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Haloarchaea from the Andean Puna: Biological Role in the Energy Metabolism of Arsenic

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Abstract

Biofilms, microbial mats, and microbialites dwell under highly limiting conditions (high salinity, extreme aridity, pH, and elevated arsenic concentration) in the Andean Puna. Only recent pioneering studies have described the microbial diversity of different Altiplano lakes and revealed their unexpectedly diverse microbial communities. Arsenic metabolism is proposed to be an ancient mechanism to obtain energy by microorganisms. Members of Bacteria and Archaea are able to exploit arsenic as a bioenergetic substrate in either anaerobic arsenate respiration or chemolithotrophic growth on arsenite. Only six aioAB sequences coding for arsenite oxidase and three arrA sequences coding for arsenate reductase from haloarchaea were previously deposited in the NCBI database. However, no experimental data on their expression and function has been reported. Recently, our working group revealed the prevalence of haloarchaea in a red biofilm from Diamante Lake and microbial mat from Tebenquiche Lake using a metagenomics approach. Also, a surprisingly high abundance of genes used for anaerobic arsenate respiration (*arr*) and arsenite oxidation (*aio*) was detected in the Diamante's metagenome. In order to study in depth the role of arsenic in these haloarchaeal communities, in this work, we obtained 18 haloarchaea belonging to the *Halorubrum* genus, tolerant to arsenic. Furthermore, the identification and expression analysis of genes involved in obtaining energy from arsenic compounds (*aio* and *arr*) showed that *aio* and *arr* partial genes were detected in 11 isolates, and their expression was verified in two selected strains. Better growth of two isolates was obtained in presence of arsenic compared to control. Moreover, one of the isolates was able to oxidize As[III]. The confirmation of the oxidation of arsenic and the transcriptional expression of these genes by RT-PCR strongly support the hypothesis that the arsenic can be used in bioenergetics processes by the microorganisms flourishing in these environments.

Keywords Arsenic · Haloarchaea · Chemolitotrophic growth · Bioenergetic purposes · Andean Puna

Introduction

Arsenic metabolism is proposed to be an ancient mechanism to obtain energy by microorganisms. It is known that in early earth (3800 million years ago), arsenic compounds were more abundant than in the current crust surface, presenting a biochemical challenge (and opportunity) for the development of early life in the earth [1]. Consequently, microbial life has been exposed to this compound from the beginning, so several microorganisms have developed a variety of dynamic resistance strategies to cope with the toxicity of arsenic, such as precipitation, chelation, intracellular uptake, efflux from the cell or biochemical transformation (redox or methylation) [2–4].

The most common oxidation states for soluble arsenic in nature are pentavalent arsenate (As[V]), and trivalent arsenite (As[III]), present as (AsO_{4-3}) and ($As(OH)_3$), respectively [5, 6], with As[III] being much more toxic than As[V] [5, 7, 8]. Whereas As[V] and As[III] are considered poisonous to life, several prokaryotes use these compounds for bioenergetics purposes [4, 9]. Two enzyme systems are known to mediate the use of arsenic in the bacterial metabolism, either by taking

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electrons from arsenite and oxidizing it to arsenate through the arsenite oxidase (Aio) or by catalyzing the reduction of arsenate to arsenite at the end of the respiratory chain through the arsenate respiratory reductase (Arr) [8, 10–12]. Both Aio and Arr are members of a large group of enzymes containing molybdopterin, commonly known as DMSO superfamily [13]. Genes codifying these enzymes (*aio* and *arr*) were found in members of Bacteria and Archaea domains living in hydrothermal environments where arsenic compounds are present [9, 14, 15]. Although members of the Archaea domain were demonstrated carry out arsenite oxidation in chemolithotrophic growth, this process has not yet probed in isolated haloarchaea [7].

The high-altitude Andean lakes (HAALs) consists of several shallow lakes distributed through the Central Andean region known as Puna. The region called Puna is a plateau located in the Andean mountains along the north of Argentine and Chile and south of Bolivia and Peru and can reach a height of up to 6000 m above sea level (masl). It hosts remote ecosystems, and it is characterized by hard physicochemical and environmental conditions such as low oxygen, high UV radiation, and high concentrations of arsenic, attributed to a natural geochemical phenomenon and volcanic activity, with observed concentrations of arsenic never before described in natural environments [16–28]. Recently, Rascovan et al. (2016) revealed by metagenomic analysis that a red biofilm from the Diamante Lake was widely dominated by Archaea (96%), with all of the Archaeal sequences assigned to the *Halobacteriaceae* family, *Halobacteria* class (also called haloarchaea). They also described a high relative abundance of genes related to the use of arsenic as a bioenergetic substrate and reported an extremely high content of arsenic in water: 115 to 234 mg L^{-1} [22]. Therefore, they suggested that these haloarchaeal communities could use arsenic compounds as bioenergetic substrates to sustain their growth. Besides, phylogenetic analysis of *aioA* was placed into a new monophyletic group, suggesting that the origin of arsenic metabolism in haloarchaea is ancestral [22]. Another work revealed that microbial mats and evaporites from Tebenquiche Lake (Atacama Desert, Chile), with a reported arsenic concentration between 3 and 5 mg L^{-1}, were also dominated by Archaea, with most assigned to the class *Halobacteria*. Similar to Diamante, these authors postulated that *Halobacteria* inhabiting Tebenquiche could be using arsenic for bioenergetics purposes [29].

