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Response of Sediment Bacterial Assemblages To Selenate and Acetate Amendments

FRANÇOISE S. LUCAS AND JAMES T. HOLLIBAUGH*

Department of Marine Sciences, University of Georgia, Athens, Georgia 30602-3636

We followed the response of bacterial assemblages in slurries of estuarine sediments to amendments of 100 μM sodium selenate and 10 mM sodium acetate. Selenate was removed from the dissolved phase in all slurries after an initial lag that varied depending on the source of the sediment used in the slurry. Subsequent selenate amendments were removed without a lag but with the appearance of transient peaks of selenite. We documented changes in the composition of bacterial assemblages in the slurries using PCR/DGGE and RT-PCR/DGGE of 16S rDNA and rRNA. Bands of interest were sequenced to identify organisms responding to selenate amendments. The composition of communities from the two sampling sites was different and changed but did not converge during incubations with selenate. Selenate-reducing assemblages had broad phylogenetic diversity. Most bands were related to groups of bacteria known to contain organisms capable of selenate or selenite reduction, except for *Acinetobacter* species which dominated one of the samples and has not previously been associated with selenium oxyanion reduction.

Introduction

In estuarine wetlands, selenium concentrations rarely exceed 1 μg/L in water and 2 mg/kg in sediment, except when there are anthropogenic sources (agricultural or industrial discharges) of Se, such as in the Scheldt River (Belgium) or San Francisco Bay (*1, 2*). Se is a micronutrient essential for growth and survival (*3*); however, at higher concentrations it is toxic and thus constitutes a threat to the health of the environment (*4*).

Selenium exists in different oxidation states: selenate (+VI) and selenite (+IV), which are highly soluble in water; insoluble elemental selenium (0); and selenide and methylated forms (−II) (*5*). Se cycling in estuarine sediments is not well defined (*2*). However, several studies have shown that Se undergoes oxidation and reduction reactions mediated by microorganisms (*6*). This directly affects Se oxidation state and hence its chemical properties and behavior in the environment (*7*). Microbial transformations include reduction of selenium oxyanions (SeR) with subsequent precipitation of elemental Se^0 in the sediment. Further reduction to Se^{2-} and methylation result in volatile gaseous species such as dimethylselenide (*7*−*9*). Microbial reduction of selenate and selenite is widely observed in contaminated as well as pristine sediments (*10*). This process controls the fate of Se and could be a useful strategy for bioremediation (*1, 9, 11*).

* Corresponding author phone: (706)542-3016; fax: (706)542-5888; e-mail: aquadoc@uga.edu.

A wide variety of microorganisms (common laboratory cultures or isolates from various contaminated sites) have the ability to reduce Se oxyanions, either by assimilatory reduction or dissimilatory respiration (*1, 12*−*14*). The reduction of Se oxyanions to Se^0, followed by further reduction and methylation to volatile methylated forms (dimethylselenide or dimethyldiselenide), may provide a detoxification system and does not support growth (*1, 7, 9*). Oremland et al. (*8*) demonstrated that heterotrophic bacteria were capable of dissimilatory selenate reduction (DSeR) in anaerobic sediments. Dissimilatory selenate reducing bacteria are ubiquitous; however, they are difficult to culture (*1*). So far, only a few isolates from extreme or contaminated environments are known to oxidize organic matter anaerobically using selenate as an electron acceptor: *Thauera selenatis* (*15*); *Sulfurospirillum barnesii* SES-3 (*10*); *Aeromonas hydrophila* (*16*); *Bacillus arsenicoselenatis* E1H (*17*). Only one strain has been isolated from an estuarine environment, an anaerobic Gram negative coccus (strain SES-1), which has been lost (*8*). In addition, several other DSeR isolates are in the process of being characterized (*16, 18*).

The ecological relevance of selenate reduction and the distribution of these strains or of other Se (VI) reducers is unknown, particularly in estuarine environments. Several studies using molecular techniques demonstrated that isolates often represent a negligible fraction of natural communities (*19*). Therefore, our knowledge and understanding of the diversity and composition of bacterial communities in natural environments is very limited. However, molecular biological techniques offer new opportunities to analyze bacterial assemblages, eliminating the need to culture organisms in order to identify them (*19*). Knowing the diversity and composition of selenate-reducing bacterial communities may be important for understanding the influence of environmental factors on the response of ecosystems to Se contamination.

