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Characterization of the extremely arsenic-resistant *Brevibacterium linens* strain AE038-8 isolated from contaminated groundwater in Tucumán, Argentina

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ABSTRACT

Brevibacterium linens AE038-8, isolated from As-contaminated groundwater in Tucumán (Argentina), is highly resistant to arsenic oxyanions, being able to tolerate up to 1 M As(V) and 75 mM As(III) in a complex medium. Strain AE038-8 was also able to reduce As(V) to As(III) when grown in complex medium but paradoxically it could not do this in a defined minimal medium with sodium acetate and ammonium sulfate as carbon and nitrogen sources, respectively. No oxidation of As(III) to As(V) was observed under any conditions. Three copies of the *ars* operon comprising arsenic resistance genes were found on *B. linens* AE038-8 genome. In addition to the well known *arsC*, *ACR3* and *arsR*, two copies of the *arsO* gene of unknown function were detected.

1. Introduction

Arsenic (As) is a highly toxic element involved in a number of redox reactions occurring between its As(V) and As(III) oxidation states that have environmental relevance with regard to its mobility and toxicity (Zhu et al., 2014). Despite its toxicity, there are many microorganisms capable of tolerating exposure to and growth in the presence of arsenate [As(V)] or arsenite [As(III)]. This is possible because they carry arsenic resistance (*ars*) genes (Liu et al., 2013). Arsenic in groundwater used as a source for drinking water poses a human health problem world-wide (Naujokas et al., 2013), and is of significant concern in areas of northern Argentina (Escalante et al., 2009; Lara et al., 2012). There are several regions affected, especially in the Tucumán province (located in the north-west region), where arsenic in drinking water reaches concentrations as high as 2 mg/L (~27 μM) (Rodríguez et al., 2006), or more than 200-fold higher than the current recommended MCL of 10 μg L^{-1} (~0.13 μM) (WHO, 1993).

In this study we report on the tolerance to arsenic of *Brevibacterium linens* AE038-8, an isolate obtained from well water in Los Pereyra, a village located in the eastern region of the Tucumán province in Argentina. Strain AE038-8 was found to be highly tolerant to inorganic arsenicals and capable of reducing As(V) to As(III). The genome of *B. linens* AE038-8 has recently been sequenced, and *ars* genes were identified through *in silico* methods (Maizel et al., 2015). Strain AE038-8 was capable of growth at concentrations of ~700 mM As(V) and ~75 mM As(III) in complex medium, and growth was progressively inhibited at higher concentrations. Somewhat greater sensitivity was observed in a chemically defined minimal medium. To the best of our knowledge, this is the first study which reports a bacterial strain that is resistant to such exceptionally high concentrations of inorganic arsenicals.

2. Materials and methods

2.1. Culture media

Cells were grown in sterile Luria Bertani Broth (LB) (Escalante et al., 2009), 25% strength LB ("LB_{25}") or a defined minimal

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medium SeFr1 composed of (g L^{-1}): K_2HPO_4 (0.225), KH_2PO_4 (0.225), NaCl (0.46), $(NH_4)_2SO_4$ (0.225), $MgSO_4.7H_2O$ (0.117), $CaCl_2.2H_2O$ (0.06), yeast extract (0.2), sterile additions/10 ml: vitamin stock solution (0.1 ml), sodium acetate (10 mM) (Oremland et al., 1994) and 1 ml of SL10 trace elements solution (Widdel et al., 1983). Filter-sterilized solutions of Na_2AsO_2 or Na_2HAsO_4 were added as indicated.

2.2. Analytical

Arsenic chemical speciation was determined by high-performance liquid chromatography (HPLC) using a Shimadzu VP series chromatograph with a UV–visual detector (SPD- 10AVP) set at 210 nm. Arsenate and arsenite were separated by using two columns in series (Bio-Rad Aminex HPX-87H and Hamilton PRP X300) with a 0.016 N H_2SO_4 eluent (flow rate, 0.6 ml/min). Retention times for arsenate and arsenite under these conditions were 11.7 and 16.4 min, respectively (Hoeft et al., 2004). Na_2AsO_2 solution and $Na_2HAsO_4.7H_2O$ (0.05 M), >99% purity) used in all experiments 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.