Based on the metagenomics results reported by Rascovan et al. (2016) and Fernandez et al. (2016), we suggest the hypothesis that haloarchaea from HAALs obtain energy from arsenite oxidation and arsenate reduction. So, in this work, we isolated new strains of haloarchaea from Diamante and Tebenquiche samples and evaluate their role in the arsenic cycle. These results were verified in vitro to determine if they use arsenic as a bioenergetics substrate to support their growth.

Materials and Methods

Description of Sampling Site

Samples were obtained from two lakes located in the Puna region (Fig. 1). Diamante Lake is located inside of Galan Volcano crater at 4589 (masl) in the Catamarca province (Argentina) (26.01°S, 67.05°W). This lake presents multiple extreme conditions including high UV radiation (84 W m^{-2} of UV-AB at noon), high salinity (270 g L^{-1}, 217 mS cm^{-1}), high pH (9 to 11), high day–night temperature range (−20 to + 20 °C), low O_2 pressure, and a hydrothermal vent input, but the most important characteristic is the unusually high arsenic content (115 to 234 mg L^{-1}) [22]. Tebenquiche Lake is located in the northern part of the Salar de Atacama, in northern Chile (Antofagasta Region II), close to the Atacama Desert's hyperarid core zone. The Salar de Atacama lies within a closed basin, which receives water from the San Pedro de Atacama river and many other mountain streams. Tebenquiche, located at 2300 masl, is characterized by sulfates-rich brines, due to its central location, with a high UV–AB radiation (53.4 W m^{-2} at noon), high salinity (38–150 g L^{-1} brine), a slightly alkaline pH (7.4–8.6), a temperature that reached 21.5 °C in March and 31.0 °C in November, and high arsenic content (3 to 5 mg L^{-1}) [29–32].

In this work, samples of a red biofilm from Diamante and microbial mats and evaporites from Tebenquiche were used. These samples were obtained in October 2014 and December 2013 respectively. Subsamples for DNA and RNA extraction were stored in RNA Later™ (Sigma Aldrich^{R}) in the dark at 4 °C and processed within 2 weeks. Subsamples for microbial isolation were stored at 4 °C.

Enrichment and Isolation of Haloarchaea

To perform the isolation of haloarchea strains, samples of biofilm, microbial mat, and evaporite were used. Selective enrichments were carried out using WJK medium [33] containing per liter of deionized water: NaCl 252 g L^{-1}; $MgCl_2 \cdot 6H_2O$ 0.5 g L^{-1}; $MgSO_4 \cdot 7H_2O$ 0.5 g L^{-1}; KNO_3 1 g L^{-1}; KCl 5.84 g L^{-1}; peptone (oxoid) 5 g L^{-1}; yeast extract (oxoid) 1 g L^{-1}; trace element solution 1 mL (HCl, 25% 7.7 M 10 mL, $FeCl_2$ 1.5 g, $ZnCl_2$ 70 mg, $MnCl_2 \cdot 4H_2O$ 100 mg, H_3BO_3 6 mg, $CoCl_2 \cdot 2H_2O$ 190 mg, $CuCl_2 \cdot 2H_2O$ 2 mg, $NiCl_2 \cdot 6H_2O$ 24 mg, $Na_2MoO_4 \cdot 2H_2O$ 36 mg). Solid media were prepared with 1.45% agar (oxoid). Enrichments were achieved as follows: flasks containing 2 g of each sample (biofilm, mat, and evaporite) and 20 mL of WJK. These were added with As[V] at a final concentration of 20 mM and 1 mM of As[III], added as sodium arsenate dibasic heptahydrate ($Na_2HAsO_4 \cdot 7H_2O$) and sodium arsenite ($NaAsO_2$), respectively. Flasks were incubated under stirring at 37 °C for 30 days at 120 rpm and at intervals, and enrichments were

