Molecular methods to detect and monitor dissimilatory arsenate-respiring bacteria (DARB) in sediments

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Abstract
Dissimilatory arsenate-respiring bacteria (DARB) reduce arsenate to arsenite and may play a significant role in arsenic mobilization in aquifers and anoxic sediments. Many studies have been conducted with pure cultures of DARB to understand their involvement in arsenic contamination. However, few studies have examined uncultured DARB in the environment. In order to investigate uncultured DARB in anoxic sediments, genes encoding arsenate respiratory reductases (arr) were targeted as a genetic marker. Degenerate primers for the α-subunit of arr genes were designed and used with PCR amplification to detect uncultured DARB in the sediments collected from three stations (upper, mid and lower bay) in the Chesapeake Bay. Phylogenetic analysis of putative arrA genes revealed the diversity of DARB with distinct community structures at each of the three stations. Arsenate reduction in sediment communities was confirmed using enrichment cultures established with sediment samples from the upper bay. In addition, terminal restriction fragment length polymorphism analysis of the putative arrA genes showed changes in the community structure of DARB in the enrichment cultures while reducing arsenate. This was also confirmed by cloning and sequence analysis of the arrA genes obtained from the enrichment cultures. Thus, we were able to detect diverse uncultured DARB in sediments, as well as to describe changes in DARB community structure during arsenic reduction in anoxic environments.

Introduction
Arsenic is a ubiquitous contaminant associated with natural weathering of rocks and anthropogenic inputs associated with mining and agricultural applications. Contamination of drinking water resources by arsenic has caused serious health problems in northeast India, Bangladesh and northwest United States (Pontius et al., 1994). The predominant forms of dissolved arsenic in natural water systems are arsenate [As(V)] and arsenite [As(III)]. Arsenic is dominant in oxic waters, but under reducing conditions it is converted to arsenite (Massarcheleyn et al., 1991). In oxic environments, such as the epilimnion of lakes or surface sediments in streams, arsenate can be adsorbed onto iron or manganese oxides and transported to suboxic or anoxic environments (i.e. sedimentation into the hypolimnion or entrainment into deeper sediments). Microbial reduction of arsenate, iron oxide (III) and manganese oxide (IV) can occur in the sediments, and results in desorption of arsenite (Cummings et al., 1999; Zobrist, 2000; Herbel & Fendorf, 2006). Thus, microbial reduction of arsenate to arsenite is one of the main pathways involved in arsenic mobilization in anoxic environments.

Microorganisms involved in arsenate reduction can be classified as either arsenic resistant or dissimilatory arsenate-respiring organisms (Mukhopadhyay et al., 2002). A wide range of microorganisms use arsenate reduction as a form of arsenic resistance to survive in arsenic-contaminated environments. Arsenic-resistant microorganisms have the enzymes required to reduce arsenate to arsenite coupled to arsenite efflux systems. Alternatively, anaerobic microorganisms utilize arsenate as a terminal electron acceptor in dissimilatory arsenate respiration (DASR) while oxidizing various organic (e.g. lactate, acetate, formate and aromatic compounds) or inorganic (hydrogen and sulfide) electron donors (Stolz & Oremland, 1999; Hollibaugh et al., 2006).
Microorganisms capable of DAsR have been isolated from various environments and are widely distributed in diverse phylogenetic groups from *Proteobacteria* to *Crenarchaea* (Oremland & Stolz, 2003). Dissimilatory arsenate-respiring bacteria (DARB) are able to reduce both sorbed and dissolved arsenate to arsenite presumably because the enzymes responsible for arsenate reduction are located in the periplasmic membrane (Oremland & Stolz, 2006). The periplasmic enzymes were recently characterized from *Chrysiogenes arsenatis*, *Bacillus selenitireducens* and *Shewanella* sp. ANA-3 (Kraft & Macy, 1998; Afkar et al., 2003; Malasarn et al., 2008) and the functional genes encoding arsenate respiratory reductase (*arrAB*) were identified in several isolates of *Proteobacteria* and Gram-positive bacteria (Saltikov & Newman, 2003; Malasarn et al., 2004; Pérez-Jiménez et al., 2005). The expression of the *arrA* gene in *Shewanella* sp. ANA-3 corresponded to the reduction of arsenate to arsenite (Malasarn et al., 2004). Thus, the *arrA* gene was proposed as a good genetic marker to detect DARB and to monitor their activities in the environment.