The goals of this research were to investigate the possibility of using molecular fingerprinting methods to follow the response of estuarine bacterial communities to selenate amendments and to identify new SeR bacteria in estuarine environments. We used molecular biological techniques (PCR/DGGE and RT-PCR/DGGE) to follow shifts in the composition of bacterial communities in sediment slurries amended with selenate. SeR activity was monitored by measuring selenate and selenite removal from the dissolved phase in order to guide sampling. We also sequenced bands of interest to obtain phylogenetic information on the organisms responding to selenate amendment.

Material and Methods

Sampling Site. Intertidal, surficial (1−4 cm deep) sediment samples and water from the adjacent river channel were collected in September 1998 from estuarine marshes of the Altamaha River (lat. 31°19′N, long. 81°24′W, Georgia, USA), at distances of 20 km (AL20) and 10 km (AL10) from the sea. Samples were refrigerated until preparation of sediment slurries. Characteristics of the sampling sites and of the slurries are reported in Table 1.

Sediment Slurries. Sediment was homogenized with river water (1:4 w/w) under N_2 and amended with sodium acetate to a final concentration of 10 mM. Sodium selenate was added to a final concentration of 100 μM (slurry "S"), while control slurries (slurry "C") did not receive sodium selenate. The slurries were dispensed (100 mL) in 150 mL serum bottles, flushed with N_2 and incubated under an N_2 atmosphere at 24 °C with rotary shaking at 200 rpm. We did not replicate

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TABLE 1. Characteristics of the Sampling Sites and of Sediments and Water Samples[a]

characteristic	AL10	AL20
km to the sea	10	20
predominant vegetation	*Spartina alterniflora* *Spartina cynosuroides*	*Spartina cynosuroides* *Cladium jamaicense* *Juncus roemerianus*
water temperature (°C)	30.4	31.4
water salinity (‰)	5.6	0.5
water pH	6.6	6.6
water As	ND	ND
water Fe	0.1	0.9
water Mn	ND	3.9
water W	ND	ND
sediment C (mg.g^{-1} DW)	22.8	54.1
sediment N (mg.g^{-1} DW)	2.1	4.8
sediment C/N ratio (g:g)	10.9	11.3
sediment Se (μg.g^{-1} DW)	ND	ND
dissolved Se (μM) in the control slurry	ND	ND
slurry salinity ‰	5.9	0.0
slurry pH	7.5	7.1
slurry As	85.0	72.9
slurry Fe	*4.7	*4.4
slurry Mn	15.0	225.4
slurry W	1.4	1.4

[a] Sediment and slurry properties are expressed in dry weight (DW). Selenium concentration in sediments and dissolved phase of control slurries were at the limit of detection by the methods used. Concentrations of other elements are expressed in mass fraction in slurries (mg/kg DW) and in dissolved phase water (mg/l), unless noted by an asterisk for %. Values below the limit of detection are noted ND for not detected.

treatments because the time course experiments would have produced too many samples to process, but previous work showed good reproducibility (data not shown). Subsamples (2 mL) were periodically withdrawn and centrifuged. Selenate and selenite concentrations in the supernatant were determined, and pellets were stored frozen (−20 °C) for nucleic acid extraction. Once selenate concentrations had begun to decline, the slurries were amended a second time with the same amounts of selenate and acetate.

Chemical Analyses. Sediment organic carbon and nitrogen were measured using a CHN analyzer after treatment with diluted HCl (pH∼2.0) to remove carbonate. The concentrations of trace and major elements in the dissolved phase and in sediments (EPA method 3050) were measured by ICP (Thermo-Jarrell Ash 965, Atom Corp.). CHN and ICP measurements were made by the Chemical Analysis Lab of the University of Georgia, USA.

Dissolved inorganic selenite and selenate were measured according to the fluorimetric method of Rodriguez et al. (*20*), slightly modified. For selenite measurements, 1 mL of sample was mixed with 2 mL of 0.04 M EDTA-Na_2 and 1 mL of 0.1% 2,3-diaminonaphthalene (DAN). Sample pH was adjusted to 1.8 before incubation at 60 °C for 15 min. After cooling in ice, the piazselenol (fluorescent Se-DAN complex) was extracted at room temperature with 3 mL of cyclohexane. The fluorescence of the piazselenol extract was measured at an excitation wavelength of 377 nm and an emission wavelength of 516 nm (Shimadzu RF 5000U spectrofluorometer).