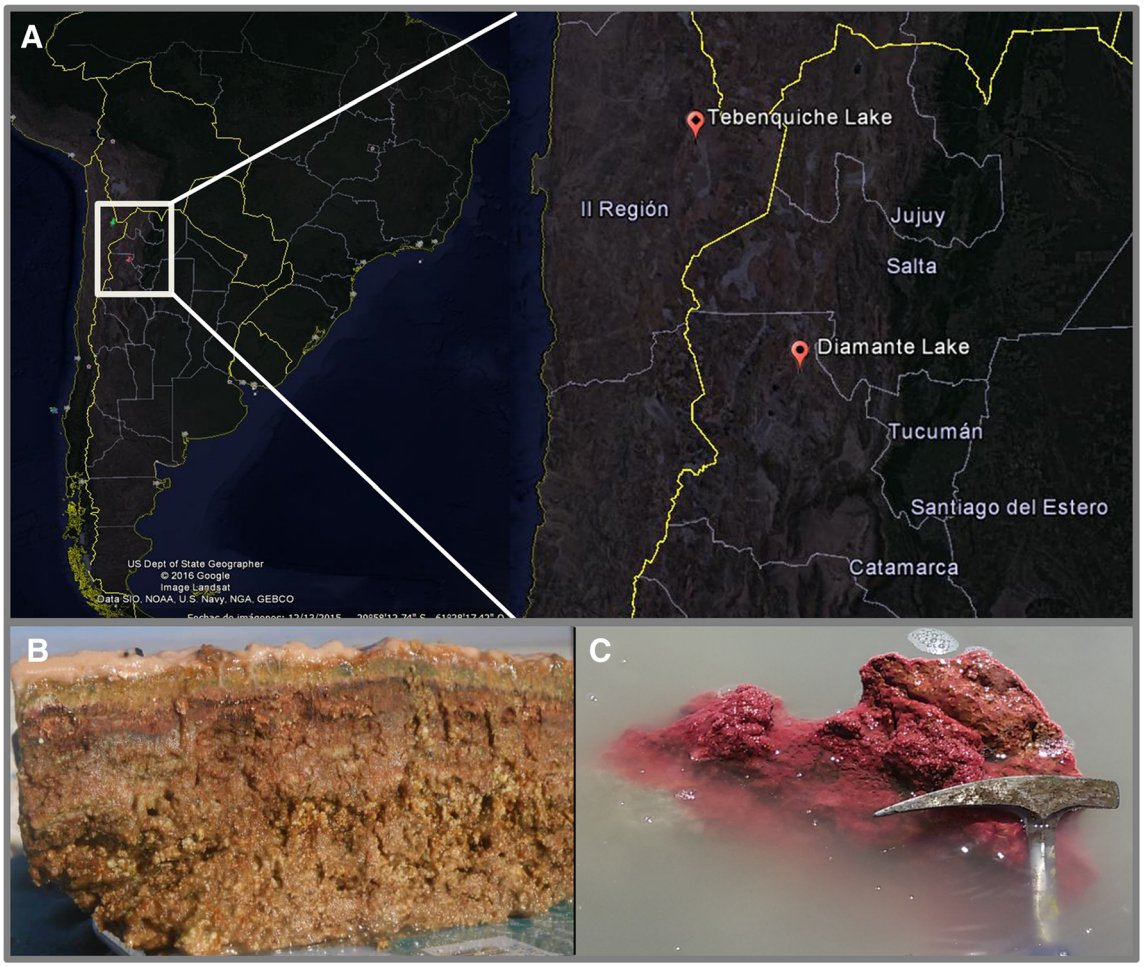

Fig. 1 Sampling sites. **a** Geographical location of the studied lakes (Diamante and Tebenquiche). **b** Mat sample at Tebenquiche. **c** Biofilm sample at Diamante

streaked onto plates of the enrichment medium containing As[III] and As[V] at 1 and 20 mM, respectively. In this way, well-isolated colonies were restreaked several times at enrichment temperatures to ensure axenic cultures, which were confirmed microscopically.

The strains were named DM and TC according to the lake where they were taken. Thus, DM strains come from Diamante and TC from Tebenquiche.

DNA Extraction from the Environmental Samples and the Isolated Strains

Total DNA from all samples (biofilm, microbial mat, and evaporite) were obtained using the Power Biofilm DNA Isolation Kit (MO BIO Laboratories, Inc.) according to the supplied protocol, while the extraction of the genomic DNA from the isolated strains was performed using the UltraClean Tissue and Cells DNA Isolation Kit Sample (MOBIO Laboratories, Inc.) following the protocol supplied by the manufacturer.

Identification of Isolates

The partial 16S rRNA gene was amplified by PCR using the oligonucleotide primers F344 and R915 described in Table 1 [34–36]. The PCR reaction was carried out in a thermocycler T1 thermoblock (Biometra). The reaction mixture consisted of 10 μl 5× buffer, 200 μM each of dNTPs, 250 nmol of each primer (Genbiotech), 2 U Taq DNA polymerase (Invitrogen) to a final volume of 50 μL with Milli-Q water. The thermal profile for PCR was as follows: denaturing at 94 °C during 4 min, and amplification reaction was performed in 30 cycles: denaturation (1 min, 94 °C), primer annealing (1 min, 60 °C), extension (1 min, 72 °C), and a final extension step of 5 min at 72 °C. The PCR products were analyzed in 1% (*w*/*v*) agarose gel and stained with SYBR Safe (Invitrogen ™). To carry out the clone sequencing, the chain termination method of Sanger, by the DNA analyzer 23ABI Prism 3730XL in Macrogen (Korea) was done. The obtained DNA sequences were compared with the data accessible through NCBI (National Centre for Biotechnology Information) using BLASTn. These sequences were also analyzed using EzBioCloud, in order to

Table 1 The primer sequences used for the amplification of *aioA*, *arrA* and 16S rRNA genes

Genes	Primers	Sequences	Size	References
16S rRNA 16S rRNA	F-344 R-915	5′-ACGGGGYGCAGCAGGCGCGA-3′ 5′-GTGCTCCCCCGCCAATTCCT-3′.	571 bp	Stahl and Amann (1991); Raskin et al. (1994); Casamayor et al. (2000b)
Respiratory arsenate reductase (*arrA*)	F-417 R-614	5′-CCC GAG TTC GAG CCS ATC TC-3′ 5′-GCR CAG ATC GMG CTG TGG GA-3′	197 bp	Designed in this work
Arsenite oxidase (*aioA*)	F-1190 R-1507	5′-GCT CMT SAC CGG CAG CGT CG-3′ 5′-YGA TCT CGT CGA TGT CGG CG-3′	317 bp	Designed in this work

find species names as the closest relatives. The sequences were registered at the GenBank database with the following accession numbers: KY290131, KY290132, KY290133, KY290134, KY290135, KY290136, KY290137, KY290138, KY290139, KY290140, KY290141, KY290142, KY290143, KY290144, KY290145, KY290146, KY290147, KY290148 (Table 2).

PCR and Identification of aioA and arrA Putative Genes

In order to detect and identify putative *aioA* (partial sequence codifying the large subunit of arsenite oxidase) and *arrA* (partial sequence codifying the large subunit of respiratory arsenate reductase) genes, degenerate primers (F417-R614 and F1109-R1507) were designed (Table 1). These primers match with a conserved region of the *aio* genes of 7 sequences from (i) scaffold00014 genome of strain AJ67, (ii) contig 79 of the genome of *Halorubrum tebenquichense* DSM14210, (iii) contig 12 of the genome of *Halorubrum kocurii* JCM14978, and (iv) four contigs from Diamante L. metagenome (01149, 01223, 01361, 02851) [22], and to *arrA* gene were used the conserved regions of 10 sequences belonging to (i) genome of *Natronobacterium gregoryi* SP2 (CP003377), (ii) genome of *Halobiforma nitratireducens* (006671188), and (iii) seven contigs of Diamante L. metagenome (00107, 02621, 06381, 05573, 08496, 16,033, 20,150, 04372) [22].

