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Arsenic Mobilization by Epilithic Bacterial Communities Associated with Volcanic Rocks from Camarones River, Atacama Desert, Northern Chile

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Abstract The arsenic biogeochemical cycle is greatly dependent on microbial transformations that affect both the distribution and mobility of arsenic species in the environment. In this study, a microbial biofilm from volcanic rocks was characterized on the basis of its bacterial composition and ability to mobilize arsenic under circumneutral pH. Biofilm microstructure was analyzed by scanning electron microscopy (SEM)–energy-dispersive spectroscopy (EDS). Strains were isolated from biofilms and identified by 16S rDNA sequences analysis. Arsenic oxidation and reduction capacity was assayed with high-performance liquid chromatography coupled to gaseous formation performing the detection by atomic absortion in a quartz bucket (HPLC/HG/QAAS), and polymerase chain reaction was used to detect *aox* and *ars* genes. Bacterial communities associated with volcanic rocks were studied by denaturing gradient gel electrophoresis (DGGE). The SEM–EDS studies showed the presence of biofilm after 45 days of incubation. The relative closest GenBank matches of the DNA sequences, of isolated arsenic-resistant strains, showed the existence of four different genus: *Burkholderia*, *Pseudomonas*, *Erwinia*, and *Pantoea*. Four arsenite-resistant strains were isolates, and only three strains were able to oxidize >97% of the As(III) present (500 uM). All arsenate-resistant isolates were able to reduce between 69 and 86% of total As(V) (1000 uM). Analysis of 16S rDNA sequences obtained by DGGE showed the presence of four bacterial groups (∝-proteobacteria, γ-proteobacteria, Firmicutes, and Actinobacteria). Experiments demonstrate that epilithic bacterial communities play a key role in the mobilization of arsenic and metalloids speciation.

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Arsenic is often found in relatively high concentrations in sediments and groundwater as a consequence of natural dissolution and weathering of arsenical minerals. Arsenic mobility amongst various environmental compartments and chemical speciation is known to be mediated by complex chemical as well as microbial interactions (Tamaki and Frankenberger 1992; Quinn and McMullan 1995). Indeed, microorganisms are responsible for arsenic oxidation reduction and methylation. The mobility of arsenic commonly increases as reducing conditions are established within sediments or flooded soils. Although the reduction of arsenic increases its solubility at circumneutral pH, hydrous ferric oxides strongly sorb both As(V) (arsenate) and As(III) (arsenite), the two primary inorganic species (Cummings et al. 1999).

Heterotrophic and chemoautotrophic microorganisms have been reported to be able to oxidize As(III) under aerobic and anaerobic conditions (Oremland and Stolz 2003). These microorganisms have been isolated from arsenic rich environments, such as gold mine wastewater (Ilialetdinov and Abdrashitova 1981; Santini et al. 2002), arsenic-contaminated soils, lake water or sediment (Oremland et al. 2002; Campos et al. 2009; Escalante et al. 2009; Valenzuela et al. 2009), and

geothermal environments (Gihring et al. 2001). In addition, *Acidithiobacillus ferrooxidans*, an arsenic-tolerant strain, was observed to mobilize arsenic from pyrite in acid-mine drainage tailings (Duquesne et al. 2003), and a few strains of *Thiomonas* have been shown to oxidize As(III) released from pyrite in acid-mine drainage (Bruneel et al. 2003). However, there are few reports of mobilization of arsenic from minerals under circumneutral pH. Rhine et al. (2008) reported a strain able to mobilize and transform arsenic from arsenopyrite mineral under circumneutral pH. These results showed microbially enhanced mobilization of arsenic achieving complete oxidation of released arsenic and sulfur to stoichiometric amounts of arsenate and sulfate.

Northern Chile, particularly the Atacama Desert, is known to be an arsenic-rich region. Arsenic and other metallic sulfides present in the Andes Mountains are dissolved by rainfall, thus affecting the quality of surface and ground drinking water sources (Yañez et al. 2005). It is expected that microorganisms present in such an environment should play a key role in arsenic transformations and mobilization. The aim of the present study was to describe the ability to mobilize arsenic from volcanic rocks by a natural bacterial community at circumneutral pH.

Materials and Methods

Analytic Methods

Chemical oxygen demand (COD) and biologic oxygen demand (BOD_5) were measured according to standard methods (APHA-AWWA-WPCF 1985). Total nitrogen, phosphorous, and nitrogen-like ammonia were measured with Merck Spectroquant kits (Merck KGaA, Darmstadt, Germany) and quantified in a spectrophotometer (Spectroquant NOVA 60; Merck KGaA).