2.3. Enrichment and isolation of strain AE038-8

Bacteria were isolated from drinking water wells from Los Pereyra (Lat: 26° 59′ 15.3″, Long: 64° 53′ 56.1″) in north-east Tucumán province. The following physicochemical characteristics were determined for the water sample: conductivity (μS/cm), 5020; temperature (C), 17.8; TDS (mg L^{-1}), 4350; chemical components (mg L^{-1}); total As (1.24); Na (740); P (95); Ca (250); Mg (83); Cl (95); sulfate (865); nitrate (750); alkalinity (as milligrams $CaCO_3$/L) (672). Enrichment procedures consisted of successive cultivation on LB_{25} liquid medium amended with increasing concentrations of As(III) (1–20 mM) or As(V) (10–400 mM). All the enrichments were incubated aerobically at 30 °C in an orbital shaker at 150 rpm. After incubation, 100 μl of the resulting cell suspension was plated on LB_{25} agar, and incubated at 30 °C. A single colony was picked from the agar plate and streaked to purity. Representative colonies with different morphotypes were selected for further characterization. Strain AE038-8 was isolated from the water sample 038 that contained 300 mM As(V). The strain was later identified as *B. linens*, according to the partial sequencing of its 16S rDNA gene (NCBI Acc. Num. KJ019204.1).

2.4. Growth conditions for arsenic tolerance and arsenic speciation assays

Both LB_{25} and SeFr1 ("minimal medium") media were utilized for arsenic speciation analysis. Each was supplemented with either As(III) or As(V), both at 5 mM. Incubations were carried out in 125 ml conical flasks containing 50 ml of the liquid media. An inoculum (100 ml) was grown overnight on LB_{25} without arsenic at 30 °C on a rotary shaker at 125 rpm. Flasks with LB_{25} or SeFr1 media amended with As(III) or As(V) were inoculated (10%) with this culture. All flasks were incubated for 32 h at 30 °C and 125 rpm. Each experimental growth condition was run in triplicate. Samples of 0.5 ml were taken every 2 h for cultures in LB_{25} and every 4 h for cultures in SeFr1 and filter-centrifuged at 10,000 rpm at 8 °C for 15 min using 0.45 μm filters. The resulting supernatant was stored at −80 °C for later arsenic speciation analysis. Tolerance of *B. linens* AE038-8 during growth to As(III) and As(V) was determined using the two liquid media, LB_{25} and SeFr1 supplemented with either arsenite or arsenate at increasing concentrations. As(III) concentration tested were 10, 15, 20, 25, 30, 40, 50, 75 and 100 mM for SeFr1 medium, and 25, 30, 40, 50, 100, 250 and 500 mM for LB_{25}. The As(V) concentrations tested were 100, 200, 300, 400 and 500 mM for SeFr1 and 150, 200, 250, 500, 750, 1000 and 1200 mM for LB25 medium. One ml samples were taken every 4 h to measure O.D. 600. Arsenic-free controls were carried out for each condition.

2.5. DNA preparation, genome sequencing and bioinformatic analysis

DNA extraction from AE038-8 strain was performed by the CTAB method according to Ellis et al. (1999) using 2 ml of a pure culture. The quality and quantity of DNA were checked by a 0.8% agarose gel electrophoresis after staining with ethidium bromide. Genome sequencing was performed using Illumina HiSeq platform and gene prediction and annotation was performed at Oak Ridge National Laboratory as described previously (Brown et al., 2012). *B. linens* AE038-8 draft genome is available at DDBJ/EMBL/GenBank under the accession number JTJZ00000000 (Maizel et al., 2015). Graphics of the arsenic-related genes detected on *B. linens* AE038-8 full genome were designed to scale with SnapGene Viewer software (version 2.5).