In a first set of experiments, total DNA was extracted from the biofilm, microbial mat, and evaporite samples and used as template for amplification of putative *aioA* and *arrA* whose expected sizes are ~ 197 and 317 bp, respectively. In a second set of experiments, these genes were searched for in the isolated strains (genomic DNA). PCR reaction containing 20 ng of DNA, 0.25 mM of each primer, 200 μM dNTPs (Genbiotech, Argentina), 2.5 μl of PCR buffer 10×, and 1.5 U of Taq DNA polymerase recombinant (Invitrogen Brasil Ltda.) were performed. The final volume (25 μl) was reached by adding Milli-Q water. The PCR thermal profile for both genes was as follows: 94 °C for 5 min and 30 cycles at 94 °C for 30 s, 68 °C for 45 s, and 72 °C for 50 s, followed by 8 min of extension time at 72 °C. The PCR product sizes were determined by comparison with 100 bp DNA ladder (Invitrogen by Thermo Fisher Scientific) on 2% agarose gel by electrophoresis.

Amplification products from genomic DNA (isolates DNA) were also sequenced in Macrogen (Korea). The obtained sequences were analyzed using Chromas version 2.6.1 and aligned by DNAMAN 1.55 v4.03. Identity of sequences was determined by BLASTX against NCBI database at the NCBI website.

Then, all the sequences of the putative *aioA* and *arrA* genes were registered at the GenBank database under the following accession numbers: KY290154, KY290155, KY290156, KY290157, KY290158, KY290159, KY290160, KY290161, KY290162, KY290163, KY290164, KY290165, KY290166, KY290167, KY290149, KY290150, KY290151, KY290152, KY290153.

Arsenic Tolerance Assay

In order to evaluate the arsenic tolerance of the isolated strains, the drop plate technique was used. All isolated strains were grown at 37 °C in flasks containing WJK medium at 120 rpm until optical density (OD) at 600 nm of 0.7. Then, 10 μL of five dilutions were plated (10^{-1}–10^{-6}) in different plates containing solid chemically defined medium (CDM) [37], with 1, 3, 5 and 7 mM of As[III] as sodium arsenite ($NaAsO_2$) and 20, 80, 150, and 250 mM of As[V] as dibasic heptahydrate arsenate sodium ($Na_2HAsO_4.7H_2O$). A plate with CDM without adding arsenic was used as control. All plates were incubated at 37 °C for 15 days.

Effect of Arsenic on DM2 and TC1 Growth

Based on the results obtained in arsenic tolerance assay, one strain from each lake (DM2 and TC1) was chosen for study of growth patterns in the presence of As[III] and As[V].

The strains were grown in WJK medium (50 mL) until exponential phase. Pellets were collected, washed twice, and inoculated to an initial optical density (OD) at 600 nm of 0.08 into four conditions: (1) CDM (control), (2) CDM + As[III] (1 mM), (3) CDM + As[V] (20 mM), and (4) WJK (control). Cultures were incubated at 37 °C under stirring (120 rpm) for 7 days. Growth was monitored in culture medium by

Table 2 Phylogenetic relationship (using 16S rRNA gene), source, and putative *arrA* and *aioA* sequence detection of the isolates

ID	Taxonomic affiliation (closest sequence relative)	Top-hit strain	Identity	Accession number	Origin		Genes	
			%		Site	Source	*aioA*	*arrA*
DM1	*Halorubrum lacusprofundi*	ATCC 49239(T)	97.54	KY290131	Diamante L.	Biofilm	+	+
DM2	*Halorubrum sp.*	AJ67	97.94	KY290132	Diamante L.	Biofilm	+	+
DM3	*Halorubrum aidingense*	31-hong(T)	96.80	KY290133	Diamante L.	Biofilm	+	+
DM4	*Halorubrum xinjiangense*	CGMCC 1.3527(T)	97.53	KY290134	Diamante L.	Biofilm	+	+
DM5	*Halorubrum xinjiangense*	CGMCC 1.3527(T)	97.53	KY290135	Diamante L.	Biofilm	+	+
TC1	*Halorubrum aidingense*	31-hong(T)	97.79	KY290136	Tebenquiche L.	Microbial mat	+	+
TC3	*Halorubrum sodomense*	DSM 3755(T)	94.60	KY290137	Tebenquiche L.	Microbial mat	+	+
TC5	*Halorubrum aidingense*	31-hong(T)	99.14	KY290138	Tebenquiche L.	Evaporite	+	–
TC7	*Halorubrum sp.*	AJ67	96.64	KY290139	Tebenquiche L.	Microbial mat	+	–
TC8	*Halorubrum sodomense*	DSM 3755(T)	98.36	KY290140	Tebenquiche L.	Microbial mat	+	+
TC9	*Halorubrum aidingense*	31-hong(T)	98.30	KY290141	Tebenquiche L.	Microbial mat	+	+
TC11	*Halorubrum aidingense*	31-hong(T)	96.06	KY290142	Tebenquiche L.	Microbial mat	+	+
TC12	*Halorubrum rubrum*	YC87(T)	99.14	KY290143	Tebenquiche L.	Microbial mat	ND	ND
TC16	*Halorubrum Tebenquichense*	DSM 14210(T)	94.04	KY290144	Tebenquiche L.	Microbial mat	+	–
TC18	*Halorubrum aidingense*	31-hong(T)	96.09	KY290145	Tebenquiche L.	Microbial mat	ND	ND
TC24	*Halorubrum aidingense*	31-hong(T)	99.03	KY290146	Tebenquiche L.	Microbial mat	ND	ND
TC28	*Halorubrum aidingense*	31-hong(T)	96.30	KY290147	Tebenquiche L.	Microbial mat	+	+
TC29	*Halorubrum sodomense*	DSM 3755(T)	90.40	KY290148	Tebenquiche L.	Evaporite	ND	ND

