Arsenic hyper-tolerant and reducing bacteria isolated from wells in Tucumán, Argentina

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Abstract

Arsenic hyper-tolerant bacteria were isolated from arsenic-contaminated well water from the village of Los Pereyra in Tucumán province, Argentina. Microorganisms that biotransform arsenic are a major factor in arsenic mobilization in contaminated aquifers. Groundwater analyses showed a level of arsenic contamination (average concentration of 978 $\mu g.L^{-1}$) that exceeds the safe drinking water limit of 10 $\mu g.L^{-1}$ recommended by the World Health Organization (WHO) and the Argentine Food Code (AFC). There was considerable spatial variability in the concentration of arsenic in each of the wells analyzed, as well as in the distribution of the major anions HCO_3^-, SO_4^{2-} and Cl^-.

Eighteen bacterial strains were characterized. Six strains belonging to the Actinobacteria phylum, were able to grow in media with 20 mM As(III) or 200 mM As(V) and were also highly resistant to Cr, Cd and Cu. Their ability to biotransform arsenic was examined by speciation of the products using high performance liquid chromatography (HPLC) inductively coupled plasma mass spectrometry (ICP-MS). In addition, two strains, *Brevibacterium* sp. AE038-4 and *Microbacterium* sp. AE038-20, were capable of aerobic arsenate reduction, which suggests that these strains could increase the mobility of arsenic by formation of more mobile As(III).

Keywords: Arsenic hyper-tolerant bacteria; domestic water wells; arsenic contamination; arsenic-reducing bacteria.

1. Introduction

Arsenic, a toxic metalloid widespread in nature, causes serious health problems world-wide (Cervantes et al. 1994; Smedly and Kinniburgh 2002). Its presence in well water is a threat to public health (Jain and Ali 2000). Arsenic toxicity depends on both its chemical form and oxidation state. As(III) (arsenite) and As(V) (arsenate) are the most common oxidation states found in natural waters. Methylated arsenicals are also produced by microbes and are introduced anthropogenically (Cervantes 1994; Cullen and Reimer 1989). Countries with the highest arsenic concentrations in water are Mexico, Argentina, China, India and Bangladesh (Mandal and Suzuki 2002; Smedley and Kinniburgh 2002).

In Argentina approximately 1,000,000 people are estimated to be affected by daily ingestion of arsenic-contaminated water (Galindo et al. 2005). The populace of Los Pereyra, a village located in the eastern region of Tucumán province, drink well water that is contaminated with arsenic. The arsenic concentration in these wells exceeds the limit of 10 $\mu g.L^{-1}$ permitted by the World Health Organization for arsenic in drinking water (WHO 2003). In some cases the concentrations are as much as 200-fold higher than the WHO limit (Bundschuh et al. 2012).

In arsenic-rich environments, arsenic-tolerant microorganisms are capable of diverse arsenic biotransformations that contribute to the arsenic biogeochemical cycle (Mukhopadhyay and Rosen 2002; Oremland and Stolz 2003; Zhu et al. 2014; Yang and Rosen 2016). In addition, there is a relationship between tolerance to arsenic and to heavy metals such as cadmium; thus, bacterial resistance to heavy metals may also affect the arsenic biogeocycle (Carrasco et al. 2005).

The aim of this study was to characterize heterotrophic arsenic-resistant bacterial strains isolated from arsenic-contaminated well water from Tucumán, Argentina. The strains are hyper-tolerant to inorganic arsenic, as well as to other toxic metals. Two strains, *Brevibacterium* sp. AE038-4 and *Microbacterium* sp. AE038-20, reduce arsenate aerobically, suggesting they could affect environmental arsenic mobilization.

2. Materials and Methods

2.1. Chemicals, sampling and measurements

Arsenite (As[III]) was added as Na_2AsO_2, and arsenate (As[V]) as $Na_2HAsO_4.7H_2O$ (>99% purity). Reagents were purchased from Fluka Analytical (Sigma Aldrich Co., St. Louis, MO, US) and Anedra (Bs As, Argentina), respectively. All chemicals used in this study were analytical grade or better.

Water from drinking water wells was acquired from Los Pereyra village, Tucuman province, Argentina (26º 57' 4.5", 64º 53'09.4").

Groundwater samples were collected from four selected domestic water wells belonging to four farming families. Water samples were taken from dug wells or open pits at shallow depths (3–6 m). For sampling, 2 L plastic bottles were rinsed with a 20% v/v HNO_3 solution for 24 h, and then five times with distilled water (McCleskey et al. 2004). Approximately 2 L of water were filtered through several nitrocellulose membranes (0.22 µ pore size, 47 mm diameter) (Millipore, Billerica, MA, USA). Arsenic concentrations were determined by electrothermal atomic absorption spectrometry (ETAAS) (APHA, 1992) using a Perkin Elmer atomic absorption spectrometer AAnalyst 100 with graphite furnace HG 800 equipped with a deuterium lamp background corrector and autosampler AS70. A hollow cathode lamp was used as radiation source with lamp current of 18 mA and a 0.7 nm slit. Pyrolytically-coated graphite tubes with L`vov platforms and a hollow cathode lamp were employed. Arsenic was quantified by calibration against aqueous standards using peak area measurements determined at 193.759 nm. The calibration curve was linear to 150 $\mu g.L^{-1}$ (r=0.9985). A mixed Pd and Mg $(NO_3)_2$ matrix modifier solution was used. Each sample was injected in 20 µL of 5% (v/v) nitric acid, Argon (high purity 99.9%) was used as purge gas (250 mL min^{-1}). A Mettler Delta 320 pH meter was used to measure temperature and pH *in situ*. Salinity, conductivity, and total dissolved solids (TDS) were determined using a Tacussel CD 78 conductivity meter. Na^+, K^+, Ca^{++}, Mg^{++}, Cl^-, HCO_3^- and SO_4^- were determined with standardized methods (Greenberg and Clesceri 1992). NO_3^- was determined with a Visocolor ECO Nitrate Test kit (Macherey-Nagel).