3. Results

3.1. Arsenic resistance

When grown in LB_{25} supplemented with various concentrations of either As(V) or As(III) (Fig. 1), *B. linens* AE038-8 proved highly tolerant to both. At concentrations of As(V) ≥50 mM, growth became progressively impaired (Fig. 1A). Nonetheless, low but detectable growth (OD_{600}) was still observed at As(V) concentrations in excess of 700 mM. Growth of *B. linens* AE038-8 in minimal SeFr1 medium was about half of that achieved in LB_{25} broth in the absence of arsenate, and the cells were more sensitive to arsenate in minimal medium than in complex medium. Nonetheless, there was still evident cell growth in minimal medium with 500 mM arsenate. Arsenite was more toxic than As(V) (Fig. 1B). In LB_{25} medium, the maximum biomass was reduced by almost 60% at 30–50 mM As(III) compared to the one obtained without As(III). There was a sharp decrease in growth when the medium contained 75 mM As(III), and no growth at 100 mM As(III). The results were qualitatively similar for cells grown in SeFr1 minimal medium, although greater sensitivity to As(III) was observed.

3.2. Arsenic speciation

Cells of *B. linens* AE038-8 reduced As(V) to As(III) in LB_{25} culture medium during growth (Fig. 2A). When the strain was grown with 5 mM As(V) for up to 32 h, about 40% was recovered as As(III), and the remaining As(V) was less than 20% of the initially added arsenic. The remainder of the arsenic is unaccounted for but might have been bound to cellular constituents. No organoarsenicals were detected in the medium by ICP-MS analysis (data not shown) even though a putative *arsM* gene for an As(III) methyltransferase was identified in the genome of the organism (Maizel et al., 2015). Cells incubated with As(III) did not oxidize it to As(V) in LB_{25} medium (not shown). Interestingly, no reduction of As(V) was observed during growth in SeFr1 minimal medium (Fig. 2 B), nor was As(III) oxidized in this medium (data not shown). It is possible that the *ars* genes were not induced in cells grown in minimal medium.

3.3. Genome annotation of arsenic resistance

The annotated genome for *B. linens* strain AE038-8 showed the presence of two copies of the well-studied *ars* operon, *ars1* and *ars3*