For selenate measurements, Se(VI) was first reduced to Se(IV) by adding 1 mL of a solution containing 2 M hydrobromic acid and 2.5 M perchloric acid to 1 mL of sample. After heating at 150 °C for 5 min, the samples were cooled and treated as for selenite measurements. Selenate concentrations were determined by subtracting selenite concentrations. Linear regressions of selenate concentration vs time were used to estimate the lag phase for selenate removal from sediment slurries.

Nucleic Acid Extraction. Nucleic acids were extracted from sediment (0.3 to 0.4 g DW) according to Créach et al. (*21*). Crude extracts were purified by high salt ethidium bromide treatment and phenol-chloroform extraction (*22*), followed by ultrafiltration through Centricon 100 (Amicon) spin filters.

Reverse Transcription (RT). Nucleic acid extracts were divided into two aliquots. DNA was removed from one of them by treatment with DNase, as described by Teske et al. (*23*). The DNase was removed by phenol-chloroform extraction. Reverse transcription of RNA to complementary DNA (cDNA) was performed according to Teske et al. (*23*). In addition, control reactions (without reverse transcriptase) were run to check for DNA contamination of RNA samples.

PCR/DGGE. The variable region V3 of the 16S rRNA gene was amplified with primer sequences complementary to positions 341 to 358 (primer 358f, Eubacterial) and positions 517 to 534 (primer 517r, universal) corresponding to *Escherichia coli* 16S rRNA numbering (*24*). The 358f primer possessed a 40 bp GC clamp (*25*), and the 517r primer was labeled at the 5′-end with fluorescein. DNA, cDNA samples and RT-controls were amplified under PCR conditions similar to those used by Ferrari and Hollibaugh (*26*). Denaturing Gradient Gel Electrophoresis (DGGE) was performed according to Ferrari and Hollibaugh (*26*), using a CBS Scientific DGGE system. After electrophoresis, band positions were determined by measuring fluorescein fluorescence using a Hitachi FMBIO II gel scanner (505 nm filter). The gel image was then processed using image analysis software (Molecular Analyst version 1.12, Bio-Rad). Band patterns of samples were compared by cluster analysis (Jaccard index (*27*), UPGMA method).

Sequencing. Bands of interest and containing sufficient quantities of DNA were excised from the gel and frozen at −20 °C for 1 h. DNA was eluted in 100 μL of EP1 buffer (0.5 M sodium acetate, 1 mM $MgCl_2$, 0.1 mM EDTA, 0.1% SDS, pH 7.7) at 37 °C for 3 h and then purified using Qiaquick PCR purification kits (Qiagen). DNA was amplified by PCR using ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems) and the 517r primer. An ABI PRISM 377 sequencer (PE Applied Biosystems) was used for sequencing. With the exception of Band 1 (AL20b1), which yielded a poor quality sequence, all band sequences

TABLE 2. Name and Accession Number of the Band Sequences and Similarity to Related Organisms

band	accession no.	% similarity	related species	base pair difference
AL20b2	AF312786	100.0	*A. junii* DSM 1532	0/152
AL20b3	AF312788	100.0	*A. junii* DSM 1532	0/152
AL20b4	AF312783	99.2	*A.nitrofigilis* CCUG 15893	1/125
AL20b5	AF312784	93.0	*G. algens* ACAM 551	9/134
AL20b6	AF312777	96.7	*P. putida* NCIMB 9816	5/146
AL20b7	AF312785	97.4	*A. baumannii* DSM 30008	9/152
AL20b8	AF312787	98.7	*A.haemolyticus* DSM 6962	4/152
AL20b9	AF312789	98.0	*A.haemolyticus* DSM 6962	5/152
AL20b10	AF312792	97.4	*A.haemolyticus* DSM 6962	5/152
AL20b11	AF312790	98.0	*A.haemolyticus* DSM 6962	5/152
AL20b12	AF312791	97.4	*A.haemolyticus* DSM 6962	5/152
AL20b13	AF312780	93.0	*Polaribacter sp.* MED18	9/134
AL20b14	AF312781	100.0	*E. coli* ATCC4 3895	0/125
AL20b15	AF312782	96.0	*P. putida* NCIMB 9816	7/146
AL20b16	AF312779	100.0	*E. coli* ATCC 43895	0/125
AL20b17	AF312778	94.0	*P. putida* NCIMB 9816	8/146