2.2. Enrichment cultures and screening of arsenic-resistant bacteria

After water filtration, membranes were cut aseptically and added to 125 mL bottles containing 30 mL of sterile LB_{25} medium at pH 7.0 (Maizel et al. 2016). Na_2AsO_2 [As(III)] at either 5 or 10 mM, or $Na_2HAsO_4.7H_2O$ [As(V)] at either 25 or 100 mM, was added as noted. The flasks were incubated at 30°C on a

rotatory shaker at 150 rpm for 48 h. After incubation, serial dilutions were prepared, and 0.1 mL portions were spread onto LB_{25} agar plates at pH 7.0 containing As(V) or As(III) at the same concentration used for enrichment cultures. The plates were incubated at 30°C for 48 h. Single colonies with visibly different morphology were picked from the plate and streaked onto fresh medium with the same arsenic species and concentrations. This procedure was repeated several times to ensure purity of the strains. The pH of the LB_{25} medium was checked before plating.

2.3. DNA preparation, PCR amplification and phylogenetic analysis

DNA was extracted from pure cultures using cetyl trimethylammonium bromide (CTAB) (Ellis et al. 1999). The quality of the DNA was determined by gel electrophoresis with 0.8% agarose after staining with ethidium bromide. DNA purity was assessed from the A_{260}/A_{280} and A_{260}/A_{230} ratios (Johnson and Whitman 1997). Universal primers 8f (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492r (5'-GGTTACCTTGTTACGACTT-3') (corresponding to position 8-27 and 1492-1509, respectively in the 16S rRNA sequence of *Escherichia coli*) were used to amplify the 16S rRNA by PCR, as previously described (Quillaguamán et al. 2004). Sequencing was performed directly on PCR amplicons using Macrogen sequencing service (Macrogen Inc., Korea). The sequences were analysed with Chromas software (Technelysium, Tewantin, Australia). The identity and similarity to the nearest neighbor of sequences were obtained by using the BLAST (Basic Local Alignment Search Tool) algorithm (Altschul et al. 1990) through alignments performed with BLASTn (http://www.ncbi.nlm.nih.gov/BLAST).

A phylogenetic tree was constructed using MEGA 7 software based on 16S rRNA sequences obtained from each of the six bacterial isolates that had been selected for further characterization (Kumar et al. 2016). A bootstrap consensus tree was inferred from 1000 replicates to represent the evolutionary history of the taxa analyzed (Felsenstein 1985). The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei 1987). The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al. 2013).

2.4. Characterization of arsenic-resistant strains

Tolerance of the bacterial isolates to arsenic was tested on LB_{25} agar plates at neutral pH containing 5, 10, 15 or 20 mM) As(III) or 25, 50, 75, 100 or 200 mM As(V), as indicated. LB_{25} agar plates without arsenic were used as control. An inoculum of each strain (100 mL) was grown overnight on LB_{25} broth without arsenic at 30°C on a rotary shaker at 150 rpm. Five µl drops from this culture were deposited on the plates at several As(III) and As(V) concentrations until completely absorped. The plates were incubated 48 h at 30 °C. *Shewanella* sp. A33 (Saltikov et al. 2005) was included as a reference strain. The experiment was conducted in duplicate. Isolates able to grow at the highest concentrations of As(III) (20 mM) and As(V) (200 mM) were selected for further characterization.

Arsenic-resistant isolates were further characterized for tolerance to inorganic arsenic in liquid medium, tolerance to heavy metals (Cr, Cu and Cd), antimicrobial activity, temperature or pH. Inocula were prepared as previously described. Bottles of 125 mL with 20 mL of LB_{25} were used for all experiments. Bottles were inoculated (10%) with the inoculum culture and incubated for 24 or 48 h at 30 °C and 150 rpm.

Tolerance to heavy metals was assayed in LB_{25} agar plates at neutral pH. Isolates were streaked onto agar plates containing the indicated concentrations of heavy metals (Polti et al. 2007). Tolerance to the corresponding metal was determined semi-quantitatively by measuring length of growth along the streak. Each assay was performed in triplicate.

The ability of each bacterium to produce antimicrobial activity was examined using a deferred antagonism method (Gratia and Fredericq 1946; Fredericq et al. 1947). The pH of the LB_{25} medium was confirmed in each case before plating.

2.5. Arsenic biotransformation

Two isolates with the highest resistance to As compounds were examined for their ability to transform either As(III) or As(V). Each strain was assayed in 15 mL Falcon tubes containing 2 mL of LB_{25} medium, amended with 1 µM of either arsenic species. Tubes without bacteria were included as abiotic control, to confirm species stability in the liquid medium, as described above. After incubation, aliquots of 0.5 mL were collected in triplicate from the stationary phase and were centrifuged at 13,000 rpm for 5 min at 4 °C.