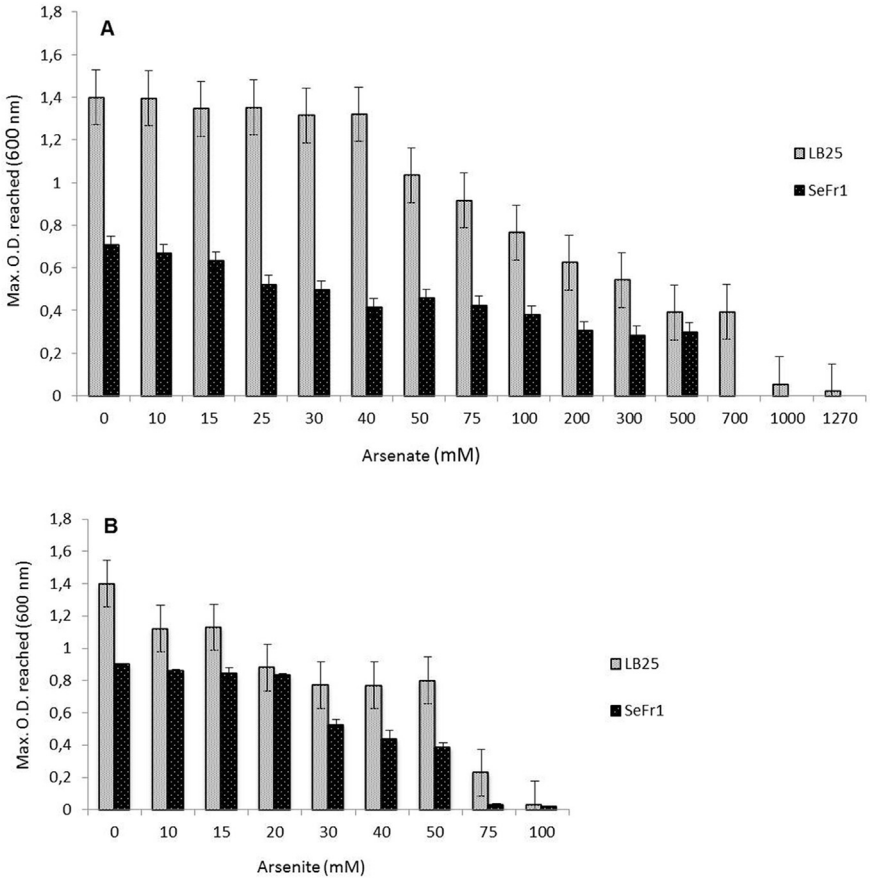

Fig. 1. Maximum OD_{600nm} reached by *B. linens* sp. AE038-8 at different As(V) concentrations (A), or As(III) concentration (B) using either LB_{25} or minimal medium $SeFr_1$ as indicated. The symbols represent the experimental mean of three separate cultures. Vertical bars represent +1 standard error (n = 3). Where no bars are evident, error was smaller than the symbols.

(Fig. 3A and C respectively), and a third *ars* operon (*ars2*) with genes less well represented than in other *ars* operons (Fig. 3B). As expected, each of the three *ars* operons included an *arsR* gene encoding an As(III)-responsive transcriptional repressor. It is not clear how many of the genes shown in Fig. 3 are actually transcriptionally coupled, but their proximity to each other suggests that they are in three distinct operons. Two copies of the *ACR3* gene (encoding a membrane efflux permease for arsenite) were contained in *ars1* and *ars3*. Alignment of the two ACR3 protein sequences (corresponding to ACR3-1 and ACR3-2) was performed through the protein BLAST search tool of the NCBI database (http://blast.ncbi.nlm.nih.gov/Blast.cgi) (Fig. 4). ACR3-1 showed 92% identity with the arsenite efflux pump ACR3 of an uncharacterized transporter in another strain of *B. linens* (acc. Number WP_009885352). Protein ACR3-2, on the other hand, was 93% identical with an arsenic transporter of *B. linens* strain VCM10 (acc. Number WP_025780034). These two proteins share the same size (365 amino acids) and an alignment performed between both sequences through ClustalO software (http://www.ebi.ac.uk/Tools/msa/clustalo/) showed that they are highly conserved, with 65% identity with each other.

Three copies of *arsC* genes (encoding for a cytoplasmic arsenate reductase) were also found on the genome. *arsC1* and *arsC3* were located in *ars1* and *ars3* (Fig. 3A and C). *arsC2* was located on the opposite strand from *ars2*, so it is not clear if it is part of the transcriptional unit (Fig. 3B). Multiple alignment of the three ArsC protein sequences against the NCBI protein database showed high similarity with the ArsC family related to the low molecular weight protein phosphotyrosine phosphatases (LMW-PTPases) (data not shown), represented by the enzyme characterized in *Bacillus subtilis* (Bennett et al., 2001) and the one in the pI258 plasmid of *Staphylococcus aureus* (Messens and Silver, 2006). These enzymes are widely found in microbes, and their role as arsenate reductases has been well documented (Li et al., 2003; Mukhopadhyay and Rosen, 2001; Musumeci et al., 2005). This class of arsenate reductases uses thioredoxin as the source of electrons for reduction, and in *ars1* genes encoding a thioredoxin and thioredoxin reductase (Thx/TxR) were found immediately upstream of ACR3-1 (Fig. 3A). Thioredoxin genes have been observed in other *ars* operons. *arsO* genes are found in many *ars* operons and were represented in *ars2* and *ars3,* even though the role of this putative oxidoreductase in arsenic resistance is not known.