TABLE 3. Name and Accession Numbers of the Related Organisms

organisms	accession no.
Acinetobacter anitratus ATCC 15308	U10874
Acinetobacter baumannii DSM 30008	X81667
Acinetobacter calcoaceticus DSM 30009	X81668
Acinetobacter haemolyticus DSM 6962	X81662
Acinetobacter johnsonii DSM 6963	AJ247202
Acinetobacter junii DSM 1532	X81658
Acinetobacter lwoffii DSM 2403	X81665
Acinetobacter venetianus B1/II	U71006
Arcobacter butzleri 28197	U34388
Arcobacter cryoaerophilus 28198	U34387
Arcobacter nitrofigilis CCUG 15893	L14687
Arcobacter skirrowii CCUG 10374	L14625
Cytophagal clone MD9	AF025552
Enterobacter gergoviae JCM 1234	AB004748
Erwinia chrysanthemi ATCC 11663	U80200
Escherichia coli ATCC 43895	Z83205
Estuarine clone CRE-PA44	AF141524
Flavobacterium aquatile ATCC 11947	M62797
Flavobacterium psychrophilum ATCC 49418	AF090991
Gelidibacter algens ACAM 551	U62916
Marine clone MBE7	AF191758
Polaribacter sp. MED18	AF025561
Pseudomonas alcaligenes IAM 12411	D84006
Pseudomonas azotoformans ANT3	AF025573
Pseudomonas fulva IAM 1529	D84015
Pseudomonas gessardii CIP 105469	AF074384
Pseudomonas monteilii CIP 104883T	AB021409
Pseudomonas pseudoalcaligenes JCM 5968T	AB021379
Pseudomonas putida NCIMB 9816	D86000
Pseudomonas stutzeri ATCC1 7682	AJ006107
Serratia marcescens 615	AF076038
Shigella disenteriae ATCC 13313	X96966
Shigella flexneri ATCC 29903	X96963
Sulfurospirillum arsenophilus MIT-13	U85964
Sulfurospirillum barnesii SES-3	U41564
Thiomicrospira denitrificans DSM 1251	L40808

were submitted to Genbank (accession numbers are listed in Table 2).

Sequences were aligned and compared using the Genetics Computer Group Inc. package (Madison, WI). Related organisms (name and accession numbers) used for phylogenetic analysis are listed in Table 3. Phylogenetic trees were inferred from Jukes-Cantor distances using the neighbor-joining method (PHYLIP 3.572 (*28*)); the branching pattern was checked by 100 bootstrap replicates. To ensure reliable phylogenetic positioning, a complete sequence (at least 400 bp) is desirable; however, it is possible to use partial sequences to identify organisms or to assign them to well-established phylogenetic groups, as long as the database contains sequences of close relatives (*29*).

Results and Discussion

The relatively undisturbed Altamaha is the largest river of the Georgia coast. It originates in the Piedmont region and has a high carbonate content and relatively high pH values (*30*). None of the samples we collected contained abnormal levels of selenium (Table 1), which suggests that no selection for selenate reducing bacteria should have occurred before adding selenate.

Selenate Reduction in the Slurries. Selenate was removed from the dissolved phase under anaerobic conditions in AL20 and AL10 slurries (Figure 1A). We attribute this to reduction of Se (VI) to insoluble Se^0 by a microbial population adapted to reduce SeO_4^{2-} anaerobically, for the following reasons. First, selenate is weakly adsorbed by soil or sediment particles (*31*). Second, chemical precipitation and reduction of selenate is unlikely in these neutral sediments (Table 1), since selenate coprecipitates with aluminum or iron oxides only at low pH *(32),* and chemical reduction of selenate is a slow process requiring acidic conditions and high temperature (*33*). Benson (*33*) reports that the reduction of selenate by H_2S was not observed over a period of days at 100 °C, and according to White and Dubrovsky (*11*) Fe^{2+} and Mn^{2+} do not directly reduce SeO_4^{2-}. Se(VI) reduction by "green rust" has been demonstrated in laboratory (*34*), but it is probably not a significant process in the sediment (*35*).

Third, several previous studies demonstrated that SeO_4^{2-} reduction (SeR) in sediment is a direct result of biological activity and is not due to reaction with reductants (like sulfide) produced by bacteria (*8, 13, 36*−*38*). Moreover, in our experiment the second selenate addition was removed immediately (no lag phase in Figure 1A), strongly suggesting that selenate removal from the dissolved phase is due to Se(VI) reduction by an adapted microbial community.

Fourth, we observed strong qualitative evidence (garlic-like odor) of the formation of volatile reduced alkylated Se compounds (*9, 35, 39*) in both of the selenate-amended slurries. Methylation of Se compounds follows assimilatory reduction of selenite and allows microorganims to detoxify their environment (*1, 7, 9*). Thus, we conclude the Se(VI) removal in the slurries was performed by bacterial reduction.