Supernatants were filtered through ultracentrifugation membranes (Amicon®) for speciation by HPLC ICP-MS, as described (Zhang et al. 2015). A C18 reverse phase column (Jupiter 300) was isocratically eluted with a mobile phase composed of 3 mM malonic acid, 5 mM tetrabutylammonium hydroxide and 5% of methanol (pH 5.6) at a flow rate of 1.0 $mL.min^{-1}$. The retention times of 1 µM of each arsenical species [As(III), As(V), MAs(V) and DMAs(V)] were used as standards.

3. Results

3.1. Physicochemical characterization of water samples from Los Pereyra

The physicochemical parameters of water samples were determined from four wells at Los Pereyra. The total arsenic content in the four samples ranged between 241 and 2098 ($\mu g.L^{-1}$). The local groundwater showed slightly elevated pH, between 7.4 and 8.3, and high electric conductivity (EC) between 1570 and 5020 $\mu S.cm^{-1}$. Considerable variation was observed for the distribution of major anions: HCO_3^- (740–1303 $mg.L^{-1}$), SO_4^{2-} (73– 865 $mg.L^{-1}$) and Cl^- (21–588 $mg.L^{-1}$). Nitrate concentrations were high in samples 036 and 038. Total dissolved solids (TDSs) were between 958 and 4350 $mg.L^{-1}$ (Table 1). The predominant cation in the waters was sodium, with values between 370 and 1140 $mg.L^{-1}$. Bicarbonate was determined as the predominant anion, with values between 740 and 1303 $mg.L^{-1}$. The levels of Na^+, K^+, Mg^{2+} and anions were considered normal according to the Argentine Food Code (CAA 2007). Values of total alkalinity ranged between 606 and 1069 mg $CaCO_3.L^{-1}$.

3.2. Identification of arsenic-resistant strains

Heterotrophic bacterial strains were recovered in a complex LB enrichment culture supplemented with 100 to 300 mM arsenate. Eighteen morphologically distinct colonies grew at these concentrations of arsenate in solid medium and were identified by amplification and sequencing of the 16S rRNA genes and comparison with the most closely related sequences in the GenBank database (Table 2). Seven of the eighteen strains belonged to the Actinobacteria phylogenetic group, which comprised the majority of the isolates. The gammaproteobacteria group was the second most predominant. Additionally, two representative members of alphaproteobacteria (*Ochrobactrum* sp. and *Brevundimonas* sp.) and one representative member of the betaproteobacteria (*Alcaligenes faecalis* strain SND_5) were found.

3.3. Characterization of arsenic-resistant strains and tolerance to arsenic compounds

Eighteen heterotrophic bacterial strains obtained from the different LB enrichment cultures supplemented with arsenate were further characterized. Samples 036 and 038 showed intermediate concentrations of total arsenic, and sample 038 showed the highest conductivity and concentration of TDS. Isolates from samples 037 and 039 grew at concentrations lower than 100 mM As(V), thus were not characterized further. However, water samples were included in the physicochemical analysis. Additionally, arsenic resistance assays were not performed at high nitrate concentration, and there was no clear relationship in resistance between the two oxyanions. When resistance to arsenicals was assayed in solid LB_{25} medium, all isolates were able to grow at 200 mM As(V), however only six of them were able to grow at the highest As(III) concentration of 20 mM: AE038-4, AE038-5, AE038-9, AE038-12, AE038-16 and AE038-20. These six strains were characterized further. They were able to grow at temperatures 10 to 30°C, but not 55°C. They could grow in media with initial pH values from 3 to 11 (Table 3). A flocculating phenotype in liquid medium was observed for the strains, suggesting biofilm formation. None showed antimicrobial activity against *E. coli* ATCC 35218 and *S. aureus* ATCC 29213.

The six bacterial strains were resistant to high concentrations of arsenic in liquid medium. Five, AE038-4, AE038-5, AE038-9, AE038-12 and AE038-16 grew at 50 mM As(III), the highest concentration of arsenite tested, and at 200 mM As(V). Strain AE038-20 was resistant to 40 mM As(III) and 100 mM As(V) (Table 4). Additionally, most strains were highly tolerant to Cu(II), Cr(VI) and Cd(II), while strain AE038-16 was sensitive to the highest concentration of Cd(II) tested (1 mM) (Table 4).

3.4. Phylogenetic analysis

An evolutionary tree was constructed based on comparative sequence analysis of the 16S rRNA genes from the six resistant strains (Fig. 1). The tree shows a well-established relationship between the six strains, even though they belong to different genera (*Brevibacterium* and *Microbacterium*) according to their 16S rRNA sequences, while the *B. epidermidis* NBRC 14811 reference strain is not directly related to the other strains and is represented by a separate branch in the tree. The strain identified as *B. epidermidis* AE038-4 has a closer evolutionary relationship with *B. linens* AE038-12 than with *B. epidermidis* AE038-9, as would be expected since they were both identified as strains of the same species according to their 16S rRNA sequences.