4. Discussion

B. linens strain AE038-8 was isolated from an enrichment culture initiated in a complex LB medium that was supplemented with a high concentration of arsenate (300 mM). Arsenic-resistance assays are often conducted in such complex media (Bachate et al., 2009; Butt and Rehman, 2011; Sunita et al., 2012). However, we also studied the behavior of our strain utilizing a minimal defined medium as well, to assure ourselves that there were no media biases in our investigations. Other studies have also employed defined minimal media (e.g., Krumova et al., 2008; Liao et al., 2011; Bahar et al., 2012). The choice of medium being one of either convenience or expedience, the presumption being that it would not

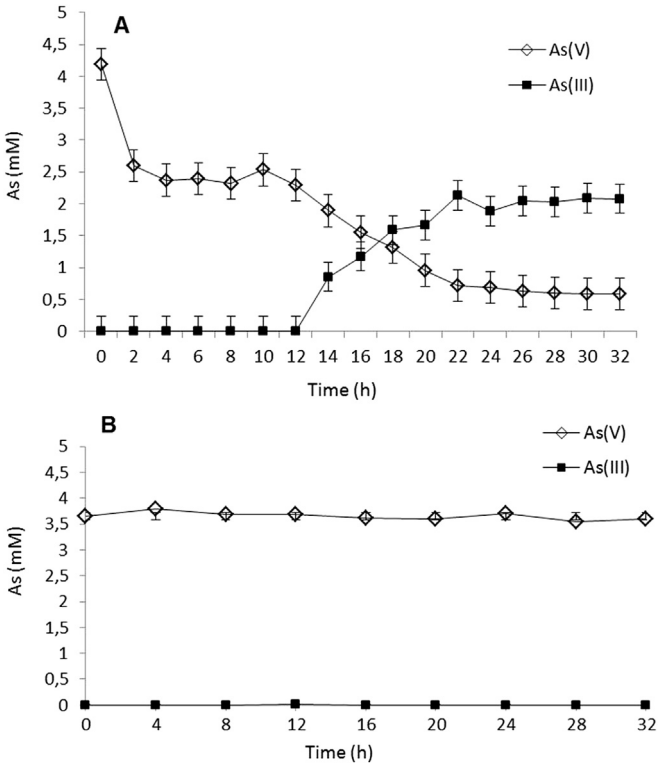

Fig. 2. Arsenic speciation changes in LB_{25} medium supplemented with either 5 mM As(V) (A) or As(III) (B), final concentrations. The symbols represent the experimental mean of three separate cultures. Vertical bars represent +1 standard error (n = 3). Where no bars are evident, error was smaller than the symbols.

make much of a difference with regard to the ability of the microbe tested to either resist application of arsenic or to carry out arsenic redox reactions between As(V) and As(III).

When the tolerance to different concentrations of As(III) and As(V) was investigated using diluted complex LB (LB_{25}) and minimal SeFr1 media, strain AE038-8 was able to grow well on both over a wide range of arsenic concentrations. The MIC, defined as the lowest concentration of arsenical that will inhibit visible growth after overnight incubation (Andrews, 2001), was determined to be 1 M for growth on LB_{25} medium amended with As(V). MIC values for SeFr1 were not determined, but the strain was still able to grow at 0.5 M, the highest concentration of As(V) tested. MIC values for As(III) were determined to be 75 mM and ~50 mM for LB_{25} and SeFr1, respectively.

