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Isolation of Arsenite-Oxidizing Bacteria from Arsenic-Enriched Sediments from Camarones River, Northern Chile

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Abstract In Northern Chile, high arsenic concentrations are found in natural water, both natural and anthropogenic sources, a significant health risk. Nine bacterial strains were isolated from Camarones river sediments, located in Northern Chile, a river showing arsenic concentrations up to 1,100 μg/L. These strains were identified as Pseudomonas and they can oxidize arsenite (As(III)) to the less mobile arsenate (As(V)). The arsenite oxidase genes were identified in eight out of nine isolates. The arsenite oxidizing ability shown by the nine strains isolated from arsenic enriched sediments open the way to their potential application in biological treatment of effluents contaminated with arsenic.

Keywords Arsenite-oxidizing · Arsenite resistant Pseudomonas · Arsenite-oxidase gene

Arsenic is a highly toxic metalloid, and considered as a carcinogenic agent by the World Health Organization.

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Arsenic is widely used in pesticide and wood preservative manufacturing, and is present in mining residues. Also, arsenic could be found in nature, due to volcanic activity, forest fires and other natural phenomena, in soils, and surface and ground water (Donahoe et al. 2004; Mandal and Suzuki 2002).

Arsenic is frequently found as arsenite [As(III)] or arsenate [As(V)] (Jackson et al. 2003). The arsenite is 100 times more toxic than As(V). Arsenate is poorly soluble in water and, therefore, less bioavailable (Mandal and Suzuki 2002).

In Northern Chile, high arsenic concentrations are found in natural water courses, from both natural and anthropogenic sources, constituting significant health risks (Arriaza 2005). As(III) removal by biological oxidation has been proposed (Lièvremont et al. 2003). In this respect, various bacterial strains able to oxidize As(III) to As(V) have been described. Such activity has been explained as a detoxification mechanism by heterotrophic bacteria, where As(III) is oxidized by a periplasmatic enzyme called arsenite oxidase. In the case of chemolithoautotrophic, arsenic oxidation is coupled to oxygen or nitrate reduction, using the energy to fix oxygen into the organic cellular material (Donahoe et al. 2004).

Arsenite oxidase genes have been described in the following bacteria: *Alcaligenes faecalis*, *Cenibacterium arsenoxidans*, *Thermus* sp., *Thermus thermophilus* and *Agrobacterium tumefaciens* (Silver and Phung 2005; Kashyap et al. 2006). Arsenite-oxidazing bacteria could play a significant role in both arsenic oxidation and mobilization and could be potentially used in bioremediation of polluted soils and water.

In such context, this paper presents experimental results on the isolation and identification of bacteria capable to oxidize As(III) to As(V), obtained from Camarones river

sediments. This river is located in Northern Chile and features arsenic concentrations up to 1,100 μg/L, due to natural arsenic rock leaching and constitutes the main source of drinking and irrigation water in the region (Yánez et al. 2005).

Materials and Methods

Sediments (upper 2 cm) were colleted from Camarones River, at the Illapata sector (18°57′S, 69°30′W) (Northern Chile). Sediments samples were maintained at 4°C during transportation to laboratory facilities. Sediments samples were inoculated in chemically defined medium (CDM) described previously by Battaglia-Brunet et al. (2002) and enriched with arsenite [As(III)] (75 μM) as NaH_2AsO_3 (Macur et al. 2004). Then, samples were incubated at 25°C for a total of 14 days. Sterile controls were performed using autoclaved sediments.

Bacteria were isolated by adding 1 g of homogenized sediments to 10 mL of NaCl (0.85%) and shaking at 100 rpm for 5 min. Sediments were diluted and 0.1 mL aliquots were plated onto MCD solidified with agar-agar (14 g/L) (CDMA) with arsenite (500 μg/L), and incubated at 25°C for 48 h (Battaglia et al. 2002). After growth, a significant number of different colonies were purified and then tested for grow in CDMA medium containing arsenite (0.5 mM).