Additionally, the isolate identified as *M. oxydans* AE038-20 does not appear to be related with *M. oxydans* DSM20578. This could be due to the use of 16S rRNA sequencing as the only method for identification, which is not always reliable (Janda and Abbott 2002). Finally, a close evolutionary relationship was observed between AE038-5 and AE038-16 (both identified as *Brevibacterium linens* strains).

3.5. Biotransformation of arsenicals

Brevibacterium sp. AE038-4, and *Microbacterium* sp. AE038-20 were examined for ability to transform inorganic arsenicals. Although *Microbacterium* sp. AE038-20 was not highly arsenic resistant (Table 4), it was included so that there would be two different genera in the analysis. *Brevibacterium* sp. AE038-4 was selected as a representative member of the other five *Brevibacterium* strains. *Brevibacterium linens* AE038-8 was included as a reference strain (Maizel et al. 2016).

When grown in LB_{25} medium containing 1 μM As(V), *Microbacterium* sp. AE038-20 completely reduced As(V) to As(III), while *Brevibacterium* sp. AE038-4 only partially reduced As(V) (Fig. 2A). When grown in LB_{25} medium containing 1 μM As(III), none of the strains oxidized As(III) to As(V) (Fig. 2B). Additionally, no methylated arsenicals such as DMAs(V) or MAs(V) were produced, indicating that these strains do not methylate As(V) (Fig. 2). The nucleotide sequences of the isolates *Brevibacterium* sp. AE038-4, and *Microbacterium* sp. AE038-20 identified in this study were deposited in the EMBL nucleotide sequence database (GenBank/EMBL/DDBJ) under accession numbers KX369589 and KX369591, respectively.

4. Discussion

Los Pereyra comprises a population of 1,000 inhabitants, living mainly in small agricultural settlements. Agriculture production is generally dependent on artificial irrigation. Drinking water is in many cases drawn from shallow aquifers. The affected populations recognize the extent of As contamination and the health effects of prolonged ingestion of As. In the last decade, efforts have been made by the local government to mitigate the problem. For example, some of these domestic water wells had been closed by 2012, when this study was conducted.

Analysis of four water samples from Los Pereyra revealed slightly alkaline pH values in all water samples (between 7.4 and 8.3), which were considered moderate compared with groundwater from Los

Pereyra (≥9.4) (Bundschuh et al. 2008). These alkaline pH values can dissolve volcanic glass and cause leaching of loess pyroclastic material, which might contribute to the high arsenic content in the water samples. In these ground waters, in which As(V) is predominant, most of the trace elements tend to be mobilized as complex anions or oxyanions and are controlled by reaction with carbonates (Litter 2009). The conductivity values (between 1570 and 5020 µS.cm-1) were considered high. The maximum value of conductivity allowed in drinking water is 1000 $\mu S.cm^{-1}$, according to the Argentine Food Code (CAA 2007), so these waters are outside the limits established as fit for human consumption.

All water samples were high in sodium bicarbonate. High values for total alkalinity (between 606 and 1069 mg $CaCO_3.L^{-1}$) are directly related to the presence of bicarbonates, which also coincides with the slightly alkaline pH values. In general, nitrate levels were higher than the maximum limits established for drinking water (40 $mg.L^{-1}$) according to the Argentine Food Code (CAA 2007), which indicates the presence of faecal matter in the water provided by farm animals that had been observed in the area where the samples were collected. However, variations of these and other ions (K^+, Na^+, K^+, Ca^{++}, Mg^{++} and SO_4^-) were observed (between 4 and 750 $mg.L^{-1}$). The chemical composition of the groundwater from the area is the result of a long contact with fine sediments, a minor interaction with the atmosphere, and the probable mixing with deeper saline water in Tertiary rocks. Dissolution of minerals such as halite and sodium sulfate in the sediments produces the observed Cl^-, K^+ and Na^+ content in the groundwater. Other reactions, such as cation exchange and weathering of aluminosilicates, also contribute to the Na^+ and K^+ content. On the other hand, the presence of SO_4^{2-} and Ca^{2+} can be attributed to gypsum dissolution (Garcia et al 2001).

Analysis of total arsenic revealed variable levels of As (between 333 and 2098 $\mu g.L^{-1}$), in some cases exceeding by more than 200-fold the WHO recommended limit of 10 $\mu g.L^{-1}$ established for arsenic in drinking water (WHO 1993) and the Argentine Food Code (CAA 2007). These values are in agreement with those obtained in previous studies carried out in the region, which have reported total As levels in water wells of around 2000 µg $As.L^{-1}$. It is worth noting that arsenic is from natural origin (Bundschuh et al. 2012).

Growth of eighteen morphologically different colonies was carried out on plates containing either As(III) (from 5 to 20 mM) or As(V) (from 25 to 200 mM). Even though all the strains were able to grow at the highest concentration of arsenate tested, only AE038-4, AE038-5, AE038-9, AE038-12, AE038-16 and AE038-20 grew at the maximum arsenite concentration of 20 mM. When growth of these six strains was

assayed in liquid medium, each was able to grow at 50 mM As(III) except for AE038-20. Similar results had been reported by Liao et al. (2011), who studied growth of diverse bacterial strains belonging to genera such as *Pseudomonas, Psychrobacter, Vibrio, Citrobacter, Enterobacter,* among others, in presence and absence of 2 to 20 mM and 2 to 200 mM As(III) and As(V), respectively. The tolerance to arsenic shown by such strains was determined in solid medium. The minimal inhibitory concentration (MIC) is often higher in solid than in liquid medium, perhaps because of non-uniform distribution of arsenicals throughout the agar (Costerton et al. 1987). The strains reported in our study can be considered "hyper-tolerant" to arsenic compounds according to other definitions of hyper-tolerance or extreme-tolerance (Jackson et al. 2005; Drewniak et al. 2008; Bahar et al. 2012).