Differences in Lag Time. It seems that the capacity for microbial selenate reduction is a common feature of a diversity of sediments in nature (*37*). However, Figure 1A shows that selenate reduction was initiated more rapidly in AL20 (22 h lag-phase) than AL10 (122 h lag phase). Several

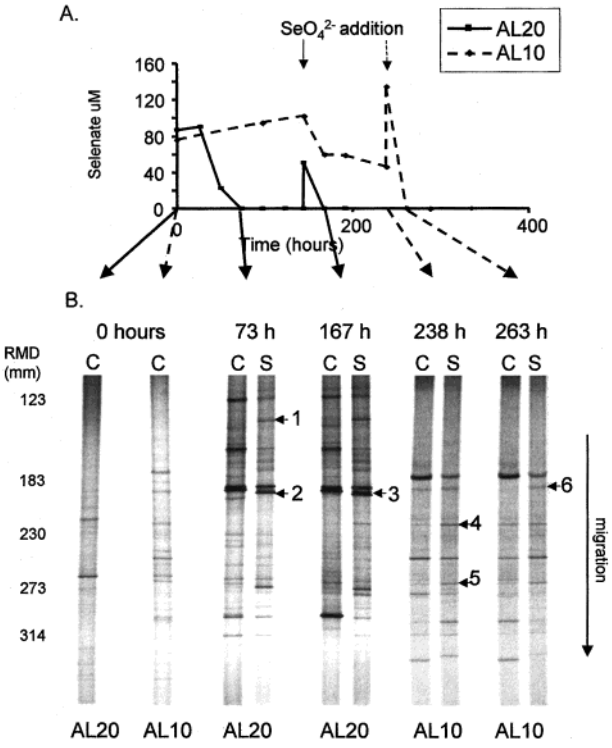

FIGURE 1. A. Dissolved selenate concentration (μM) in sediment slurries from stations AL20 and AL10. B. 16S rDNA DGGE profiles from control (C) and selenate-amended (S) slurries. Sequenced bands are numbered from 1 to 6. RMD is the relative migration distance (mm) from loading wells; gels were normalized using DNA standards.

factors may affect the lag prior to SeR and subsequent selenate reduction rates. Environmental factors (inhibiting DSeR) may play an important role (*12*). It has been demonstrated previously that salinity does not correlate with selenate reduction potential (*37*).

Nitrate or other competitors (group VI oxyanions) have an inhibitory effect on SeR when they are present at equimolar levels; however, the severity of inhibition may fluctuate with environmental conditions (*7*). Sulfate and FeOOH have no effect on SeR, while nitrate and manganese oxides are inhibitory (*13*). The physiological and enzymatic aspects of nitrate competition have been studied recently by Oremland et al. (*38*). Concentrations of potential competing elements (Cr, Mn, As, W) in AL10 and AL20 slurries were far lower than the 18.9 mg of selenate/kg we added (Table 1). Normal levels of nitrate at these stations (around 10 μM in AL10 and 20 μM in AL20 waters, Georgia River LMER data) are 5 to 10 times lower than the 100 μM of selenate we added to the slurries. Thus, it is unlikely that differences in the concentrations of these compounds explain the difference in the lags.

One possible explanation for the longer lag in AL10 slurries is that sulfate reducers may have used all of the acetate before the growth of SeR bacteria. Lag time differences may also be explained by the higher concentrations of N and C in AL20 (Table 1), favoring the growth and metabolic activity of bacteria in this slurry. Moreover, the presence of species adapted to use acetate and/or selenate such as denitrifiers (*39*) can explain the smaller lag time in AL20.

Selenate-Reducing Communities. SeR capacities of the sediment result from the activities of a number of distinct microorganisms, competing or acting in synergy (*1*). For example, *B. arsenicoselenatis* and *B. selenitireducens* sequentially reduce selenate to elemental selenium when cocultured (*17*). Besides DSeR bacteria, other types of anaerobes have been shown to reduce sub mM concentrations of Se(VI) to Se(0), without respiration or growth: *Wolinella succinogenes (40), Desulfovibrio desulfuricans* (*41*), specific strains of *Pseudomonas stutzeri* (*42*), and *Enterobacter cloacae* (*43*). To analyze the structure of SeR assemblages, DGGE profiles of 16S rDNA were compared: in the initial slurries (0 h); in the control and selenate slurries at 73 and 167 h for AL20; or 238 and 263 h for AL10 (Figure 1B).