Arsenite-oxidazing bacteria were screened using the $AgNO_3$ method described by Simeonova et al. (2004). Agar plates (CDMA) with NaH_2AsO_3 (500 μg/L) were inoculated with isolates, incubated at 25°C for 48 h and flooded with a solution of 0.1 M $AgNO_3$. A brownish precipitate revealed the presence of arsenate in the medium (Lett et al. 2001).

To test strains ability to oxidize arsenite, the isolates were inoculated in 50 mL of CDM with NaH_2AsO_3 (0.5 mM), and incubated at 25°C for 4 days with shaking (180 rpm), As(III) and As(V) concentrations in the culture media were then determined. Experiments were conducted in duplicate. Control experiments using media without inoculation, in presence of arsenite (0.5 mM), were incubated under the same conditions.

Arsenic speciation was performed using ion-pair chromatography (IP-HPLC) combined with hydride generation (HG) and inductively coupled atomic absorption spectrometric (HPLC/HG/QAAS) (Kumaresan and Riyazuddin 2001).

Genomic DNA was extracted from each isolate (10^8 UFC/mL) boiling for 10 min and centrifuged for 5 min at 14,000 rpm in a microcentrifuge. The supernatant was used as DNA template for PCR amplification of arsenite oxidase genes. Each reaction tube contained 2 ng of ADN template, PCR buffer (10 mM Tris–HCl, 50 mM KCl, pH 8,3), degenerated primer 69F (5′-TGY ATY GTN GGN TGY GGN TAY MA-3′) y 1374R (5′-TAN CCY TCY TGR TGN CCN CC-3′) (100 μM each one), $MgCl_2$ (1,5 μM), each deoxynucleotide phosphate (200 μM), *Taq* polymerase Roche (1 U), adjusted to a total volume of 50 μL with highly purified sterile water. PCR was carried out with a P × 2 Thermal cycler (Thermo, Electron Corporation). 50 μL reaction mixtures were composed of 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 2.0 mM $MgCl_2$, 200 nM each primer, 1 unit/μL of *Taq* polymerase (Roche), and 50 ng of template DNA. The protocol consisted of an initial denaturation step (95°C for 5 min) followed by 35 cycles of 94°C for 30 s, 58°C for 30 s, and 74°C for 2 min. A final extension was done for 10 min at 72°C. *E. coli* S17-1 strain, kindly provided by Dr. McDermott, was used as positive control (Kashyap et al. 2006) and *E. coli* K-12 strain as negative control The amplified products (5- to 10-μL aliquots) were separated by electrophoresis in 0.8% agarose gel (0.5 μg of ethidium bromide per mL). The bands were visualized on a UV transilluminator.

Bacterial isolates were identified by 16S rDNA sequence analysis. Bacterial colonies were suspended in nuclease-free water. Bacterial suspension were boiled for 10 min and were centrifuged for 5 min and the supernatant was used as DNA template for PCR amplification of 16S rRNA genes. Each reaction tube contained, 2 ng of ADN template, PCR buffer (10 mM Tris–HCl, 50 mM KCl, pH 8,3), first EUB I (9-27f) (5′-AGT TTG ATC MTG GCT CAG-3′) and P2 (534r) (5′-CTA GCY TTG TAG TTT CAA ACG C-3′) (1 μM each one), $MgCl_2$ (1,5 μM), each deoxynucleotide phosphate (200 μM), *Taq* polymerase Roche (1 U), adjusted to a total volume of 50 μL with highly purified sterile water. PCR conditions were of 35 cycles of denaturation at 94°C for 30 s, annealing 55.6°C for 45 s and elongation 72°C for 1.5 min. PCR products (arsenite oxidase genes and 16S rDNA) were separated by electrophoresis in 0.8% agarose gel, and visualized by staining with ethidium bromide in UV transilluminator.

PCR products of 16S rDNA were purified by using the QIA quick PCR purification kit (Qiagen) following the maker's indications. The sequence was carried out using the Dyenamic ET terminador kit (General Electric) following the maker's instructions, in a 3,100 Avant genetic Analyzer (Applied Biosystem). Analysis of DNA sequences and homology searches were completed with a MEGA-BLAST using the BLAST algorithm for the comparison of a nucleotide query sequence against a nucleotide sequence database.