measuring optical density (OD) at 600 nm periodically (24 h). Three independent experiments were run for each strain, and mean values and standard deviations were determined.

It is needed to highlight that the samples to perform RNA extraction and As[III] and As[V] detection were taken here.

RNA Extraction and RT-PCR

A microbial mat from Tebenquiche and the biofilm from Diamante were selected to extract total RNA (all the RNA from each community). Two subsets of each sample were processed independently in order to obtain more representative results. To perform the extraction of total RNA, Power Biofilm RNA Isolation Kit (MO BIO Laboratories, Inc.) was used following the protocol supplied by the manufacturer.

Also, RNA extraction from pure cultures of the isolates DM2 and TC1 were completed twice independently. Pellets were obtained at the end of the incubation in the four conditions detailed above. This extraction was performed using UltraClean® Microbial RNA Isolation kit (MOBIO Laboratories, Inc.).

The synthesis of the simple-strand complementary DNA (cDNA) from RNA (total RNA and RNA from pure cultures) was performed using Superscript III First—the Strand Synthesis system for RT-PCR (Invitrogen ™) kit following the protocol supplied by the manufacturer. *aioA* and *arrA* differential expression was assessed by RT-PCR using the same degenerate primers and program detailed above to corroborate its synthesis. The 16S primers (344F-915R) were used as housekeeping gene (Table 1).

As[III] and As[V] Detection

DM2 was grown in CDM with pyruvate as carbon source, containing 1 mM of As[III] and 20 mM of As[V] [37]. They were incubated at 37 °C for 9 days, and supernatant samples were taken at the different growth phases, filtered through a sterile 0.22-μm pore size filter (Millipore), and stored at 4 °C. As[III] and As[V] were analyzed by AEC (Series 200, Perkin-Elmer (Thornhill, Canada) with ICP-MS (Perkin-Elmer SCIEX, ELAN DRC-e; Thornhill, Canada). The selected isotope for mass monitoring by ICP-MS was As^{75} to avoid interference by polyatomic of argon. In Table S1, AEC-ICP-MS conditions and for separation of arsenospecies by reverse phase are resumed. This was performed at the Laboratorio de Espectrometría de Masas (LEMs, San Luis, Argentina).

Results

Detection and Expression of Putative *aioA* and *arrA* Genes in the Communities

In order to assess the presence of *aioA* and *arrA* genes in Diamante and Tebenquiche communities, total DNA from the Diamante's biofilm and Tebenquiche's microbial mat were

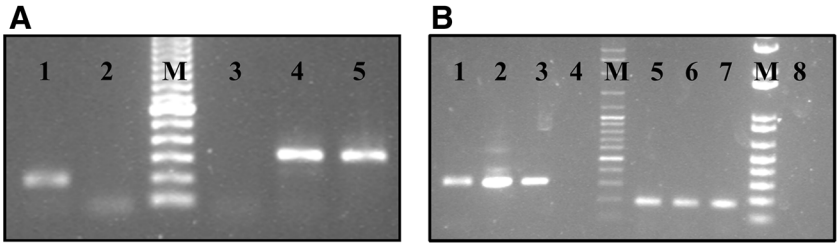

Fig. 2 Identification of *aioA* and *arrA* genes from the communities. **a** Amplification products of total DNA from Diamante. Line 1 *arrA*, line 2 negative control, line M 100 bp molecular marker, line 3 negative control, line 4 *aioA*, line 5 positive control (DNA of DM2). **b** RT-PCR of total RNA from Diamante (Dt) and Tebequiche (Tb) samples. Line 1–4 *aioA* (line 1 cDNA Tb, line 2 cDNA Dt, line 3 positive control (DNA of DM2), line 4 negative control). Line M 100 bp molecular marker. Line 5–8 *arrA* (line 5 cDNA Dt, line 6 cDNA Tb, line 7 positive control (DNA of DM2), line M 1Kb molecular marker, line 8 negative control)

amplified using degenerated primers. Amplification products to both samples were obtained with the expected sizes (197 bp to *arrA* and 317 bp to *aioA*).

Moreover, the transcriptional expression of these genes was studied in total RNA of both samples by RT-PCR. Figure 2b shows the amplification products of the cDNA to both genes in the two samples tested demonstrating they are expressing in the studied communities.

Isolation of Haloarchaea

In order to elucidate if the microorganisms present in these communities could use arsenic for bioenergetic purposes, isolations of arsenic-tolerant haloarchaea were performed. A total of 18 strains were isolated from both samples, 5 from the biofilm (DM1, DM2, DM3, DM4, and DM5) and 13 from the microbial mat and the evaporite (TC1, TC3, TC5, TC7, TC8, TC9, TC11, TC12, TC16, TC18, TC24, TC28, and TC29). Isolates were identified by 16S rRNA gene sequencing. The sequence was compared to those available in the GenBank database using the BLASTn application and also EzBioCloud database. All the reference haloarchaeal identities were confirmed with 94% to 99% similarity with corresponding species. The closest relatives of the DNA sequences revealed the existence to only one genus: *Halorubrum*, and the species names of the closest relatives were *H. aidingense*, *H. sodomense*, *H. lacusprofundi*, *Halorubrum xinjiangense*, *H. rubrum*, and *H. tebenquichense* (Table 2).