AL10 and AL20 slurries presented significantly different bacterial communities (Figure 1B) with only 23% similarity between the banding patterns at 0 h (Figure 2). Selenate-reducing assemblages and control communities (73AL20, 167AL20, 238AL10 and 263AL10) differed from these initial communities (14 to 36% similarity, Figure 2). Differences between the initial and subsequent samplings of control communities may result from adaptation to acetate as the major electron donor. We used acetate as an electron donor in these experiments following Oremland et al. (*8*). If we had used lactate or hydrogen we might have selected for other bacterial species. The addition of selenate also selected for selenate-adapted organisms, since DGGE profiles of control and selenate slurries were 48 to 60% similar (Figure 2).

The composition of AL10 and AL20 selenate-reducing communities did not converge during incubation with selenate (14% similarity between 73AL20S and 238AL10S). Bacterial communities of different taxonomic composition may have different metabolic potential (*44*), which may provide another explanation for the differences in lag phase. The original composition of the bacterial community and the length of time needed for species selection or metabolic adaptation (*45*) may also explain the differences in the lag and rate of selenate removal between AL10 and AL20.

The difference between 16S rRNA and rDNA profiles was greater for AL10 samples (only 18 to 35% similarity, Figure 2) than for AL20 samples (32 to 46% similarity). This suggests that the active part of the AL10 community was smaller and/or that growth rates were slower in AL10 than in AL20 slurries. Selenate reduction following the second amendment was as rapid and complete in AL10 as in AL20, suggesting that selenate-reducing bacteria were selected during the first addition of selenate. Indeed, no major changes in bacterial communities appeared following the second selenate addition (71% similarity for AL20, 75% for AL10, Figure 2).

Sequences of DNA Bands. To identify members of SeR communities, we sequenced significant 16S rDNA bands that were present in "S" slurries and not in "C" slurries. These bands are indicated on the gel images (Figures 1B and 2), numbered from 1 to 6. Phylogenetic affinity and accession numbers are given in Table 2. The sequences ranged from 125 to 152 bp in length (excluding primer sites).

In sample AL20, a consortium of *Acinetobacter* species (*γ-Proteobacteria*, Figure 3A) dominated the SeR assemblage. Bands AL20b2 and AL20b3 were both 100% identical to the sequence of *Acinetobacter junii* (Table 2). The Band 1 (AL20b1) sequence not only was of poor quality but also matched *Acinetobacter* species (Figure 3A). To our knowledge, SeR activity has never been demonstrated or studied in this genus before.

AL10 selenate-reducing communities had greater phylogenetic diversity, with sequences related to Cytophagales (*Flavobacteriaceae*, Figure 3B); *ϵ-Proteobacteria* (*Campylobacter* group, Figure 3C); and *γ-Proteobacteria* (*Pseudomonads*, Figure 3D).

The sequence of Band 4 (AL10b4) was 99.2% similar to *Arcobacter nitrofigilis* (Table 2), a microaerophilic nitrogen-fixing bacterium (*ϵ-Proteobacteria, Campylobacter* group). This strain has been isolated previously from *Spartina alterniflora* roots in Georgia salt marshes (*46*). *Sulfurospirillum barnesii*, an another member of the *Campylobacter* group, was isolated from a Se contaminated site and is capable of dissimilatory selenate respiration (*10*).