Molecular detection of uncultured DARB based on the *arr* genes has been conducted with environmental DNA extracted from either water or sediments from Mono Lake and Searles Lake, CA (Hollibaugh et al., 2006; Kulp et al., 2006, 2007), as well as from an arsenic-contaminated groundwater aquifer in Cambodia (Lear et al., 2007; Pederick et al., 2007). Each arsenic-contaminated site contained unique DARB communities, which were not closely associated with any of DARB isolates on the basis of the *arrA* gene analysis. In order to extend the understanding of DARB diversity and distribution in temperate environments, we used molecular methods to detect diverse DARB in estuarine sediments and to monitor the changes in their community composition during arsenate reduction. Direct PCR amplification of the *arrA* genes was performed to detect uncultured DARB in estuarine sediments collected from three locations in Chesapeake Bay, to which significant amounts of arsenic compounds have been introduced via anthropogenic sources such as urban runoff and poultry farming (Sanders, 1985; Riedel, 1993; Gupta & Karuppiah, 1996; Riedel et al., 2000). In addition, arsenate-reducing enrichment cultures were established with the sediments collected from the upper bay to confirm DARB activities. Changes in community structure of DARB in the enrichment cultures were monitored by terminal restriction fragment length polymorphism (T-RFLP) analysis of *arrA* genes and confirmed by cloning and sequence analysis.

### Materials and methods

#### Bacterial strains and growth conditions

Four DARB isolates were used as controls in methods testing (Table 3). *Bacillus arsenicoselenatis* and *B. selenitireducens* were obtained from the American Type Culture Collection and grown under arsenate-reducing conditions as described previously (Blum et al., 1998). *Sulfurospirillum barnesi* and *Desulfotobacterium hafniense* DCB-2 cultures were provided by Dr John F. Stolz and Dr François M.M. Morel, respectively.

#### Environmental samples

Estuarine sediment samples from three stations (CB1, CB2 and CB3) in Chesapeake Bay, MD, were collected in April 2001. The details of site locations and characteristics were reported in Table 1 and by Francis et al. (2003). Station CB1 is located on the upper part of Chesapeake Bay near the mouth of the Susquehanna River. Station CB2 is located on the mid bay near the Choptank River. Station CB3 is located in the lower part of the bay near the ocean. The sediment samples were obtained by deploying a boxcore sampler from the ship. Samples from the top 5 cm of the sediment core were collected using sterile 50 mL Falcon tubes and stored at −80 °C until analyzed. An additional sediment sample from the upper Chesapeake Bay (station CB1) was collected in a sterile 200 mL jar for establishing enrichment cultures. Bottom water from CB1 was also collected and stored at 4 °C until used for the enrichment cultures.

#### Genomic DNA isolation from pure cultures and environmental samples

Genomic DNA of the bacterial isolates was extracted using the Puregene DNA extraction kit (Gentra Systems Inc., Minneapolis, MN) following the manufacturer’s instructions. Environmental DNA from sediment samples from three stations was extracted using the Power Soil DNA kit (Mo Bio laboratory Inc., Carlsbad, CA) following the manufacturer’s instructions.

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<table>
<thead>
<tr>
<th>Station</th>
<th>Sampling date</th>
<th>Water depth (m)</th>
<th>Temperature (°C)</th>
<th>Salinity (psu)</th>
<th>NH₄⁺ (µM)</th>
<th>NO₃⁻ (µM)</th>
<th>DOC (µM)</th>
<th>DON (µM)</th>
<th>DOP (µM)</th>
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<tbody>
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<td>Chesapeake upper bay (CB1)</td>
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<td>10</td>
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<td>4.4</td>
<td>9.9</td>
<td>77</td>
<td>280.7</td>
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<tr>
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<td>17.5</td>
<td>7.2</td>
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<td>9.5</td>
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<tr>
<td>Chesapeake lower bay (CB3)</td>
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<td>11</td>
<td>8.7</td>
<td>23.6</td>
<td>1.9</td>
<td>1.1</td>
<td>243.8</td>
<td>2.0</td>
<td>0.1</td>
</tr>
</tbody>
</table>