Samples and Microcosm Experiment

Volcanic rocks were obtained from the Camarones river basin, Atacama Desert-Northern Chile (18°57′00.85″S/69°29′59.41″W). Samples were placed in polyethylene bags, refrigerated at 4°C, and transported to laboratory for further processing.

The rocks were fragmented in a sterile mortar, under sterile condition, in a biologic safety cabinet (1300 Series B2; Thermo Scientific). Microcosm experiments were carried in two flasks, each one containing 5 g fragmented volcanic rocks (particle diameter approximately 1–2 cm) and 250 ml chemically defined medium (CDM) (Santini et al. 2000). The biotic experiment was performed by incubating inocula from the natural community present in the volcanic rock in CDM. The abiotic control experiment was performed using heat-sterilized volcanic rock in CDM. The rocks were sterilized by dry heat at 130°C for 3 h according to the description by Jenneman et al. (1986). All experiments were performed in duplicate and incubated at 20°C for 45 days under aerobic conditions. The temperature used in all experiments was maintained according to the temperature detected in the river.

Arsenic was monitored by taking periodic samples. One milliliter supernatant was filtered through a sterile 0.22-μm pore size filter (Millipore). Arsenic species were detected by means of high performance liquid chromatography (HPLC) coupled to arsine gaseous formation performing the detection by atomic absortion in a quartz bucket (HPLC/HG/QAAS) (Kumaresan and Riyazuddin 2001).

Scanning Electronic Microscopy–Energy Dispersive Spectroscopy Analysis

Bacterial communities and arsenic present in rocks were studied by scanning electron microscopy (SEM) and energy-dispersive X-ray spectroscopy (EDS). Microcosm samples, previously incubated for 14 and 45 days, were washed repeatedly with distilled water and fixed with formalin 30%. Samples were further washed with buffer solution and fixed with 1% osmium tetraoxide. Finally, samples were washed again with buffer solution, dehydrated with alcohol and acetone (Marrie and Costerton 1984), and dried with liquid CO_2 (critical point method). Finally, samples were gold-coated in a sputter coater (Edwards S150, Sussex, UK) and examined in an Etec Austoscan SEM. (Etec Corp., CA).

Strain Isolation and Identification

After 45 days of incubation, bacteria were isolated by adding 1 g homogenized rocks to 10 ml NaCl (0.85%) and sonicated in utrasonic bath (Elma/S30) for 5 min. The mixture was serially diluted, and 0.1-ml aliquots were plated onto CDM agar and incubated at 25°C for 48 h (Battaglia-Brunet et al. 2002). After growth, arsenic-resistant isolates obtained from each simple were initially characterized in terms of colony morphology (color, shape, size), basic microscopic observations, and biochemical profiles (RapID ONE System and RapID NF Plus System; Remel). Then PCR amplification of 16S rRNA genes was performed. A bacterial suspension was boiled for 10 min and centrifuged for 5 min. Then the supernatant was used as DNA template. PCR was conducted with 16S rDNA bacteria universal primers GM3 (AGAGTTTGATCMTGGC) and GM4 (TACCTTGTTACGACTT). PCR determination followed the procedure described by Ward et al. (1997). Sequencing was conducted on the amplified fragments using the Dyenamic ET terminador kit (General

Electric) in a 3100 Avant genetic Analyzer (Applied Biosystems) according to the manufacturer's instructions. Strains were characterized on the basis of sequences stored in the GenBank database using the Basic Local Alignment Search Tool program (BLAST version 2.0).

Tolerance Test

Tolerance to arsenic for each isolate was investigated using the agar dilution method. CDM agar plates supplemented with different concentrations of As(III) or As(V), in the 0.5–80 mM range, were inoculated with cell suspensions from precultures to a density of approximately 3 × 107 colony-forming units (CFU) ml^{-1} and incubated at 25°C for 24 h (Campos et al. 2009) Agar plates without arsenic were used as a control.