When tolerance of the six strains to heavy metals was studied in LB_{25} liquid medium, most of the strains were tolerant to high levels of Cu(II), Cr(VI) and Cd(II). AE038-16 was sensitive to Cd(II) 1 mM. Other extremely arsenic-resistant bacterial strains are also tolerant to heavy metals such as Cd(II) (Dopson et al. 2003), although less than the strains in our study. Resistance to arsenic compounds and to heavy metals may be connected. A pre-treatment of the cells with arsenic has been shown to provide cross-resistance to metals such as cadmium (Carrasco et al., 2005).

From comparative sequence analysis, Actinobacteria was the dominant phylogenetic group. Many Actinobacteria are able to grow at a wide range of pH. For example *Brevibacterium* strains are able to grow between 3.5 and 8.5 (Lukacs et al. 1995), and *Microbacterium* grows between 5 to 10 (Yu et al. 2013). Additionally, *Brevibacterium* strains are producers of a large variety of bacteriocins and other substances with antimicrobial activity, such as linecine A, which inhibits growth of other *B. linens* strains (Kato et al. 1991), linocin M18 and linenscin OC2, which have antimicrobial activity against *Arthrobacter*, *Corynebacterium*, *Micrococcus* and *Listeria* (Valdes-Stauber and Scherer 1994; Maisnier-Patin and Richard 1995), among other metabolites. In contrast, none of the strains described in our study produced antimicrobial activity when tested against the two reference strains, *E. coli* ATCC 35218 and *S. aureus* ATCC 29213. It is possible that metabolites with antimicrobial activity are produced by the strains in different conditions than the ones used in our study. For example, antiviral activity against herpes simplex virus type 1 (HSV-1) was observed in supernatants of *B. linens* AE038-8 cultures when the strain was grown in LB_{25} medium amended with 2 mM arsenite or 2 M NaCl (data not shown).

Bacillus, *Microbacterium*, *Arthrobacter*, *Alcaligenes*, *Kocuria*, *Staphylococcus*, *Variovorax*, *Oceanimonas* (Shivaji et al. 2005; Bachate et al. 2009), *Aeromonas*, *Exiguobacterium*, *Acinetobacter* (Anderson and Cook 2004), *Acidovorax, Stenotrophomonas, Thiobacillus* (Muller et al. 2003) and *Herminiimonas* (Andres and Bertin 2016) have been described as arsenic resistant. Moreover, Dey et al. (2016) reported two strains of *Bacillus* sp. which were resistant to 2.8 mM arsenite and 21.6 mM arsenate. While resistance at those concentrations was described as extremely high, they are much lower than those reported in this study. Actinobacteria have been reported among the most arsenic-resistant bacteria (Jackson et al. 2005; Drewniak et al. 2008). In the case of some Actinobacteria strains such as *Salinispora tropica* and *Frankia alni*, resistance to arsenic is related to novel proteins which result from recent evolutionary events, particularly from fusion between arsenite intake channels and the C-terminal domain of an ArsC arsenate reductase (Slyemi and Bonnefoy 2012).

Strains of *Microbacterium* isolated from arsenic-rich soils have been reported to be highly resistant to As(III) and As(V). For example, strains of *Microbacterium* tolerate up to 30 mM As(III) (Bachate et al. 2009). However, those strains were resistant to only 150 mM As(V), a lower level of tolerance than the observed in the present study. *Microbacterium* sp. A33 isolated from arsenic-rich soils tolerate up to 800 mM As(V) and 28 mM As(III) (Achour et al. 2010). Similarly, a large number of strains belonging to the *Microbacterium* genus isolated from natural environments tolerate high levels of arsenic compounds (Macur et al. 2004; Abou-Shanab et al. 2007; Drewniak et al. 2008; Cai et al. 2009; Chen and Shao 2009). Nevertheless, such strains were isolated from environments such as rocks from gold mines and arsenic- rich soils and not well water. To our knowledge, arsenic-resistant *Microbacterium* strains isolated from natural water have not been reported, although a *Microbacterium lacticum* strain was isolated from water sewage (Mokashi and Paknikar, 2002).

Furthermore, strains of *Brevibacterium* sp. are particularly arsenic-resistant (Ali et al. 2012). Additionally, other Gram-positive genera were reported as extremely tolerant to arsenic compounds. It is possible that the thicker cell wall of Gram positive bacteria provides a barrier to arsenic compounds (Dey et al. 2016). Twelve *Bacillus* sp. strains isolated from arsenic-rich soils from the West Bengal region (India) showed tolerance to concentrations of 40-167 mM As(V) and 16-47 mM As(III) (Majumder et al. 2013). Strains of *Corynebacterium glutamicum* are also highly tolerant to arsenic (Hendrick et al. 1984; Ordoñez et

al. 2005; Mateos et al. 2006). Members of the Proteobacteria were not significantly resistant (Jackson et al. 2005). However, strains belonging to Proteobacteria were previously proposed as contributors to the arsenic biogeocycle in environmental soils (Macur et al. 2004). Betaproteobacteria have been reported as one of the main phylogenetic groups present in natural waters (Jackson et al. 2005). One representative of the *Alcaligenes* genus was detected between the arsenic-resistant bacteria obtained from Los Pereyra wells. Some *Alcaligenes faecalis* strains were tolerant toto 20 mM As(III) (Philips and Taylor 1976).