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Table 1. Bottom water parameters of Chesapeake Bay sampling stations.
**Primer design and PCR amplification of arsenate reductase (arrA) genes**

Four different primers were designed to amplify arrA genes in DARB and sediment samples (Table 2). Primers were designed by comparing conserved regions in the arrA genes from *B. selenitireducens, Shewanella sp. ANA-3, D. hafniense* DCB-2 and *Wolinella succinogenes* using the CODEHOP program (http://bioinformatics.weizmann.ac.il/blocks/codehop.html) (Fig. 1). The codon bias of *Escherichia coli* was used for the parameters in primer design. A nested PCR approach was used to amplify arrA genes from the environmental DNA as described in Lear et al. (2007). Two different initial PCRs (initial PCR I and II) were performed to optimize the reaction conditions and primer combinations. Both initial PCR amplifications were performed in a total volume of 25 μL containing 2.5 μL of 10 × Advantage II PCR bufferTM, 80 μM of each deoxyribonucleoside triphosphate, 0.4 μM of each primer, 1 U Advantage Taq polymerase and c. 100 ng of total DNA. The primers AS1F and AS1R were used for the initial PCR I with a cycle of 5-min denaturation at 94 °C, followed by 35 cycles of 30-s denaturation at 94 °C, primer annealing of 30 s at 50 °C, followed by a 1-min extension at 72 °C. The initial PCR II was performed with AS1F and AS2R primers with a PCR cycle of 5-min denaturation at 94 °C, followed by 35 cycles of 30-s denaturation at 94 °C, primer annealing of 30 s at 55 °C, followed by a 2-min extension at 72 °C. The nested PCR amplification was performed with AS2F and AS1R primers in a total volume of 50 μL containing 5 μL of 10 × PCR buffer (500 mM KCl, 200 mM Tris-HCl, pH 8.4), 1.5 mM MgCl2, 20 μM of each deoxyribonucleoside triphosphate, 1 μM of each primer, 1 U Taq polymerase and 1 μL of either initial PCR I or II reaction as a template. The nested PCR cycle began with a 2-min denaturation at 94 °C, followed by 30 cycles of 30-s denaturation at 94 °C, primer annealing of 30 s at 55 °C and followed by a 1-min extension at 72 °C. The amplified products were examined in 1.0% agarose gels by electrophoresis and then were purified using a QiaquickTM gel extraction kit (Qiagen, Bothell, WA) according to the manufacturer’s instructions.

### Cloning and sequencing PCR products

The amplified products of the nested PCR with AS2F and AS1R primers in combination with initial PCR I were cloned to make environmental arrA gene libraries from each site. The cleaned PCR products were used for direct cloning with the TOPO-TA™ cloning system (Invitrogen, Carlsbad, CA) following the manufacturer’s instructions. The ligated plasmids were transformed in high transforming efficiency *E. coli* TOP10™ (Invitrogen) following the manufacturer’s instructions. The transformed cells were plated on Luria agar plates containing 50 μg mL⁻¹ kanamycin with X-gal.

<table>
<thead>
<tr>
<th>Primers</th>
<th>Orientation</th>
<th>Sequences (5‘→3’)</th>
<th>(T_m)</th>
<th>Targeted amino acids</th>
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<td>CGAAGTCTGTCGCCCGATHACNTGG</td>
<td>69.2</td>
<td>122KFVPITW in <em>D. hafniense</em> DCB-2</td>
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<tr>
<td>AS1R</td>
<td>Reverse</td>
<td>GGGGTCCGCGTCYTTNARYTC</td>
<td>63.7</td>
<td>326ELKDRTP in <em>D. hafniense</em> DCB-2</td>
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<tr>
<td>AS2F</td>
<td>Forward</td>
<td>GTCCTACTASNGGGANRARGCNMT</td>
<td>64.2</td>
<td>795QGHWAY in <em>D. hafniense</em> DCB-2</td>
</tr>
<tr>
<td>AS2R</td>
<td>Reverse</td>
<td>ATANGCCARTGNCCYTGNG</td>
<td>62.2</td>
<td>795QGHWAY in <em>D. hafniense</em> DCB-2</td>
</tr>
</tbody>
</table>

**Fig. 1.** Primer targeting location on the α-subunit of arsenate reductase reductase (arrA).
Twenty-five clones from each cloning reaction were selected for sequencing using BigDye™ terminator chemistry (Applied Biosystems, Foster City, CA) and ABI 3100 automated DNA sequencer (Applied Biosystems). The BLASTX search was conducted to identify the sequenced clones to be putative arrA genes.