Detection of As(III) and As(V)

The strains were grown in CDM containing arsenite (500 μM) or arsenate (1000 μM). Cultures were incubated at room temperature for 48 h. The oxidation or reduction of arsenic was determined from culture supernatants filtered through a sterile 0.22-μm pore size filter (Millipore) as described previously.

aox Gene Detection in Isolated Strain

Arsenic-resistant strains were grown overnight on Luria Bertani (LB) medium supplemented with 0.5 mM NaH_2AsO_3 (10^8 CFU/ml). Then the samples were boiled for 10 min and centrifuged for 5 min at 14,000 rpm. The supernatant was used as DNA template for PCR amplification of arsenite oxidase genes. Each reaction tube contained 2 ng DNA template. PCR was performed according to the procedure described by Rhine et al. 2007, with primer 69F (TGYATYGTNGGNTGYGGNTAYMA) and 1374R (TANCCYTCYTGRTGNCCNCC). *Escherichia coli* S17-1 strain, kindly provided by Dr. McDermott (Department of Land Resources and Environmental Sciences, Montana State University. Bozeman, MT) was used as positive control (Kashyap et al. 2006), and *E. coli* K-12 strain was used as negative control. The amplified products were separated by 0.8% agarose gel electrophoresis and stained with 0.5 μg/ml ethidium bromide, and bands were visualized on an ultraviolet (UV) transilluminator. PCR products were purified by using the QIA Quick PCR Purification Kit (Qiagen) according to the maker's indications. The sequence was performed using the Dyenamic ET terminador kit (General Electric) according to the maker's instructions in a 3,100 Avant Genetic Analyzer (Applied Biosystems). Analyses of DNA sequences and homology searches were completed with EGABLAST using the BLAST algorithm for the comparison of a nucleotide query sequence against a nucleotide sequence database.

ars Gene Detection in Isolated Strains

Arsenic-resistant strains were grown overnight on LB medium supplemented with Na_2HAsO_4, and total DNA was isolated using the Instagene Matrix (BioRad) according to the manufacturer's instructions. Then, the supernatant was used as DNA template. PCR was conducted with primers arsC-F (3′GTAATACGCTGGAGATGATCCG′5) and arsC-1 (TTTTCCTGCTTCATCAACGAC) of the *ars* operon from the *E. coli* pUM3 plasmid (Macur et al. 2004) and arsC-Ps-R (5′-AGTCCTGTTCATGTGYAC-3′) and arsC-Ps-F (TGGCGTSGAAYGCCG) *ars* operon from the *E. coli* pUM3 plasmid *Pseudomonas aeruginosa* and *P. putida* (Saltikov and Olson 2002). *arsC* PCR for *E. coli* and *Pseudomonas* was performed according to the procedure described by Macur et al. (2004) and by Saltikov and Olson (2002), respectively. *Serratia odorifera* was used as positive control (Escalante et al. 2009). Negative controls included a deionized water reagent control and *E. coli*. Amplified products were separated by electrophoresis on 2% agarose gel, and bands were visualized on an UV transilluminator. PCR products were purified using the QIA quick PCR purification kit (Qiagen) according to the maker's indications. The sequence was performed using the Dyenamic ET terminador kit (General Electric) according to the maker's instructions in a 3100 Avant genetic Analyzer (Applied Biosystems). Analysis of DNA sequences and homology searches were completed with MEGABLAST using the BLAST algorithm for the comparison of a nucleotide query sequence against a nucleotide sequence database.

DNA Extraction, PCR, and DGGE

The total DNA from rock samples was extracted using the UltraClean soil DNA-extraction kit (MO BIO Laboratories) according to the protocol provided by the manufacturer. Total DNA were amplified with 16S rDNA universal primers EUB 9-27 and EUB 1542 (GAGTTTGATC CTGGCTCAG) and (AGAAAGGAGGTGATCCAGCC) (Brosius et al. 1978). Nested PCR was performed using the primer pair 341f and 534r (CCTACGGGAGGCAGCAG) and (ATTACCGCGGCTGCTGG) with a GC clamp (CGC CCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGG GGG) (Muyzer et al. 1993) attached to the forward primer. Hot-start PCR was performed in a 50-μl reaction mixture containing 5 μl 10× buffer provided by the manufacturer (Sigma) with 15 mmol $MgCl_2$/l, 1 μmol of each primer/l, 200 μmol deoxynucleoside triphosphates/l, 1 U *Taq* DNA polymerase (Sigma), and 0.2–1.0 μl DNA extract. The touchdown temperature program consisted of 6 min at

94°C, 30 cycles of 15 s at 94°C, 30 s at the annealing temperature, 2 min and 30 s at 72°C, and a final extension at 72°C for 3 min. During the first 20 cycles, the annealing temperature was decreased by 0.5°C in each cycle from 50 to 40°C. For nested PCR with the primer pair 341f and 534r, the temperature program consisted of 2 min at 94°C and 30 cycles of 15 s at 94°C, 1 min at the annealing temperature, and 1 min 30 s at 72°C. The annealing temperature was decreased during the first 20 cycles by 0.5°C in each cycle from 65 to 55°C, and a final extension of 3 min at 72°C was added. PCR products were checked for concentration, purity, and appropriate size by agarose gel electrophoresis and ethidium bromide staining.