Broad tolerance to arsenic over a wide range of concentrations have been previously described for many environmental isolates (Li et al., 2003; Patel et al., 2007; Pepi et al., 2007; Liao et al., 2011; Fan et al., 2008; Das et al., 2014). What makes our study remarkable is that only a few studies have reported bacterial strains that were able to tolerate arsenic at concentrations comparable to our study (Achour et al., 2007; Drewniak et al., 2008; Oller et al., 2013). It is worth noting that the arsenic-tolerance assays mentioned above were conducted using agar plates. This point is important, since growth on solid media alone may not give a true picture of arsenic tolerance for many microorganisms because of the formation of arsenic gradients in agar (Costerton et al., 1987; Jackson et al., 2005). Therefore, tolerance assays should be compared in both solid and liquid medium.

Even though there is no consensus definition of extreme arsenic tolerance, some reports define it as tolerance to 67 mM As(III) or As(V) (Baker-Austin et al., 2007), while others defined "hyper-

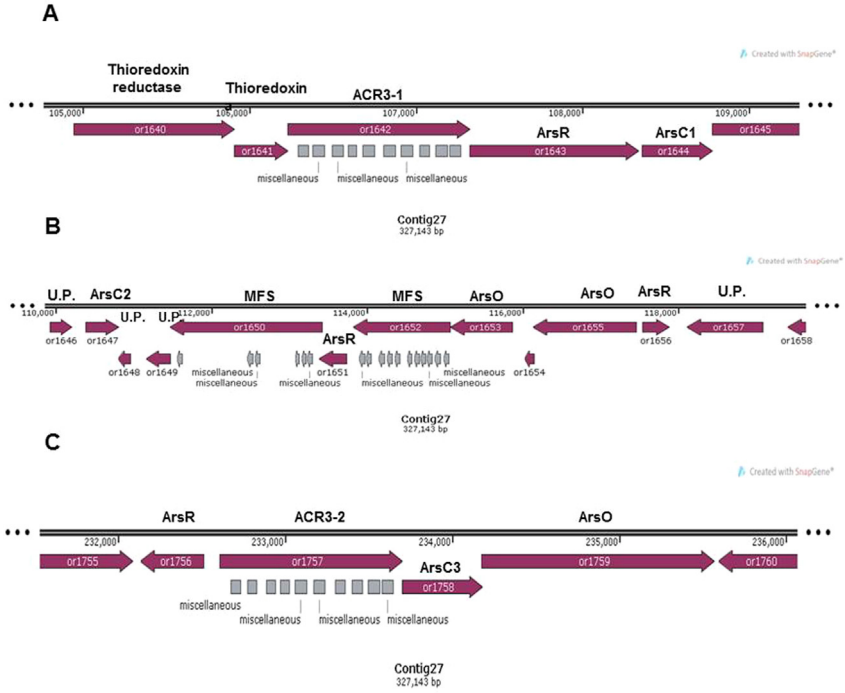

Fig. 3. The *ars* operons of *Brevibacterium linens* AE038-8. Three *ars* operons are found in the genome. These operons have genes for multiple arsenic detoxifying proteins. Two of the operons carry genes for ACR3, an As(III) efflux pump. There are three *arsC* genes encoding ArsC arsenate reductases. Each operon is controlled by an *arsR* gene product for an As(III)-responsive transcriptional repressor. Genes encoding a thioredoxin–thioredoxin reductase pair are also contained in one operon. Additional genes encoding proteins of unknown function such as ArsO or other unidentified proteins (UP) or major facility superfamily (MFS) transporters of unknown function are also encoded within these operons.