**Phylogenetic analysis of arsenate respiratory reductase (arrA) genes**

The arrA gene sequences were translated to amino acid sequences, which were aligned with reference sequences using the CLUSTALW program (http://www.ebi.ac.uk/ClustalW/). The amino acid sequences of reference arrA genes in Shewanella sp. ANA-3 (AY271310), Shewanella sp. HAR-4 (AY660886), Shewanella W3-18-1 (NC_008750), C. arsenatis (AY660883), Geobacterium uraniumreducens (NC_009483), B. arsenicoselenatis (AY660885), B. selenitireducens (AY283639), S. barnesi (AY660884), W. succinogenes (NC_005090), Desulfovporinus sp. Y5(DQ220794), D. hafniense Y51 (NC_007907), D. hafniense DCB-2 (NZ_AAAW0400004), Alkaliphilus metallicredigenes (NC_009633) and Sargasso sea environmental sequence (AACY01087453) were obtained from the GenBank database. In addition, a putative dehydrogenase gene found in the genome sequence of Magnetospirillum magnetotacticum (NZ_AAAP01003791) was used as an outgroup. Phylogenetic analyses were performed with PAUP 4.0 program (Swofford, 2002). Phylogenetic trees were reconstructed using the neighbor-joining method (Saitou & Nei, 1987) and bootstrap values were obtained from data resampling of 1000 replicates. Sequences obtained from this study were deposited under the accession numbers FJ747531–FJ747602.

**Establishment of arsenate-reducing enrichment cultures**

Arsenate-reducing enrichment cultures were established with the sediment samples and site water collected from the upper Chesapeake Bay (station CB1). Strict anaerobic techniques were followed throughout the enrichment process for medium preparation, culture handling and sampling. The enrichment cultures were established by mixing 10% (v/v) sediment slurry in site water while flushing with argon gas. One hundred milliliter aliquots of the slurry were dispensed into 160-mL serum bottles, sealed with rubber stoppers and crimped with aluminum seals under an argon headspace. 4-Hydroxybenzoate and sodium arsenate were added to the serum bottles from deoxygenated stock solutions to obtain final concentrations of 0.5 and 5 mM, respectively. 4-Hydroxybenzoate was selected as a carbon substrate because it was one of the aromatic compounds readily degraded by various anaerobes (Schink et al., 1992; Harwood & Gibson, 1997). The cultures were established in duplicate (A and B) with sterile controls (autoclaved for 1 h on three consecutive days before feeding with substrates). All cultures were incubated in the dark at room temperature.

**Ion chromatography analysis of arsenate reduction in the enrichment cultures**

The enrichment cultures were monitored for 37 days to determine arsenate reduction under anaerobic conditions. Periodically, 1 mL of well-mixed slurry was removed from each serum bottle using sterile, argon-flushed syringes. Samples were then centrifuged in a microcentrifuge and the supernatant was filtered through a 0.45-μm filter (Millipore). The pellets were stored at −80 °C for molecular analysis as described below. The concentration of arsenate was monitored using ion chromatography [ Dionex DX-500 with UV–Vis (AD20) absorbance detector, Sunnyvale, CA] with a Dionex IonPac AS12 (250 × 4.0 mm) analytical column and an InPac AG12 (50 × 4.0 mm) guard column. The eluent was 2.7 mM carbonate/0.3 mM bicarbonate. Anions were detected by suppressed conductivity detection. The detection limit of arsenate for this analysis was 10 μg L⁻¹.