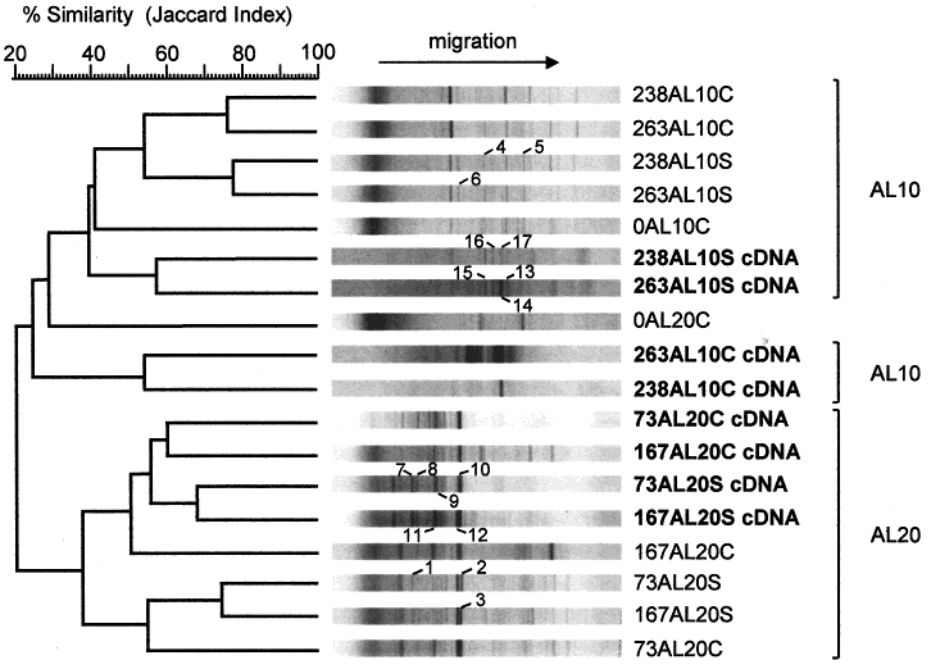

FIGURE 2. DNA and cDNA DGGE profiles and cluster analysis (Jaccard index, UPGMA) for AL20 and AL10 slurries. Sequenced bands are numbered from 1 to 17. Sample identifier numbers (for example 238AL10C) contain the duration of incubation in hours (238), the station (AL10) and the type of slurry (C for control, S for selenate-amended). cDNA sample identifiers are in boldface type.

Band AL10b5 was 99.2% similar to the sequence of Cytophagale isolate (MED9) from the northwest Mediterranean sea (Figure 3B). It was also 93.0% similar to the sequence of *Gelidibacter algens* (Table 2). *Flavobacteriaceae* strains have been shown to reduce selenite, and a *Flavobacterium* strain isolated from Kesterson Wildlife Refuge (CA) was able to reduce selenate (*47*).

The sequence of AL10b6 was 96.7% similar to the sequence of *Pseudomonas putida* NCIMB 9816 (Table 2). It was also similar to three other closely related *Pseudomonas* species (*P. azotoformans, P. fulva, P. gessardii*; Figure 3D). Other species of *Pseudomonas* (*P. fluorescens* and *P. stutzeri, 7, 42*) are reported to be able to reduce selenate; however, some confusion reigns in the identification of *Pseudomonas* isolates, and stock cultures of several *Pseudomonas* species do not reduce selenate (*48*).

In contrast to AL20 samples, bands sequenced from AL10 slurries were all related to phylogenetic groups containing known selenate-reducing bacteria: *Campylobacter* group, *Pseudomonads* and *Cytophagales*. Se reduction is widespread among various Eubacterial groups (*18*), but SeR is not present in all the members of these groups. The bands we sequenced appeared only in selenate slurries; as a consequence it seems reasonable to assume that they represent Se resistant or selenate-reducing bacteria.

Sequences of cDNA Bands. DGGE profiles of 16S rDNA do not allow us to distinguish between bacteria actively growing or metabolizing and dormant bacteria present in high numbers in the community. However, rRNA may indicate active bacteria, since a relationship between cell growth and rRNA content has been demonstrated (*49, 50*). DGGE coupled with RT-PCR will show only cells with high rRNA content that are thus likely to be actively growing (*51*). For this reason, we compared DNA and cDNA profiles for our samples (Figure 2).

Sequenced cDNA bands numbered from 7 to 17 are indicated in Figure 2, and phylogenetic affinities and accession numbers are given in Table 2. Sequencing confirmed the dominance of actively growing *Acinetobacter* species in AL20 slurries (Figure 3A and Table 2). Band 7 (AL20b7 cDNA) was 97.4% similar to the sequences of *Acinetobacter baumannii* and *A. anitratus*. Bands 8 to 12 were related to each other (99.3 to 100% similar) and were 97.4 to 98.7% similar to *Acinetobacter haemolyticus* (Figure 3A and Table 2).

AL10 cDNA bands were different from AL10 DNA bands (Figure 3 B, D; Table 2). Band AL10b13 was 93% similar to a cytophagale isolate *Polaribacter sp.* MED 18. AL10b15 and AL10b17 were respectively 96 and 94% similar to *P. putida*. None of the sequenced cDNA bands belonged to the *Campylobacter* group; however, AL10b14 and AL10b16 were 100% similar to enterobacterial species, such as *E. coli, S. flexneri, S. marcescens,* and *S. disenteriae* (Figure 3E). Several *Enterobacteria* have been shown to reduce selenate (*E. cloacae* (*1*)) or selenite as a detoxification mechanism (*E. coli* (*14*)).