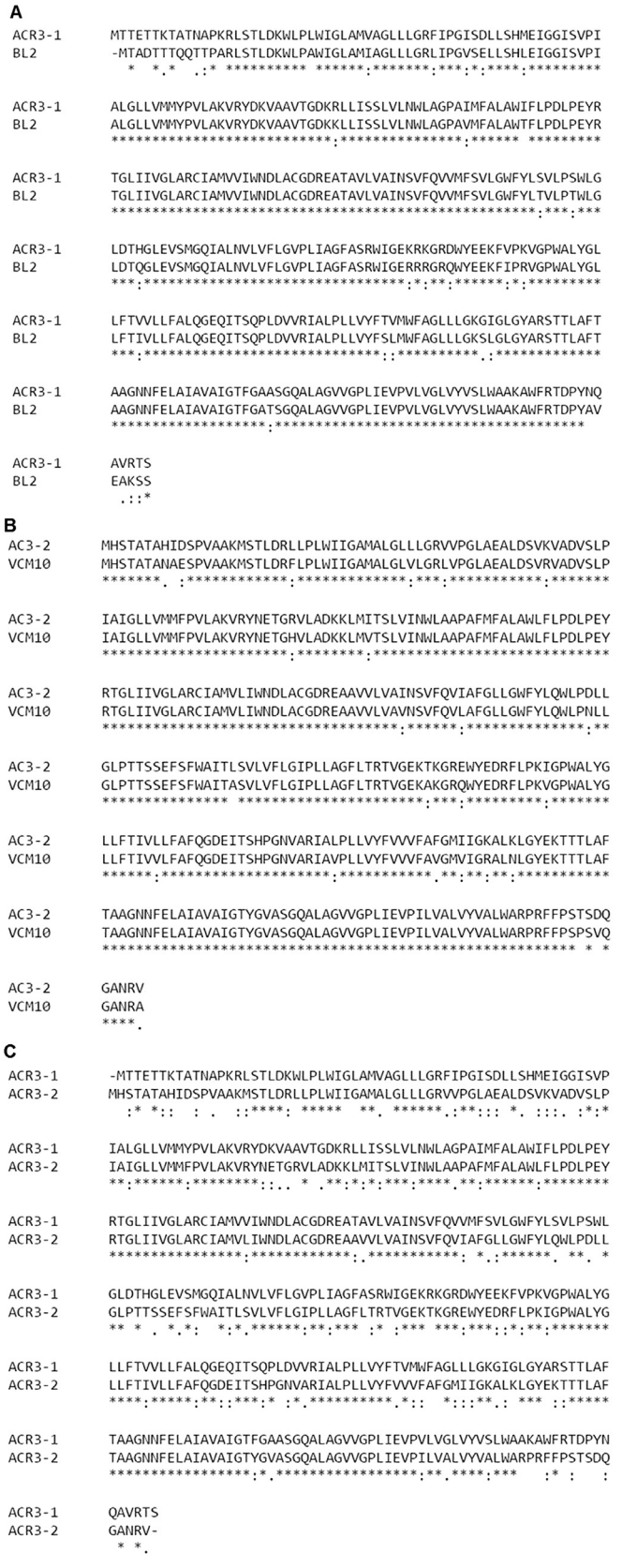

Fig. 4. Sequence alignment of both ACR3 proteins found on *B. linens* AE38-8 genome. ACR3-1 shows a 92% identity with an arsenite efflux pump from *B. linens* strain BL2 (A). ACR3-2 has a 93% identity with an arsenite export protein from *B. linens* strain VCM10 (B). The alignment performed between both ACR-3 sequences from AE038-8 strain genome shows that they are highly conserved, with a 65% identity between each other.

tolerance" as resistance to arsenic concentrations in excess of 100 mM (Jackson et al., 2005). In this case, *B. linens* AE038-8 exceeded all limits for the definition of "extreme tolerance".