**Molecular analysis of arsenate-reducing communities in the enrichment cultures**

Genomic DNA was extracted from three slurry pellets collected on day 5, 13 and 20 during the incubation using the Power Soil DNA kit (Mo Bio laboratory Inc.) following the manufacturer’s instructions. T-RFLP analysis of the arrA genes was performed to examine the changes in community structure of DARB in the enrichment cultures. The nested PCR was performed as described above using 6FAMlabeled AS2F and AS1R primers with 1 μL of the initial PCR II reaction for T-RFLP analysis with AS1F and AS2R primers. Additional PCR amplification for T-RFLP analysis was conducted with the DNA from station CB1 as a background control of DARB communities before enrichment. The PCR products were cleaned using a Qiaquick™ gel extraction kit (Qiagen). The concentration of the purified PCR amplicons was measured by ND-1000 Spectrophotometry (NanoDrop) following the manufacturer’s instruction. A total of 50 ng of PCR amplicons were mixed with 1 U ofMspI restriction enzyme (Promega Co., Madison, WI) and incubated at 37 °C overnight. The digested products were precipitated with 75% isopropanol and resuspended with Hi-Di formamide. The samples were run on an ABI3130X genetic analyzer and analyzed with the GENEMAPPER program (Applied Biosystems). Only fragment lengths in the range of 30–500 nucleotides were considered for analysis. In order to distinguish real peaks from noise in each T-RFLP profile, the peak heights of each fragment in a T-RFLP profile were added, and then the height of each fragment was divided by total heights of the T-RFLP profile. The calculated ratio of each height was multiplied by 100 to...
generate % of fragment height in each T-RFLP profile. The fragments with > 1% of total heights were included in further analysis.

The DARB communities at day 5 were further examined by cloning and sequence analysis as described above. Phylogenetic analysis of the arrA gene sequences from the enrichment cultures was conducted as described above to investigate structure variation in DARB communities. Each arrA gene sequence was subjected to in silico digestion of the restriction enzyme MspI to determine the fragment sizes from the 5'-ends, which were used to match the T-RFs from the T-RFLP analysis. In addition, the arrA genes in several clones were PCR amplified with [6'TAM]AS2F and AS1R primers and digested with the MspI enzyme as described above. T-RFLP analysis of the selected clones was conducted to confirm which arrA gene sequences corresponded to the T-RFs in the fingerprints.

Results

PCR detection of arsenate respiratory reductase genes (arrA) in sediments

Initial PCR I with AS1F and AS1R primers amplified 630-bp fragments of expected size from B. arsenicobacter, B. selenitireducens and D. hafniense DCB-2, and yielded a smear of around 630 bp from S. barnesii (Table 3). However, initial PCR II with AS1F and AS2R primers yielded a 2-kb fragment from only D. hafniense. Nested PCR with AS2F and AS1R primers performed with either the initial PCR I or II reaction generated 625-bp fragments (Table 3). The amplified products were confirmed to be arrA genes by sequencing. The arrA genes in environmental DNA extracted from three sediment samples could only be amplified using the nested PCR (Table 3). Twenty-five clones from each sediment clone library were sequenced and BLAST searched to determine their identities. A total of 52 clones were identified as putative arrA genes out of 75 sequenced clones. Specificity of the nested PCR detection from the initial PCR reactions using AS1F and AS1R primers was 69.3% based on the number of arrA genes retrieved out of the total number of clones screened.

Table 3. PCR amplification of arsenate respiratory reductase genes

<table>
<thead>
<tr>
<th></th>
<th>AS1F+AS1R</th>
<th>AS1F+AS2R</th>
<th>AS2F+AS1R*</th>
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</thead>
<tbody>
<tr>
<td>Bacterial isolates</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Desulfitobacterium</td>
<td>+</td>
<td>+</td>
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</tr>
<tr>
<td>hafniense DCB-2</td>
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<td>+</td>
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<tr>
<td>Bacillus arsenicobacter</td>
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<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Sulfurospirillum barnesii</td>
<td>±</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Environmental samples</td>
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</tr>
<tr>
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<td>-</td>
<td>+</td>
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<td>+</td>
</tr>
<tr>
<td>Chesapeake lower bay sediment (CB3)</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Arsenate-reducing enrichment cultures (CBAS)</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

*Nested PCR was used with the primers.
†The enrichment cultures were established with the sediments from Chesapeake upper bay.
+, positive PCR amplification; -, no PCR amplification; ±, smear PCR amplification.