When *Brevibacterium* sp. AE038-4 and *Microbacterium* sp. AE038-20 were grown in LB_{25} medium containing 1 µM As(V), *Microbacterium* sp. AE038-20 completely reduced arsenate, while *Brevibacterium* sp. AE038-4 only partially reduced arsenate. In contrast, none of the strains were able to oxidize arsenite, perhaps due to the absence of arsenite oxidase genes. Similar results were reported by Bachate et al. (2009) for twenty bacterial isolates obtained from agricultural soils. It is worth noting that the ability to reduce arsenate might vary between different bacterial strains, and the genera here described frequently exhibit low reductive capacity (Simeonova et al. 2004). In addition, the time required for complete reduction of As(V) also varies among different microorganisms. Some bacteria require more than 48 hours in order to completely reduce arsenate (Bachate et al. 2009). Future experiments will examine if the ability of *Microbacterium* sp. AE038-20 to completely reduce As(V) in 24 h could be attributed to the presence of multiple copies of the *ars* operon, as we previously observed with *Brevibacterium linens* AE038-8 (Maizel et al. 2016). Microbial arsenate reduction has been reported to contribute to arsenic contamination since As(III) is more mobile in water than As(V) (Mukherjee et al. 2008). Thus, the ability of *Brevibacterium* sp. AE038-4 and *Microbacterium* sp. AE038-20 to reduce As(V) to the more toxic As(III) could contribute to the high arsenic content in waters from Los Pereyra.

Conclusions

We obtained a relatively limited diversity of genera and phylogenetic groups from wells in Los Pereyra. This is not particularly surprising considering the oligotrophic conditions and high concentration of As in the samples. Six members of the *Brevibacterium* and *Microbacterium* genera were able to grow in the presence of high concentration of As(III) and As(V) and could be considered “hyper-tolerant” to inorganic

arsenic. They were also able to grow at a wide range of temperatures and pH and were highly tolerant to heavy metals. Additionally, *Brevibacterium* sp. AE038-4 and *Microbacterium* sp. AE038-20 reduced As(V) to the more toxic species As(III). Oxidation of As(III) to As(V) was not observed at the same condition. Future studies will include detection of genes and enzyme activities of arsenic tolerance to evaluate their contribution to the arsenic biogeocycle in waters of Tucumán, Argentina.

ACKNOWLEDGEMENTS

This study was conducted as a part of the Project PICT2008-312 of the Ministerio de Ciencia y Tecnología (MINCyT), Argentina and PIUNT 26/G518). The authors acknowledge financial support from NIH grant R01 GM55425 to BPR. DM and PB are postdoctoral fellows from the Consejo de Investigaciones Científicas y Técnicas (CONICET), Argentina. The authors would like to thank Dr. Nancy I. López from IQUIBICEN-UBA for her valuable collaboration with the phylogenetic analysis.

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558 Figure legends

559 Fig 1: Evolutionary relationships of arsenic-resistant bacterial strains. The evolutionary history was
560 inferred using the Neighbor-Joining method. The bootstrap consensus tree inferred from 1000
561 replicates was taken to represent the evolutionary history of the taxa analyzed. The evolutionary
562 distances were computed using the Maximum Composite Likelihood method. *B. epidermidis* NBRC
563 14811, *B. linens* DSM 20425 and *M. oxydans* DSM 20578 were included as reference strains.
564 Evolutionary analyses were conducted in MEGA7 software.

565 Fig 2: Reduction of As(V) to As(III) (A) and oxidation of As(III) to As(V) (B) in LB_{25} medium by
566 selected bacterial strains. *B. linens* AE038-8 was included as reference strain. Standard solution was
567 prepared with aqueous solutions of each arsenical at the final concentration of 1μM. Cps: counts per

second, corresponds to the number of arsenic atoms that are introduced to the ICP-MS nebulizer at the flow rate of 1.0 mL.min-1 used for this study.

Table 1: Physicochemical properties of water samples obtained from different domestic water wells at Los Pereyra village.

Parameter	Sample 036	Sample 037	Sample 038	Sample 039
Arsenic ($\mu g.L^{-1}$)	333	241	1241	2098
pH	8,1	8,3	7,4	7,7
Conductivity ($\mu S.cm^{-1}$)	2290	1570	5020	4780
TDS ($mg.L^{-1}$)	1528	958	4350	3320
Sodium ($mg.L^{-1}$)	510	370	740	1140
Potassium ($mg.L^{-1}$)	13	8	95	21
Calcium ($mg.L^{-1}$)	19	8	250	35
Magnesium ($mg.L^{-1}$)	7	1	83	9
Chloride ($mg.L^{-1}$)	53	21	95	588
Bicarbonate ($mg.L^{-1}$)	740	910	820	1303
Sulfate ($mg.L^{-1}$)	73	59	865	565
Nitrate ($mg.L^{-1}$)	600	40	750	4
Total alkalinity (mg $CaCO_3.L^{-1}$)	606	746	672	1069
Total hardness (mg $CaCO_3.L^{-1}$)	74	25	1074	124

Table 2: Phylogenetic affiliation of the isolated strains according to 16S rRNA gene partial sequencing.

