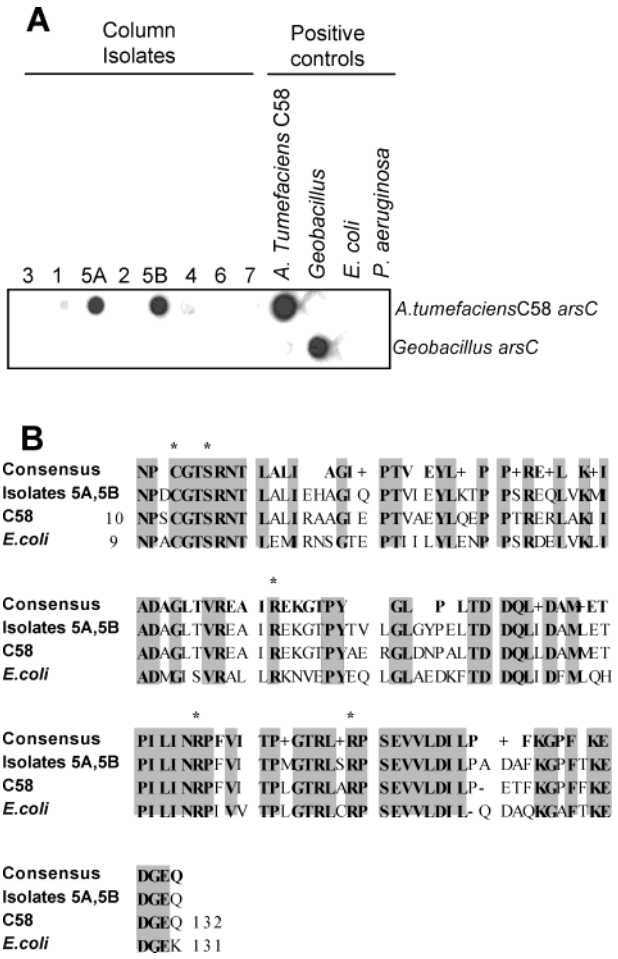

FIGURE 4. (A) Dot blot hybridizations of total DNA from the eight column isolates probed with selected *arsC* genes (*A. tumefaciens* strain C58, *Geobacillus*, *E. coli*, *P. aeruginosa*). Positive hybridizations are shown for the *A. tumefaciens* soil column isolates (5A and 5B) probed with the *A. tumefaciens* strain C58 *arsC* gene. Results with the *Geobacillus arsC* gene probe are shown as an example of the negative hybridizations obtained for all other isolate−probe combinations but which are not shown for brevity. (B) Inferred amino acid alignments of the *arsC* homologue PCR cloned from the *A. tumefaciens* soil column isolates (5A and 5B) as compared to the *arsC* gene from *A. tumefaciens* strain C58 and the *arsC* gene from *E. coli* p773. The consensus sequence denotes positions of homology between the *A. tumefaciens arsC* genes; the amino acids in bold and gray boxes show positions of conservation across all three *arsC* genes, and asterisks (*) note conservation of amino acids experimentally shown to be essential for enzyme function (*44*).

(*E. coli* p773 *arsC* numbering) found to be essential for activity in several ArsCs (*44*). Phylogenetic analysis of the cloned partial *arsC* genes unambiguously placed both as sharing a common node with the *A. tumefaciens* strain C58 *arsC* and separate from the other closely related *arsC* genes (Figure 5).

No other soil isolates obtained in this study hybridized to any of the four *arsC* probes under low stringency wash conditions (65 °C, 2× SSC; data not shown). Conversely, positive controls using total DNA extracts from *A. tumefaciens*, *P. aeruginosa*, *Geobacillus*, and *E. coli* produced strong hybridization signals with their respective probes. The failure of these *arsC* probes to hybridize with DNA from the soil isolates is consistent with the enormous diversity of known *arsC* sequences (e.g., refs *9* and *23*−*27*; Figure 5) and the fact that this diversity may preclude detection of *arsC* homologues using probes designed from phylogenetically different organisms.