Phylogenetic analysis of arrA genes detected from Chesapeake Bay sediments

The DNA sequences of the 52 putative arrA genes from Chesapeake Bay sediments were translated into amino acid sequences for further analysis. Phylogenetic analysis of the ArrA amino acid sequences showed the community structure of uncultured DARB in Chesapeake Bay sediments (Fig. 2). None of the sequences detected from the Chesapeake Bay sediments was closely associated with those found in DARB isolates. Fifty ArrA sequences grouped in seven clusters and two singletons based on their phylogenies. All of the ArrA sequences from station CB1 grouped into three clusters (IV, V and IX) with one exception (clone CB1 F1). Most of the sequences from CB1 (12 out of 17) grouped in cluster IV as the dominant ArrA sequences at CB1. A total of 17 ArrA sequences from station CB2 grouped in three clusters (II, VII and IX). Cluster VII had the sequences only from CB2 while clusters II and IX contained ArrA sequences from either CB1 or CB3. Most of the sequences (14 out of 18) from CB3 grouped in cluster III and two (2) clones, unique to CB3, were assigned to cluster VIII. Thus, each station has unique and dominant ArrA sequences, which might represent the biogeographical distribution of DARB communities in Chesapeake Bay sediments.

Arsenate reduction in sediment-enrichment cultures

Arsenate reduction in the enrichment cultures was assumed to be responsible for the decrease in arsenate concentration during the incubation. More than 90% of the spiked arsenate was reduced during 37 days of incubation in the duplicate enrichment cultures A and B (Fig. 3). The average rate of arsenate reduction was 0.16 ± 0.03 mM day-1, demonstrating the presence of active DARB communities in Chesapeake Bay sediments.
DARB communities in sediment-enrichment cultures

The DARB communities in the enrichment culture A were characterized on the basis of the *arrA* genes. T-RFLP analysis of *arrA* genes was conducted with the slurry samples obtained from the incubation experiment at days 5, 13 and 20 as described in Materials and methods. The DARB communities in the CB1 sediment were also examined to compare the changes in communities during the incubation. Based on the criteria used for fragment analysis, five different T-RFs were recognized in the CB1 sediment (Fig. 4). The T-RFs of 54 and 61 bp were dominant *arrA* genes found in this sediment community. Once the sediment communities were incubated with arsenate and 4-hydroxybenzoate under anaerobic conditions, the shift in DARB communities was observed based on the *arrA* gene fingerprints (Fig. 4). After 5 days of incubation, the T-RF of 54 bp disappeared, and new dominant DARB populations appeared as represented by the T-RFs of 385 and 410 bp. The DARB carrying the *arrA* gene for the 61-bp T-RF still remained as one of the dominant populations. In addition, the DARB represented by the 208-bp T-RF increased. Several new populations of DARB appeared as shown in Fig. 4. Once the incubation was extended to 13 days, the community was dominated with the DARB carrying the *arrA* genes.

Fig. 2. Phylogenetic analysis of ArrA sequences detected from Chesapeake Bay sediments and enrichment cultures. CB1, upper Chesapeake Bay; CB2, mid Chesapeake Bay; CB3, lower Chesapeake Bay; CBAS3 and CBAS6, arsenate-reducing enrichment cultures.

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corresponding to the T-RFs with 385 and 410 bp. The dominant 61-bp T-RF and other small RFs at day 5 disappeared in day 13. After 20 days of incubation when 60% of the spiked arsenate had disappeared, the community was dominated by DARB with the arrA gene represented by the 385-bp T-RF. A decrease in the strength of the 410-bp T-RF signal was observed at day 20. Thus, the changes in sediment community were clearly detected based on the arrA gene fingerprints while arsenate reduction occurred in the enrichment culture.

The DARB community in the enrichment culture was also examined by cloning and sequencing the arrA gene amplicons from the sediment slurry obtained on day 5. A total of 20 clones were sequenced and included in phylogenetic analysis (Fig. 2). The ArrA sequences from the enrichment culture formed two unique clusters (I and VI) and also grouped in clusters IV and V with the sequences from CB1 sediments. The presence of similar sequences in the enrichment culture and the source environment suggests that these might represent active arsenic reducers in the environment. The presence of several clones in clusters I and VI, and five clones (CBAS3 A6, CBAS3 E1, CBAS6 A1, CBAS3 F6 and CBAS6 B1) only in the enrichment culture provides additional evidence of the changes in the DARB communities in the enrichment culture as observed in the T-RFLP analysis. Thus, phylogenetic analysis of the arrA genes confirmed the

Fig. 3. Measurement of arsenate reduction in duplicate enrichment cultures A and B established with the sediments from the upper Chesapeake Bay (CB1). The arrow indicated the sampling day of the slurry pellets used for DARB community analysis.