DGGE was performed with a DGGE 1001 system (C .B. S. Scientific). Fifteen microliters PCR products, V3 region, were applied directly onto 6% (wt/vol) polyacrylamide gels in 13 TAE (40 mM Tris, 20 mM acetate, 1 mM ethylene diamine tetreaacetic acid) with denaturant gradient from 20 to 60% (where 100% denaturant contains 7 M urea and 40% formamide). Electrophoresis was performed at a constant voltage of 200 V at 60°C for 6 h. After electrophoresis, gels were stained for 20 min with SYBR gold nucleic acid gel stain (Molecular Probes) as specified by the manufacturer and visualized in a transiluminator (UVP, Inc.). Bands were excised, reamplified, purified, and sequenced in an ABI3100 genetic analyzer using the sequencing kit BigDye v.1.1 (Applied Biosystems). Sequences were identified by BLAST.

Results and Discussion

Results showed that the Camarones River presented oligotrophic characteristics, with low concentrations of phosphorus (0.8 μg/l) and nitrogen (0.4 μg/l) and low COD and BOD_5 values (1.55 and 3.67 mg/l, respectively) found in the water samples. HPLC/HG/ASS analyses showed arsenic levels pf approximately 1100 and 500 μg/l in the water column and sediments, respectively. Northern Chile, in particular the Atacama Desert, has been described as an arsenic-rich environment because metallic sulphide minerals with a high concentration of arsenic are dissolved in the Andes Mountains, which is contaminated with arsenic in both superficial and ground waters (Yañez et al. 2005).

The volcanic rocks were classified as ignimbrites (Schmid 1981). They erupted intermittently during elevation of the Andes in the northern Atacama Desert region due to volcanic activity from the Miocene period to the present (Guest 1969). Figure 1a and b show the analysis of volcanic rocks by SEM-EDS. EDS analysis of volcanic rocks detected the presence of arsenic (27%) and other trace metals. Although igneous rocks are generally poor in