Arsenic Tolerance Assay

To evaluate the tolerance of the isolates to arsenic compounds, a screening in different arsenic concentrations was performed (Fig. 3). All of the studied strains presented a high tolerance to both arsenic compounds. They were able to grow in culture media containing up to 250 mM of arsenate (As[V]). In contrast, the tolerance profiles on arsenite (As[III]) were strain dependent. Strains DM2, TC1, TC7, TC9, TC11, TC12, TC16, TC18, TC24, TC28, and TC29 were able to tolerate and grew to the maximum concentration of As[III] tested (5 mM), while DM1, DM3, DM4, and DM5 and TC3, TC5, and TC8 did not grow at concentrations higher than 3 mM of As[III].

Screening of aioA and arrA Genes in the Isolates

Amplification products of putative *aioA* and *arrA* genes were observed in 11 strains (DM1, DM2, DM3, DM4, and DM5 and TC1, TC3, TC8, TC9, TC11, and TC28). These products had the expected size corresponding to 317 and 197 bp,

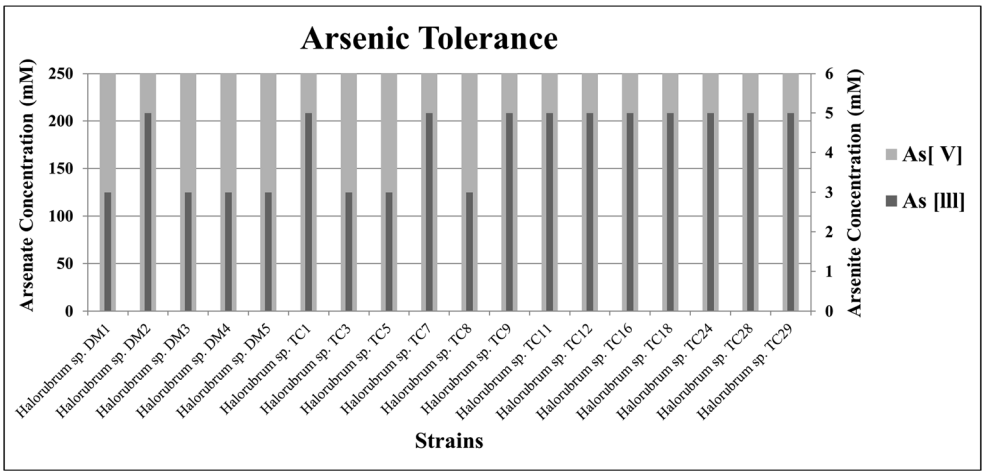

Fig. 3 Maximum concentration of arsenic tolerated by the isolated strains. Their resistance profiles are shown in light gray to As[V] and dark gray to As[III]

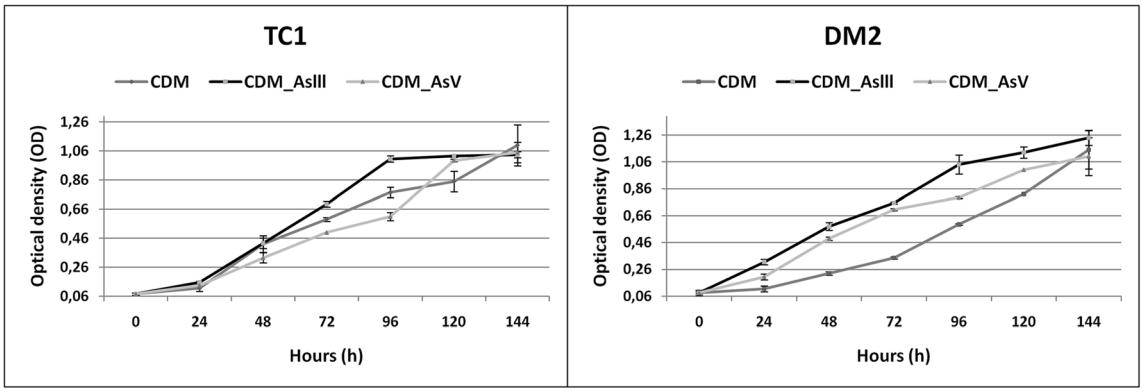

Fig. 4 Effect of arsenic on archaeal growths. Growth curves in CDM and CDM containing As[III] (1 mM) and As[V] (20 mM), monitored by absorbance (600_{nm}) each 24 h. **a** TC1. **b** DM2

respectively. Whilse DNA from three isolates (TC5, TC7, TC16) were successfully amplified using specific primers for *aioA* gene, neither amplification product was observed in four strains (TC12, TC18, TC24, and TC29) (Fig. S1).

It is important to note that all sequences of the amplification products corresponding to the putative *aioA* gene aligned with AioA protein sequences deposited in the NCBI database. Regarding the amplification products corresponding to *arrA* were not aligned with ArrA protein sequences in all cases. Thus, only sequences obtained from Diamante strains (DM1, DM2, DM3, DM4, and DM5) showed identity over 70% with sequences of ArrA in NCBI database (BLASTx), while sequences obtained from the Tebenquiche strains (TC1, TC3, TC8, TC9, TC11, and TC28) gave no hit with ArrA protein sequences.