Fig. 4. T-RFLP analysis of arrA genes in arsenate-reducing enrichment cultures established with the upper Chesapeake Bay sediment. The numbers indicated the sizes of T-RFs.
changes in community structure of DARB in the CB1 sediments while reducing arsenate in enrichment cultures.

The arrA gene sequences were matched with the different sizes of T-RFs based on in silico digestion of the MspI restriction enzyme. The clones from CB1 sediment belonging to the cluster IV and IX yielded 65- and 57-bp fragments, respectively. The cluster IX corresponds to the 54-bp T-RF, and the cluster IX is related to 61-bp T-RF although the in silico digestion generates 3 or 4 bp longer fragments. The mismatches between T-RFs and sequences were previously reported with the variation of up to 7 bp (Kitts, 2001). In order to confirm the matches between sequences and T-RFs, T-RFLP analysis was conducted with the clones in the clusters IV and IX, and showed that the fragment sizes of the clones matched with the T-RFs in Fig. 4 (data not shown). Thus, two fragments with the sizes of 54 and 61 bp dominated in the CB1 sediment communities were matched with the arrA sequences in Cluster IX and IV, respectively. The fragment with 300 bp was recognized as the sequence of the clone CB1_F1. However, the sequences corresponding to fragments with 208 and 258 bp were not found in the sequenced clones. The T-RFLP profiles generated from the enrichment culture were also compared with the arrA gene sequences in Fig. 2. The arrA genes grouped in cluster VI matched with 410-bp T-RF, and the clone CBAS3_A6 was corresponded to a 385-bp T-RF. The enrichment cultures also showed the presence of dominant arrA genes in cluster IV as the T-RF at 61 bp appeared. Cluster I matched with a 128-bp T-RF, which was detected in the enrichment culture sampled on days 5 and 13. A total of four T-RFs in the enrichment culture were matched with the detected arrA genes from the sequence analysis. The clones in cluster V were not detected in T-RFLP analysis of CB1 and the enrichment cultures. In addition, three clones (CBAS3_F6, CBAS6_A1, and CBAS6_B3) detected in the enrichment cultures by cloning and sequencing analysis were not found in the T-RFLP analysis.

Discussion

Detection of DARB is important to understand their involvement in arsenic mobilization in anoxic environments. PCR conditions and the primers for arrA gene detection from environmental samples were previously reported (Malasarn et al., 2004; Hollibaugh et al., 2006; Kulp et al., 2006, 2007). However, we were not able to amplify the arrA genes from the Chesapeake Bay samples using the reported PCR primers and protocols (data not shown). In addition, a larger size of PCR amplicon was needed to conduct community fingerprint analysis such as T-RFLP because the previous protocols yielded fragment sizes of 112 bp (Malasarn et al., 2004; Hollibaugh et al., 2006) and 389 bp (Kulp et al., 2006, 2007). Thus, we developed new primers and nested PCR protocols to amplify 625-bp amplicons from environmental DNA. Using the primers AS1F and AS1R with the initial PCR I condition, and nested PCR with AS2F and AS1R primers as described in this study, the DARB communities in the Cambodian aquifer sediments were previously examined by Lear et al. (2007) and Pederick et al. (2007). In addition, this PCR method was successfully used to detect arrA genes from arsenic-contaminated ground water samples collected from the Piedmont aquifer, NC (Oates & Song, 2008). However, specificity of this PCR detection was found to be 69.3% as calculated by the number of putative arrA genes detected over total number of the clones sequenced. The degeneracy of both AS1F and AS1R primers might generate this reduced specificity of the detection (Fig. 1). Thus, we designed additional primer (AS2F) and developed new initial PCR conditions (initial PCR II) to enhance detection specificity. Once initial PCR II and nested PCR were conducted for the arrA gene detections in the CB1 sediment, most of the clones (> 98%) were identified to be putative arrA genes based on the BLAST search analysis (data not shown). The primer AS2R has higher specificity and less degeneracy than the primer AS1R. This enhances the success of arrA gene detection from sediment samples. Thus, the PCR methods developed in this study were highly specific for the arrA gene detection in environmental samples.