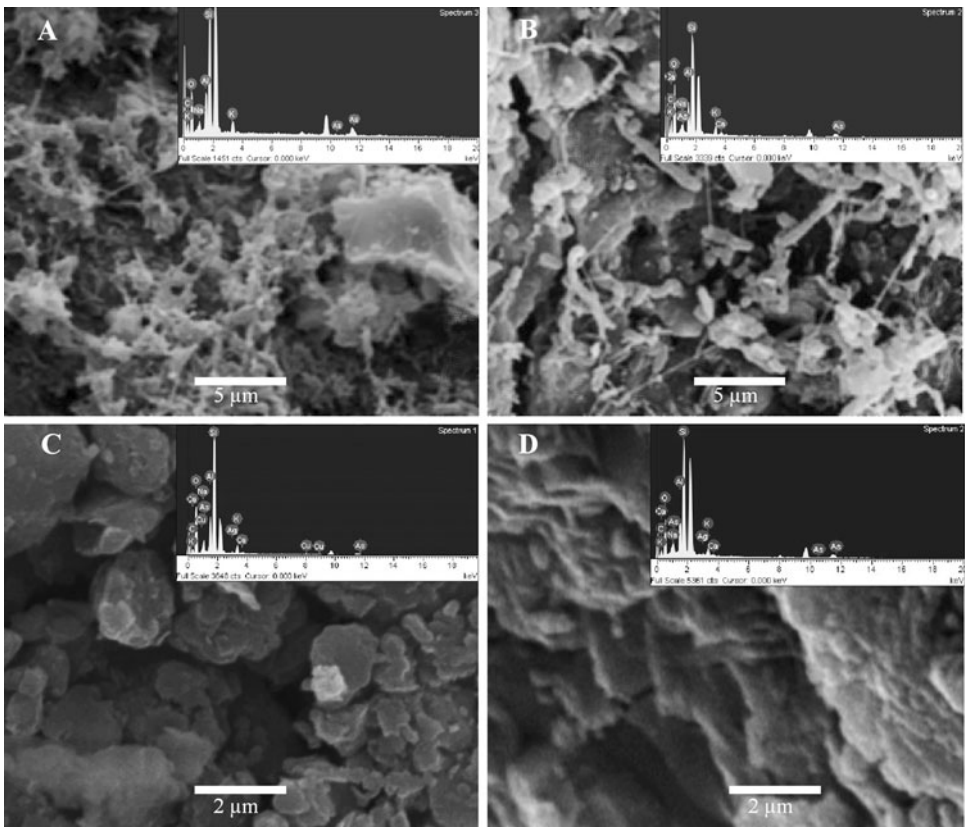

Fig. 1 SEM-EDS analysis of rock from the valley of Camarones River, Atacama Desert, Northern Chile. **a** Zero days of incubation. **b** After 45 days of incubation. **c** Sterile rock at 0 days of incubation. **d** Sterile rock at 45 days of incubation

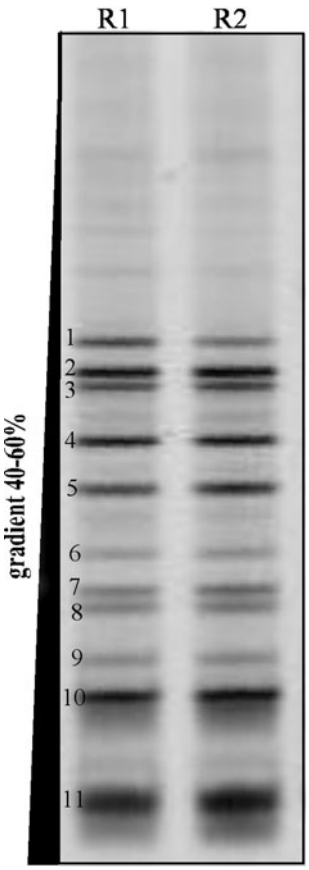

Fig. 2 DGGE of 16S rDNA products amplified with the primers P2 and P3 (GC-clamp). The gel was stained with SYBR gold, and the bands indicated in the figure were excised, reamplified, purified, and sequenced in an ABI3100 genetic analyzer using the sequencing kit BigDye v.1.1 (Applied Biosystems). The analyses were performed in duplicate (R1 and R2)

arsenic, volcanic tuffs can lead to element-rich waters (Smedley et al. 2002). In contrast, SEM showed the presence of an established bacterial biofilm on the volcanic rocks at 0 and 45 days of incubation. Figure 1a and b show the presence of thin fibrillar pili at the cells surfaces, similar to the curli structures described by Olsen et al. (1989) and extracellular polysaccharides (polymeric matrix) previously described in other microorganisms (Branda et al. 2006; Kobayashi 2007; Lerner et al. 2009). Curli is the structure of major importance for surface colonization (Saldaña et al. 2009; Gualdi et al. 2008). These microfibrils firmly anchor bacteria, thus this structure seems to provide a remarkable stability to the biofilm, which can withstand strong hydrodynamic conditions during prolonged culture without disruption (Donlan and Costerton 2000). The control rocks were not detected the presence of biofilm at 0 and after 45 days of incubation (Fig. 1c and d).

The band profile of the bacterial community associated with the volcanic rock biofilm, as obtained by DGGE, showed the presence of 11 bands (Fig. 2). The closest GenBank matches of 16S rDNA sequences obtained by DGGE showed the presence of four bacterial groups, namely, alphaproteobacteria, gammaproteobacteria, Firmicutes, and Actinobacteria, of which the gammaproteobacteria group was the most abundant. All of the reference bacteria identities were confirmed with 98–100% similarity (Table 1).