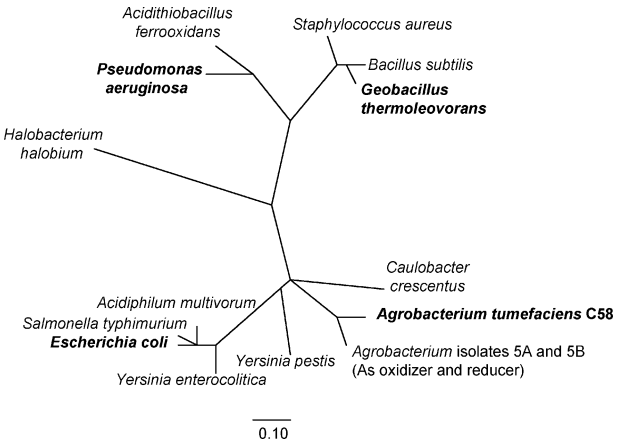

FIGURE 5. Unrooted phylogenetic tree showing relationships among *arsC* genes obtained from GenBank and from isolates cultivated in this study. The tree was constructed using the neighbor joining method in the ARB software package. Species names in bold text indicate organisms from which *arsC* genes were PCR amplified and used as probes for hybridization experiments. Bar represents 0.1 changes per nucleotide.

Interestingly, both of the *A. tumefaciens* isolates were shown to contain a putative *arsC*, despite the fact that isolate 5A did not exhibit As(V) reduction. The apparent lack of As(V) reduction in the As(III)-oxidizing *A. tumefaciens* isolate may be due to a variety of reasons including point mutations in *arsC* or separation of *arsC* from its As derepressible promoter. Given that the two *arsC* genes cloned from the different *A. tumefaciens* isolates shared 100% amino acid identity across that portion of the gene amplified and sequenced in this study, any putative mutation would have to be external to this part of the *arsC* coding region. An alternative explanation may be that the *arsC* gene in the As(III)-oxidizing isolate became separated from an As derepressible promoter due to a genomic rearrangement event that are now known to be common in bacteria (*45*, *46*), including the Rhizobiaceae (*47*, *48*). It is also possible that the opposite As redox activity of the two *A. tumefaciens* strains is due to the presence, absence, or differential expression of an As(III) oxidase gene. The As(V)-reducing strain either may lack the genes required for As(III) oxidation or may not have significantly expressed these genes under the conditions studied herein. Studies are currently under way to examine the genetic differences between these *A. tumefaciens* strains and to assess whether mutation, lateral gene transfer events, or other factors may explain the different As phenotypes.

Implications for As Cycling in Soils. Eight heterotrophic, aerobic As-transforming bacteria representing diverse genera in the Gram-positive, flavobacteria, and proteobacteria kingdoms were isolated from soil column enrichments and characterized. None of the five As(V)-reducing isolates grew on media designed for dissimilatory As(V) reduction, and the three As(III)-oxidizing isolates did not grow in media with As(III) as the primary electron donor for chemolithotrophic metabolism. On the basis of the reported mechanisms of As oxidation−reduction activity among microorganisms (*44*, *49*), these results suggest that both the As(V)-reducing and As(III)-oxidizing isolates were transforming As via detoxification mechanisms as opposed to energy generation. Although we were able to cultivate both As(V)-reducing and As(III)-oxidizing bacteria from the column environments, As(III)-oxidizing populations apparently dominated the observed net As redox activity and represented the primary 16S rDNA sequences that were detected in column samples using molecular methods.

The coexistence of both As(III)-oxidizing and As(V)-reducing aerobic populations in the same soil suggests that

the relative numerical and or metabolic dominance of these populations will influence the predominant As valence state. The isolation of both As(III)-oxidizing and As(V)-reducing *A. tumefaciens* strains from the same column enrichment (Table 1) shows that the ability to either oxidize As(III) or reduce As(V) is variable even among strains that proliferate under the same environmental conditions. Because of such variation in phenotype within similar organisms, phylogenetic identification of microorganisms based on 16S rRNA sequence analysis is not sufficient to predict the As-transforming capabilities of specific bacterial populations. Further, As contamination in oxic environments may not select for microorganisms capable of utilizing As in energy metabolism but rather may shift the microbial community structure to favor organisms capable of detoxification either via As oxidation or reduction. Specifically, the broad phylogenetic distribution of *arsC* genes suggests that the importance of nondissimilatory As(V) reduction may be underestimated as a mechanism of As redox cycling in natural systems. Results from the current study as well as other recent examples (*17*, *18*) support the hypothesis that the oxidization and reduction of As occurs in phylogenetically diverse soil bacteria via mechanisms that are not directly associated with respiration or chemolithotrophic metabolism.

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