Phylogenetic analysis of the arrA genes demonstrated the diversity of arsenate-respiring systems in prokaryotes (Fig. 2). The presence of arsenate-respiring systems in a wide range of bacteria has implications for the evolution of anaerobic respiration systems in prokaryotes. Duval et al. (2008) proposed that ArrAB evolved from an enzyme related to polysulfide reductase, and lateral transfer of the arrAB genes was occurred within either the Bacteria or Archaea after both domains were diverged. This study as well as the previous work by Hollibaugh et al. (2006) and Kulp et al. (2007) detected arrA genes originated from bacteria, which supported high divergence of arrAB genes between bacteria and archaea. The arrA genes found in Chesapeake Bay sediments were mostly different from those found in the soda lakes in California except the clones CBAS3E and CBAS6A1, which have > 60% sequence similarities with eight clones reported by Hollibaugh et al. (2006). This might imply distinct biogeography or environmental influences on DARB diversity in various ecosystems.

Sequence analysis of the arrA genes detected from the Chesapeake Bay sediments showed a biogeographical distribution of unique DARB communities at three different sampling stations. This might be related to various physical and chemical parameters along the bay. The communities at three sampling stations were exposed to gradients of salinity, ammonium, nitrate, DOC, DON and DOP (Dissolved organic carbon, nitrogen and phosphorus (Table 1)). Francis et al. (2003) found that ammonia-oxidizing bacterial
diversity was greatest at CB1, the freshwater station, and least at CB3, the highest salinity station. Salinity effects on arsenate reduction were reported by Kulp et al. (2007). The rates of arsenate reduction varied inversely with salinity. Variation in total bacterial community structures in the arsenate-reducing slurries from the Mono Lake and the Seales Lake were observed based on denaturing gradient gel electrophoresis analysis of 16S rRNA genes. Thus, distinctive community structure of the DARB found in Chesapeake Bay might be influenced by different salinities present at three different stations.

Differences in available organic carbon can be considered another factor influencing DARB communities in Chesapeake Bay. The annual average concentrations of DOC in the water column of the upper bay was 151 µM, and decreased seaward to 43 µM in the lower bay (Fisher et al., 1998). The range of DOC concentrations found in sediment pore waters was highest in the mid Bay (200–1500 µM) and slightly lower in upper bay (200–900 µM) (Burdige, 2001). The enrichment cultures established with the CB1 sediments from the upper bay showed the effect of available organic carbon (4-hydroxybenzoate) on the DARB community structures. 4-Hydroxybenzoate is one of the central intermediates from phenolic compounds (Harwood & Gibson, 1997), which comprise a large and diverse group of organic carbon in estuaries (Mitra et al., 2000; Harvey & Mannino, 2001). Estuaries receive different amounts of diverse phenolic compounds from terrestrial inputs, which could influence microbial communities capable of utilizing those compounds as carbon and energy sources. Because anaerobic degradation of 4-hydroxybenzoate was observed in denitrifying, iron-reducing, sulfate-reducing and phototrophic conditions (Schink et al., 1992), DARB in Chesapeake Bay sediments were expected to utilize the selected substrate as a carbon and energy sources. T-RFLP profiles from the CB1 sediment and the enrichment cultures showed a clear shift of DARB communities, which provided an evidence of 4-hydroxybenzoate utilization by selective DARB (Fig. 4). Sequence analysis also identified that the community dominated with the arrA genes in the clusters IV and IX was converted to the communities with the genes in cluster VI and the clone CBAS3_A6 (Fig. 2). This shift of community structures might be related to the utilization of 4-hydroxybenzoate as a carbon and energy sources. The DARB capable of degrading 4-hydroxybenzoate became dominant as the incubation time was extended. The predominant DARB populations in the original CB1 sediment may have diminished if they were not able to use 4-hydroxybenzoate. Using molecular techniques, the changes in DARB communities can be clearly demonstrated as seen in this study. Thus, this study provides a potential application of molecular tools to detect and monitor uncultured DARB communities, which can be used to understand the significance of DARB in arsenic mobilization in anoxic environments.

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References