Cytoplasmic reduction of arsenate to arsenite by ArsC, with coupled efflux of As(III) by ArsB or Acr3 is the primary mechanism of detoxification in microorganisms (Yang et al., 2012). *B. linens* AE038-8 was able to reduce As(V) to As(III) in LB_{25} culture medium but could not oxidize As(III) to As(V). Similar results were reported by Anderson and Cook (2003), who isolated two bacterial strains, *Aeromonas* sp. CA1 and *Exiguobacterium* sp. WK6, which were able to reduce As(V) to As(III) when grown aerobically in complex medium, though no oxidation of As(III) was observed. Reduction capability of such strains was determined via ArsC enzyme activity, according to method described by Ji and Silver (1992). Several bacterial strains isolated from arsenic contaminated environments have been reported in other works as capable of reducing As(V) but in the majority of cases such reduction activity was based either on enzyme activity assays, or through qualitative methods for the rapid detection of arsenic transformation in the culture medium (Simeonova et al., 2004; Fan et al., 2008; Krumova et al., 2008; Bachate et al., 2009; Liao et al., 2011). It is also worth mentioning that in all cases above, these arsenic redox experiments were only carried out using complex media.

When As(V) reduction was carried out in LB_{25} medium by strain AE038-8, a substantial fraction of the total arsenic was unaccounted for. One possibility is that it could be released from the medium as the volatile trimethylarsine gas as a detoxification mechanism. This has been shown for the bacterium *Rhodopseudomonas palustris* (Qin et al., 2006) and the alga *Cyanidia merolae* (Qin et al., 2009), which are able to release volatile trimethylarsine through the methylation of inorganic arsenic by an As(III) S- adenosylmethionine methyltransferase (ArsM). The genome of strain AE038-8 contains a putative *arsM* homolog (Maizel et al., 2015), which makes the explanation of arsine volatilization plausible for the lack of recovery of total arsenic. However, the absence of any detection of soluble methylated arsenic species during our study make this an unlikely possibility. Another possibility is that arsenic is sequestered in the cells by binding to proteins and small thiols such as glutathione.

In contrast to the results in complex medium, no reduction of As(V) to As(III) was observed when the cells were grown in minimal medium (Fig. 1B). The reason for this disparity between complex and defined media is not readily evident. It could be possible that under nutrient-limited conditions more time is needed for cells to synthesize the enzyme machinery required for reduction of arsenate to arsenite and coupled As(III) extrusion. Alternatively, arsenate reduction in a complex medium (LB-type) could be triggered by the presence of growth factors, which might enhance the reduction mechanism during growth. Its is also possible that rapid growth itself caused a large influx of As(V) that accompanied the abundant nutrients into the cytoplasm.

Various *ars* operons have been reported in a wide variety of bacteria (Diorio et al., 1995; Cai et al., 1998; Rosen, 1999; Butcher et al., 2000). The number of *ars* genes can vary in different organisms and details about their function can be very different as well. The full genome sequence of strain AE038-8 reveals the presence of three *ars* operons, two of which, *ars1* and *ars3*, are very typical with genes for a transcriptional repressor, ArsR, an arsenite efflux permease, *ACR3*, and an arsenate reductase, ArsC. A third arsenate reductase is found in or near *ars2*. Additionally, genes encoding thioredoxin and thioredoxin reductase, which are required for ArsC activity, were also found upstream of the *ars* genes in the *ars1* operon. This multiplicity of efflux permeases and reductases may explain why strain AE038-8 is so highly resistant to As(V). Additionally, two copies of an *arsO* gene encoding for a putative oxidoreductase found in the AE038-8 genome may contribute to

the hyper-resistance of this organism.

5. Conclusions

B. linens AE038-8 is able to tolerate extreme concentrations of As(III) and As(V) in both complex and minimal medium. The primary mechanism of resistance appears to be arsenate reduction, and three genes for the ArsC arsenate reductase are found in the genome (Maizel et al., 2015). We considered the possibility that arsenic was volatilized, perhaps as trimethylarsine gas since this organism has a putative *arsM* gene. The tolerance to high concentrations of As(V) suggested the existence of multiple copies of *ars* operons typically involved in the arsenic resistance system of most microorganisms. *B. linens* AE038-8 is the first bacterial strain reported to date that is able to tolerate such extreme concentrations of As(III) and As(V). Further physiological studies will provide a deeper understanding of the arsenic biogeocycle in waters of the Tucumán province.

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