Table 2 lists results of the characterization of strains isolated from volcanic rocks. A total of 17 morphologically different isolates were initially obtained from the natural bacterial biofilm. Subsequent microscopy, biochemical profiles and 16 s rRNA analysis suggested that some of these isolates were likely the same taxa; thus, a final total of six different isolates were identified. The biochemical profiles and closest GenBank matches of the DNA sequences showed the existence of four different genus, namely, *Burkholderia*, *Pseudomonas*, *Erwinia*, and *Pantoea*. All of the reference bacteria identities were confirmed with 98–100% similarity with the corresponding species. As listed in Table 2, only two bacterial groups, betaproteobacteria and gammaproteobacteria, were identified in

Table 1 Analysis of 16S rDNA sequences obtained by DGGE

Band	Closest sequence relative	GenBank access no.	Bacterial groups
1	Uncultured *Anaerococcus* sp.	GQ179680	*Firmicutes*
2	Uncultured gamma proteobacterium	EF621534	Gammaproteobacteria
3	*Pantoea* sp. Mp285	FJ528275	Gammaproteobacteria
4	*Pseudomonadales* bacterium	AM931147	Gammaproteobacteria
5	Unculture Firmicutes bacterium	GQ180977	*Firmicutes*
6	Uncultured beta proteobacterium	EF698301	Betaproteobacteri a
7	*Psychrobacler* sp.	FJ596500	Gammaproteobacteria
8	Uncultured bacterium clone B9	DQ104271	Actinobacteria
9	Uncultured *Psychrobacler* sp.	DQ274142	Gammaproteobacteria
10	Uncultured gamma proteobacteria	AF440839	Gammaproteobacteria
11	Uncultured *actinobacterium*	EU417749	Actinobacteria

Table 2 Characterization of strains isolated from volcanic rocks

Strain	Closest sequence relative	GenBank accession no.	As^{3+} (mM)[a]	As^{5+} (mM)[a]
R-1	*Pseudomonas migulae*	DQ377758	40	2
R-2	*Burkholderia. cepacia*	AY741354	40	4
R-3	*P. putida*	AM285012	40	2
R-4	*Pseudomonas* sp. *B08*	AM285023	40	≥80
R-5	*Erwinia* sp.	AMI 17463	4	≥80
R-6	*Pantoea* sp. *WPCB006*	FJ006863	4	≥80

[a] Tolerance level

culturable strain, which is comparable with the results obtained by DGGE.

All bacterial strains isolated from biofilm were able to grow in the presence of arsenic. Resistance to arsenic was defined as the ability to grow on agar containing either 7 mM As(III) or 20 mM As(V) at 25°C (Rokbani et al. 2007). Arsenic-resistance experiments showed that R1, R2, and R3 strains were resistant to As(III) (40 mM), and R5 and R6 strains were resistant to As(V) (≥80 mM). Only R4 strain was resistant to both metalloids (Table 2). Jackson et al (2005) isolated 40 arsenic-resistant strains from sediments contaminated with the metalloid that presented resistance ≤400 mM for arsenate. However, most of these strains presented a low level of resistance to arsenite (between 0 and 2 mM); three of these strains presented resistance to 5 mM arsenite; and only one strain presented resistance to 10 mM arsenite. These bacteria were isolated from samples with low concentrations of arsenic (1.33 μM), which would not allow a selection of high-level resistant bacteria.

The capacity to transform arsenic by isolates under aerobic condition, as assessed by HPLC/HG/QAAS, showed that three of the four arsenite resistant isolates were able to oxidize >97% of As(III) present in the culture medium. In contrast, all strains of arsenate-resistant isolates were able to reduce between 69 and 86% of As(V) present in the culture medium under aerobic conditions (Table 3). Valenzuela et al. (2009) reported similar oxidation capacity (80–93%) from sediments of Camarones River with high concentrations of arsenic. In addition, Campos et al. (2009) reported oxidation activity of approximately 95% in strains isolated of Camarones valley using bicarbonate (HCO_3^-) as unique carbon source. Arsenate reduction and arsenite oxidation, under aerobic conditions, has been reported as detoxification mechanisms in several aerobic bacteria isolated from different arsenic-contaminated sites (Jones et al. 2000; Macur et al. 2001), suggesting that arsenic (As(V)/As(III)) resistance plays an important role in the biogeochemical cycling of this element in nature (Inskeep et al. 2002).