Information obtained from cDNA sequences confirmed the presence and dominance of active *Acinetobacter* species in selenate-reducing assemblages in AL20. However, in AL10 the active bacteria, as indicated by cDNA profiles, differed from the dominant bacteria in DNA profiles and consisted of *Cytophagales, Pseudomonads,* and *Enterobacteria.*

Although studies of the role of SeR in Se biogeochemistry in estuarine systems have been conducted previously, there are few data on the types of microbes likely to be involved in this process or of factors that regulate selenate-reducing assemblages. Environmental factors may have an effect on the ability of a bacterial community to adapt to Se contamination and the capacity of an ecosystem to remove selenate. This study suggests that the composition of selenate-reducing assemblages and the interactions between bacterial species may also affect the rate of Se reduction. Assemblages presenting low diversity at the genus level such as AL20 (dominance of *Acinetobacter* species) showed high activity and adaptability potential.

This work compliments the more classical enrichment/isolation approach usually carried out in geomicrobiology. Diversity studies (using DGGE or other fingerprinting tools) may help us identify and isolate new and useful SeR bacterial species from natural systems. For example, the dominance of active *Acinetobacter* species in AL20 may focus research on the selenate-reduction capacity of isolates and strains belonging to this genus. However, phylogenetic analysis

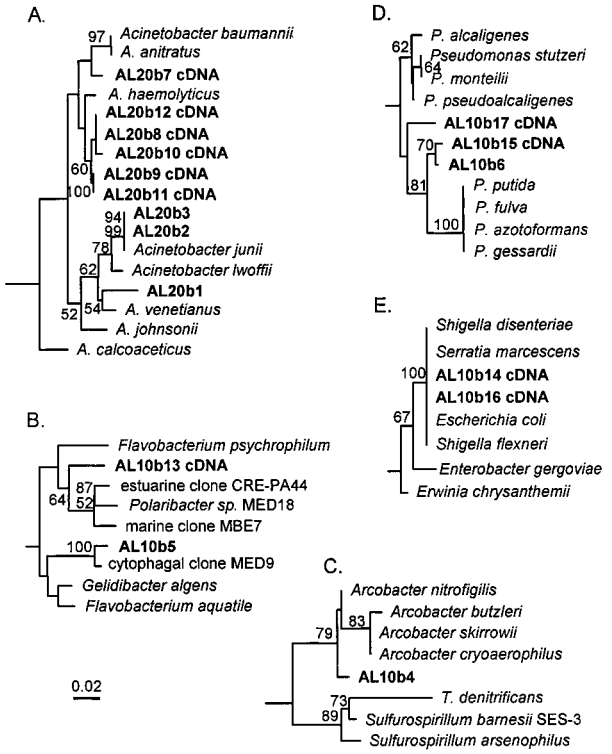

FIGURE 3. Phylogenetic relationships of the fragments sequenced from DGGE bands. Bands identifiers (for example AL20b1 cDNA) indicate the station (AL20), band number (b1 for Band 1) and the nature of the template (cDNA). The bar indicates a Jukes-Cantor distance of 0.02 and bootstrap values greater than 50% are indicated. The trees are unrooted with *Microcystis aeruginosa* as the outgroup. A. Phylogenetic position of Bands 1−3 and 7−12 among members of the genus *Acinetobacter* of the γ-*Proteobacteria*. B. Phylogenetic position of Bands 5 and 13 within the *Flavobacteriaceae* of the Cytophagales. C. Phylogenetic position of Band 4 within the *Campylobacter* group of the ϵ-*Proteobacteria*. D. Phylogenetic position of Bands 6, 15, and 17 among the *Pseudomonas* group of the γ-*Proteobacteria*. E. Phylogenetic position of Bands 14 and 16 within the *Enterobacteriaceae* of the γ-*Proteobacteria*.

based on 16S rRNA or rDNA does not allow us for distinguishing between DSeR bacteria and selenate-tolerant SeR bacteria. Since DSeR bacteria are polyphyletic, it is impossible to design a group-specific 16S rRNA probe. The development of probes based on functional genes responsible for SeR will be useful to study the distribution and dynamics of these organisms in the environment. It seems that several selenate reductases exist in nature, since *T. selenatis* and *S. barnesii* SES-3 possess different enzymes (*18*). The design of useful probes will thus require further investigation, such as collecting new SeR bacterial isolates and characterizing their selenate reductases.

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