Effect of Arsenic on DM2 and TC1

Two haloarchaeal isolates were exposed to arsenite and arsenate, and their growth was evaluated (Fig. 4). Both strains presented a better growth in media added with As[III] in comparison to their growth in presence of As[V] and the control culture (CDM without As). Regarding TC1, it reached the stationary phase 24 and 48 h earlier in presence of As[III] than added with As[V] and control cultures (Fig. 4a). Moreover, DM2 also grew better in presence of As[III] and As[V] than in the control, showing growth rates 1.7 and 1.2 times higher in CDM enriched with As[III] and As[V], respectively (Fig. 4b).

aioA and arrA Gene Expression Analyses

To assess the expression of *aioA* and *arrA* genes, DM2 and TC1 were exposed to different culture conditions (CDM, CDM + As[III], CDM + As[V], and WJK). RT-PCR revealed *aioA* gene is expressed by both strains in three conditions (CDM + As[III], CDM + As[V], and CDM), but no expression was observed in the culture media WJK (Fig. 5).

Regarding *arrA* gene expression, it was expressed in none of the assayed conditions to both strains.

As[III] Oxidation by DM2

Quantitative oxidation assays by AEC-ICP-MS, showed an evident transformation of As[III] to As[V] by DM2. It was able to oxidize 10 to 15% of arsenite between 72 and 120 h of incubation (exponential phase), with this oxidation more notable at 216 h (stationary phase).

Discussion

It has been suggested that arsenic, a highly toxic element to most life, is, paradoxically, also an element that can support diverse microbial life. Arsenic can accumulate to high concentrations in selected environments, and preliminary evidence suggests that it may have been of importance as an early means of energy generation during the primordial Earth [1].

HAALs represent unusual and rare environments owing to extreme conditions, such as extremely high arsenic concentration, oligotrophic conditions, high UV, and others stressors. These conditions would resemble those of the ancient Earth lacustrine habitats.

Isolation and Tolerance to Arsenic of Haloarchaeal Strains

In this paper, we further advanced the findings of Rascovan et al. [22], in which metagenomics results revealed haloarchaeal biofilms from Diamante Lake that grow under extreme conditions, whose development seems to be conditioned by an arsenic-based bioenergetic metabolism. In addition, samples of another extreme lake in which a high prevalence of haloarchaea was also revealed by Fernandez et al. [29] were also included in our present study. Our study, proceeding from the metagenomics to the metabolism of single

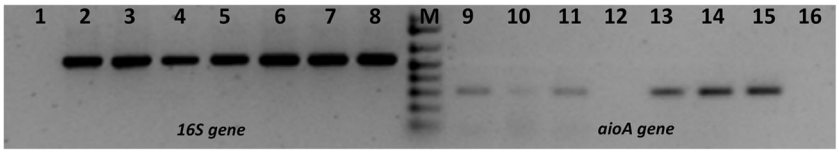

Fig. 5 Transcriptional expression of *aioA* gene in *Halorubrum* sp. DM2. Lines 1–8 Amplification products of 16S cDNA (housekeeping gene) and lines 9–16 amplification products of *aioA* from cDNA of the DM2. Line 1 negative control, line 2 positive control (DNA Tebenquiche), line 3 positive control (DNA Diamante), line 4 positive control (DNA DM2), line 5 cDNA DM2 (WJK), line 6 cDNA DM2 (CDM + As[V]), line 7 cDNA DM2 (CDM + As[III]), line 8 cDNA DM2 (CDM), line M 100 bp (molecular marker), line 9 cDNA DM2 (CDM), line 10 cDNA DM2, (CDM + As[III]), line 11 cDNA DM2 (CDM + As[V]), line 12 cDNA DM2 (WJK), line 13 positive control (cDNA Diamante), line 14 positive control (cDNA Tebenquiche), line 15 positive control (DNA DM2), line 16 negative control

strains, isolated several haloarchaeal strains from both environments that were tolerant to a high concentration of As compounds (at least 0.5 mM As[III] and 20 mM As[V]). Similar observations were described before in strains obtained from the Andean Puna [16, 17, 19, 32, 38–41], which highlights the importance of arsenic in the HAAL. The primers used here gave the expected taxonomic results since all the strains belonged to the haloarchaeal group. Some of them could be the same species, according to the data obtained by NCBI and EzBioCloud database; therefore, to obtain a more precise taxonomic assignation, the complete 16S RNA gene must be sequenced.

The isolation results from both lakes sampled are in agreement with the observations obtained by Rascovan et al. [22] and Fernandez et al. [29], where the class *Halobacteria* was the major contributor to the communities.

Putative *aioA* and *arrA* Genes

In this work, the detection of *aioA* gene in environmental samples from Diamante and Tebenquiche, in addition to the detection and identification of this gene in 14 isolated strains, strongly support the observations of Rascovan et al. [22], where a high relative abundance of genes related to the use of arsenic as a bioenergetic substrate was reported in Diamante samples. Genes encoding arsenite oxidase are widespread in Bacteria and Archaea [42, 43]. In that way, *Herminiimonas arsenicoxidans*, arsenite oxidase *aioBA* genes are located in an arsenic genomic island, which also contains genes involved in arsenic resistance and biosynthesis of a molybdenum cofactor of the aio enzyme [44, 45]. Such a genetic organization has also been observed in *Thiomonas arsenitoxydans* [46, 47], and the presence of *aio* genes on a plasmid has been reported in *Nitrobacter hamburgensis* and *Thermus thermophilus* str. HB8 [47]. Moreover, Rascovan et al. [22] also reported *aioA* gene in haloarchaea is often joined to *aioB* and a chaperon coding gene, the FKBP-like peptidylprolyl isomerase. These observations suggest *aioBA* genes were acquired by horizontal gene transfer [48].