Isolate	Enrichment condition	Closest relative (acc. num.)[a]	Identity	Phylogenetic group
AE038-1	As(V) 300 mM	*Pseudomonas* sp. FGI182 (CP007012)	99%	Gammaproteobacteria
AE038-3	As(V) 300 mM	*Pseudomonas* sp. HN5 (KF135229)	93%	Gammaproteobacteria
AE038-4	As(V) 300 mM	*Brevibacterium epidermidis* (KJ019204)	99%	Actinobacteria
AE038-5	As(V) 300 mM	*Brevibacterium linens* (AY243345)	98%	Actinobacteria
AE038-8	As(V) 300 mM	*Brevibacterium linens* (KJ019204)	98%	Actinobacteria
AE038-9	As(V) 300 mM	*Brevibacterium epidermidis* (GU576981)	97%	Actinobacteria
AE038-12	As(V) 300 mM	*Brevibacterium linens* (KJ019204)	99%	Actinobacteria
AE038-16	As(V) 300 mM	*Brevibacterium linens* (EU046495)	99%	Actinobacteria
AE038-17	As(V) 300 mM	Not determined	-	-
AE038-18	As(V) 300 mM	Not determined	-	-
P036-200/VB	As(V) 200 mM	*Ochrobactrum anthropi* strain S21808 (KF956631)	86%	Alphaproteobacteria
P036-200/VA	As(V) 200 mM	Not determined	-	-
P038-200/VA	As(V) 200 mM	*Alcaligenes faecalis* strain SND_5 (KJ555096)	99%	Betaproteobacteria
P038-200/VC	As(V) 200 mM	*Stenotrophomonas maltophilia* strain faro4_39 (KF792180)	100%	Gammaproteobacteria
P038-200/VB	As(V) 200 mM	*Stenotrophomonas maltophilia* strain faro4_39 (KF792180)	100%	Gammaproteobacteria
P036-100/VA	As(V) 100 mM	*Brevundimonas* sp. SCU-B236 (KJ000846)	100%	Alphaproteobacteria
P036-100/VB	As(V) 100 mM	*Stenotrophomonas maltophilia* strainYNA104-1 (JN867123)	100%	Gammaproteobacteria
AE038-20 (18)	As(V) 300 mM	*Mycrobacterium oxydans* (AB365061)	99%	Actinobacteria

[a] The nearest GenBank neighbors for nearly complete 16S rRNA sequences obtained from isolates and accession numbers. The sequences were aligned with related sequences retrieved from NCBI database.

Table 3: Characterization of arsenic-resistant bacterial isolates regarding their growth at different conditions (temperatures and pH) and ability to produce metabolites with antimicrobial activity.

		Brevibacterium sp. AE038-4	*Brevibacterium* sp. AE038-5	*Brevibacterium* sp. AE038-9	*Brevibacterium* sp. AE038-12	*Brevibacterium* sp. AE038-16	*Microbacterium* sp. AE38-20
[1] **Temperature (°C)**	10	+	+	+	+	+	+
	20	+	+	+	+	+	+
	30	+	+	+	+	+	+
	55	-	-	-	-	-	-
[2] **pH**	3	+	+	+	+	+	+
	5	+	+	+	+	+	+
	7	+	+	+	+	+	+
	9	+	+	+	+	+	+
	11	+	+	+	+	+	+
[3] **Production of antimicrobial metabolites**	*E. coli* ATCC 35218	-	-	-	-	-	-
	S. aureus ATCC 29213	-	-	-	-	-	-

[1] Growth at each condition is reported as + (growth) and - (absence of growth) in LB_{25} at pH 7

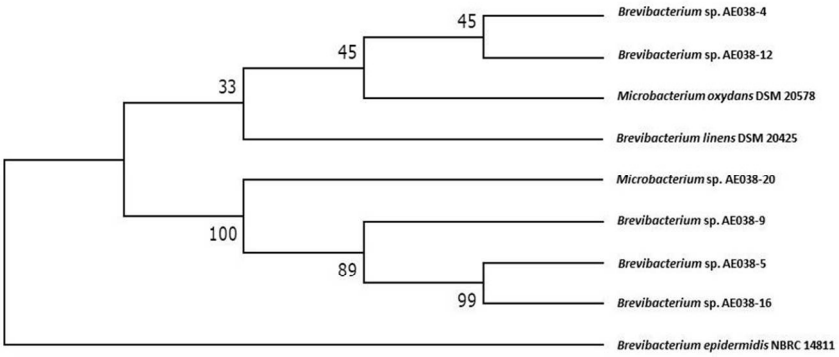
[2] Growth at each condition is reported as + (growth) and - (absence of growth). pH was adjusted accordingly using NaOH 1M and HCl 1M solutions

[3] Antimicrobial activities were tested against control strains *Escherichia coli* ATCC 35218 and *Staphylococcus aureus* ATCC 29213.

Table 4: Characterization of bacterial strains regarding their tolerance to arsenicals in liquid medium and tolerance to heavy metals.