PCR *aox* gene analyses show that three of the six isolates presented a PCR product of approximately 1200 bp (expected size) corresponding to arsenite oxidase genes

Table 3 Characterization of strains isolated from volcanic rocks

Strain	*arsC* gene[a]	*aox* gene[b]	% Reduction(As^{5+})	% Oxidation(As^{3+})
R-1	−	+	0	97.6
R-2	+	+	69	96.8
R-3	−	−	78	0
R-4	−	+	0	88
R-5	+	−	86	0
R-6	+	−	84	0

[a] (+) PCR product approximately 370 bp

[b] (+) PCR product approximately 1200 bp

(Rhine et al. 2007). The three positive amplifications corresponded to arsenite-oxidizing strains. Similar results were found for the gene *arsC*; however, only in one arsenate-reducing strain (R3) was the *arsC* gene not detected (see Table 3). Various researchers have already reported that amplification of the *ars* genes is influenced by many factors, such as the type of primer used, conformational variation in the extracted DNA, thermal cyclic conditions, and the composition of buffers or agents (Chang et al. 2007). In addition, Ford et al. (2005) failed to correlate the level of arsenate resistance with the prevalence of *ars* genes in a set of aerobic and anaerobic bacteria (Escalante et al. 2009).

In addition, *aox* and *arsC* PCR products were sequenced, and nucleotide sequences of arsenite oxidase and arsenate reductase obtained were compared with sequences available in the GenBank using BLAST. The sequences analyses demonstrated that arsenite oxidase and arsenate reductase were 65.4–88.4% and 89.5–99.7% similar to the other arsenite oxidase sequences deposited in GenBank. The diversity of the arsC and aox genes has been reported by others investigators from different environmental bacterial strains. These investigators have reported that bacterial arsenite oxidase and arsenate reductase genes are not only phylogenetically diverse but also ecologically widespread (Inskeep et al. 2007; Kaur et al. 2009).

Biotic arsenic mobilization experiments were performed incubating the natural bacterial community associated with volcanic rocks for 45 days in a CDM under aerobic

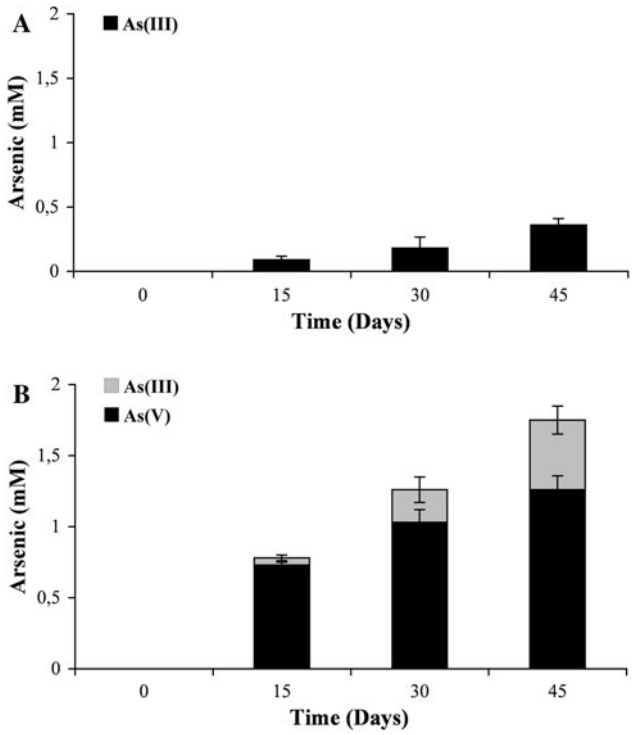

Fig. 3 Mobilization and transformation of arsenic from volcanic rocks after 45 days of incubation. Arsenic species concentration was measured by HPLC/HG/ASS. **a** Abiotic experiments (control). **b** Biotic experiments

conditions. Sterile rocks were used as negative control (abiotic experiments). In the sterile cultures at the end of 45 days, 0.37 mM total arsenic was detected (Fig. 3a). This increase in total arsenic concentration is due to chemical dissolution of soluble As(III) compounds. In contrast, in the presence of natural bacteria a final arsenic concentration of approximately 1.31 mM was obtained. Moreover, both arsenite and arsenate species were detected in the case of the biotic experiments (Fig. 3b). The presence of arsenate is due to the oxidative activity of the bacterial community, which is capable of mobilizing the metalloid from volcanic rock. In addition, the presence of arsenate-reducing bacteria enhanced arsenite formation because they are able to transform soluble arsenate to arsenite. Similar results were reported by Rhine et al. (2008) using WAO strains and arsenopyrite mineral.

Conclusion

The results suggest that microorganisms naturally present within the volcanic rock subsurface could enhance arsenic mobilization under circumneutral pH. Moreover, these bacteria play an important role in arsenic speciation once it has been mobilized. In addition, understanding such biologic processes will support modelling of arsenic mobilization from bedrocks into drinking water sources.

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