Regarding *arrA*, the only sequences found in haloarchaea belong to *Halorubrum* sp. AD156, *Halobiforma nitratireducens*, and *Natronobacterium gregoryi*. Here, we not only revealed the presence of *arrA* in total DNA but also that this was detected in genomic DNA of 11 haloarchaea strains. Only amplification products of the strains isolated from Diamante could be identified as *arrA* sequences, and those from Tebenquiche could not be identified as *arrA*. This may be because the primers to amplified *arrA* used here were designed based on the metagenomics results from Rascovan et al. [22], which belong to the Diamante lake sample. They gave successful results when environmental samples and isolates from Diamante lake were proved, while these primers did not work in Tebenquiche samples (environmental and isolates). Similar observations were found in other systems [49, 50]. The sequencing project of the Tebenquiche's metagenome is ongoing.

Effect of Arsenic on DM2 and TC1

Not only did DM2 and TC1 show better growth in media added with As[III] in comparison to its growth in control culture (CDM without As) but also that both strains were able to grow at high concentrations of arsenic compounds. Moreover, considering the high concentration of arsenic in the Tebenquiche and especially Diamante environments, it was expected that strains isolated from these sites would exhibit high tolerance. Similar data were described by Dib et al. [17], where most of the isolates from lagoons from Argentinean Puna were able to grow at high arsenic concentration, especially As[III] [17]. Strains isolated from similar environments, *Sulfolobus shibatae* and *Acidithiobacillus caldus*, also improved their growth in the presence of As[III], compared to the control medium [51–53]. Although the potential mechanisms of arsenic resistance have not been studied in this work, the presence of arsenic resistance genes in microorganisms isolated from lagoons of the Andean Puna has been studied in previous works of our group [20, 38, 41, 54].

aioA and arrA Gene Expression Analyses

In order to determine if the detected genes (*aioA* and *arrA*) were expressed in DM2 and TC1, these strains were grown in four different conditions. The fact that the *aioA* gene was the

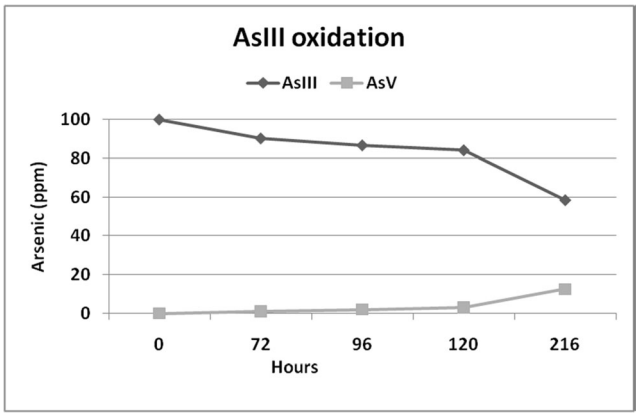

Fig. 6 Oxidation of As[III] by DM2. Arsenite oxidation measured at different time of growth

only transcript in minimal medium and not in rich medium suggests that this gene is regulated by conditions of nutritional stress. The regulation of this gene was studied in some microorganisms, so it is known that the expression of the operon encoding the AioAB enzyme is inducible and activated in presence of As[III] in the medium [9, 48, 55]. Nonetheless, it has only been practically described and there is no experimental data on its genetic expression in haloarchaea. Our report represents the first study to show the genetic expression of *aioA* gen by RT-PCR in pure cultures of haloarchaeal strains, as well as in environmental samples.

Within this study, we also investigated the presence and expression of *arrA* gene in the DM2 and TC1 strains. No expression was observed, which may be due to the study being performed in aerobic conditions. Nevertheless, a positive expression was obtained when total RNA (RT-PCR) was processed. This last result suggests the possibility that respiratory ArrA is being expressed in the system and may play an important role in these niches. Some authors suggest the presence of the *arrA* gene may be a reliable indicator of arsenate anaerobic respiration [49]. In fact, anaerobic experiments are currently conducted about this subject and preliminary results are very relevant.

As[III] Oxidation by DM2

Although bacterial As[III] oxidation had been known for decades [56–58], the chemoautotrophic link of this process could only be described in bacterial domain. Here, the ability of oxidate As[III] under aerobic conditions was evaluated in the haloarchaeal isolate DM2. Our results demonstrate it was able to oxidize As[III] in CDM medium using pyruvate as a unique carbon source (Fig. 6). Further, the DM2 isolated not only showed a greater tolerance to arsenic concentrations compared to the rest of the haloarchaea isolated from Diamante but also revealed better growth in presence of As[III] with respect to TC1.

All the results obtained here support the idea that haloarchaea DM2 could use As to bioenergetic purposes, although more assays are being carried out to further confirm this hypothesis.

Conclusion

In this work, we identified isolated strains from biofilms and mats from lakes Diamante and Tebenquiche that are members of the genus *Halorubrum* ssp. are a group that is generally considered heterotrophic and/or phototrophic. We further confirmed that these strains have the metabolic tools necessary to enable them to use arsenic to maintain their growth.

Therefore, based on all the results obtained here, we suggest that the poisonous arsenic metalloid is undoubtedly playing a substantial role not only in providing tolerance but also as a source of energy to the communities that flourish in these harsh environments. Since many of these microorganisms were likely present during early earth, when conditions of the primitive land were hostile, scarce in nutrients (especially organic compounds), and high in toxic compounds such as arsenic, our studies open a universe of possibilities to elucidate these ancient metabolic pathways that provide critical information about early life in extreme environments.

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