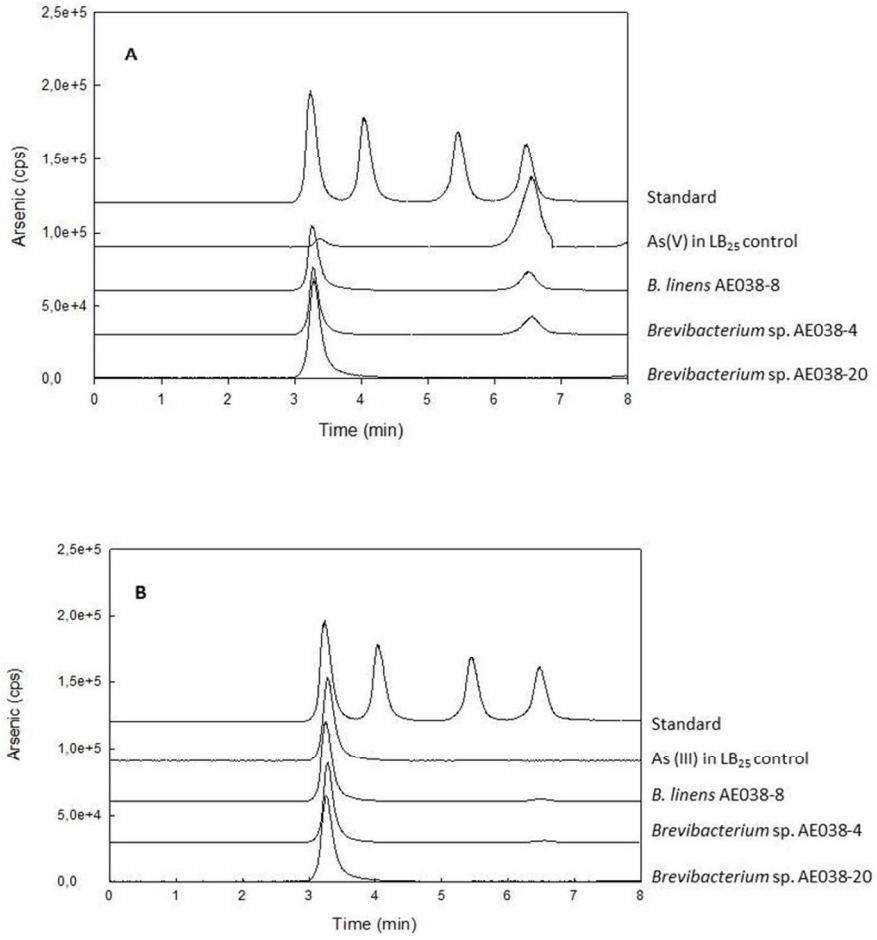
			Brevibacterium sp. AE038-4	*Brevibacterium* sp. AE038-5	*Brevibacterium* sp. AE038-9	*Brevibacterium* sp. AE038-12	*Brevibacterium* sp. AE038-16	*Microbacterium* sp. AE38-20
[1]Tolerance to inorganic arsenic	As(III)	0	+	+	+	+	+	+
		5	+	+	+	+	+	+
		10	+	+	+	+	+	+
		15	+	+	+	+	+	+
		20	+	+	+	+	+	+
		40	+	+	+	+	+	+
		50	+	+	+	+	+	-
	As(V)	0	+	+	+	+	+	+
		25	+	+	+	+	+	+
		50	+	+	+	+	+	+
		100	+	+	+	+	+	+
		200	+	+	+	+	+	-
		300	-	-	-	-	-	-
[2]Tolerance to heavy metals	Cr(VI)	1 mM	+++	+++	+++	+++	+++	+++
		2 mM	+++	+++	+++	++	+++	+
	Cu(II)	2 mM	+++	+++	+++	+++	+++	+++
		4 mM	++	++	+++	+++	+++	+++
	Cd(II)	0.5 mM	++	++	++	++	++	+++
		1 mM	++	++	++	+	-	++

[1] Growth of the strains at the different arsenic concentrations tested is reported as + (growth) or – (absence of growth).
[2] Growth of the strains in the presence of heavy metals was semi-quantitatively determined as + (poor growth), ++ (normal growth), +++ (abundant growth) and – (absence of growth).



Evolutionary relationships of arsenic-resistant bacterial strains. The evolutionary history was inferred using the Neighbor-Joining method. The bootstrap consensus tree inferred from 1000 replicates was taken to represent the evolutionary history of the taxa analyzed. The evolutionary distances were computed using the Maximum Composite Likelihood method. *B. epidermidis* NBRC 14811, *B. linens* DSM 20425 and *M. oxydans* DSM 20578 were included as reference strains. Evolutionary analyses were conducted in MEGA7 software.

99x39mm (300 x 300 DPI)



Reduction of As(V) to As(III) (A) and oxidation of As(III) to As(V) (B) in LB_{25} medium by selected bacterial strains. *B. linens* AE038-8 was included as reference strain. Standard solution was prepared with aqueous solutions of each arsenical at the final concentration of 1µM. Cps: counts per second, corresponds to the number of arsenic atoms that are introduced to the ICP-MS nebulizer at the flow rate of 1.0 $mL.min^{-1}$ used for this study.

90x90mm (300 x 300 DPI)