Results and Discussion

Results show that, after 7 days incubation, enriched sediments were able to oxidize 100% As(III) to As(V). Under

similar conditions, sterile sediments (control) did not have any effect on As(III) (data not shown), suggesting that bacteria are responsible for As(III) oxidation in sediments. These results agree with those reported by Macur et al. (2004) who found arsenic oxidizing activity in soil samples enriched with As(III).

A total of 40 arsenic resistant bacterial strains were isolated, and their ability to transform As(III) to As(V) was screened using the $AgNO_3$ (0.1 M) assay. Nine out of the forty selected isolates can oxidize arsenite aerobically. Such arsenite oxidizing capacity was confirmed by HPLC/HG/AAS. Indeed, Table 1 presents the oxidizing capacity recorded when the nine strains were incubated in presence of arsenite (0.5 mM), showing that all tested strains were able to oxidize more than 95% of As(III) present in the culture medium.

Figure 1 shows that eight out of the nine isolates presented a PCR product approximately 1,200 bp, expected size corresponding to arsenite oxidase genes (Rhine et al. 2007). No amplification was obtained from the isolate FN-57. The results suggest that the arsenite oxidase gene in these isolate differ from the other described arsenite oxidase. Similar results were reported by Rhine et al. (2007) who isolated two bacterial (DAO1 and MLHE-1) that did not amplify any genes for arsenite oxidase, despite showing arsenite oxidizing activity under autothrophic conditions.

The analysis of the 16S rDNA (27–534) gene partial sequences indicates that all isolated strains belong to the *Pseudomonas.* According to the BLAST program, the FN-13, FN-15, FN-66, FN-70, and FN-71 isolates would correspond to the same species, with base sequence identical to *P. fluorescens* P69 (Access number AY973265) y *P. putida* (Access number DQ178233), isolated from a river contaminated with phenolic compounds and featuring the ability to degrade such contaminants (Merimaa et al. 2006). In this case, the sequenced fragment does not allow to discriminate between these two bacterial species since they present differences in bases 558, 615 and 1,104, and cannot be included in the analysis. The FN-41 strain showed greater similarity (99%) to *P. marginalis* JH8 (Access number DQ232737), which is a bacteria cataloged as fitopathogenic; however, the JH8 strains corresponds to a metal-resistant strain (Moberly 2006). The sequences of FN-57 and FN-58 strains showed a 99% similarity to different sequences of *P. putida* isolated from hazardous residues, featuring the ability to use vinil chloride as carbon source (Mohn et al. 1999). The FN-48 strain resulted 99% identical to *P. vancouverensis* (Access number DQ232737), isolated from cellulose effluents (Cámara et al. 2007) (Table 2).

Results presented in this paper constitute the first reported of arsenite oxidizing activity for *Pseudomonas marginalis* and *Pseudomonas vancouverensis*. This work described the first reported about arsenite oxidase genes in *Pseudomonas.* Moreover, the arsenite oxidizing ability shown by the nine strains isolated from arsenic enriched sediments opens the way to their potential application in biological treatment of effluents contaminated with arsenic.

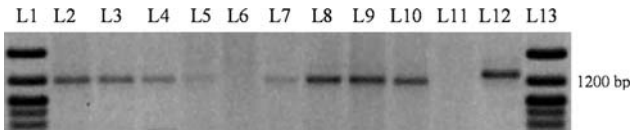

Fig. 1 DNA products derived from PCR amplification of genes coding for arsenite oxidase enzyme. L1: DNA Ladder, L2:NF-13; L3: NF-15; L4: NF-41; L5: NF-48; L6: NF-57; L7: NF-58,; L8: NF-66; L9: NF-70; L10: NF-71; L11: negative control (*E. coli* K-12); L12: positive control (*E. coli* S17-1, pDK402), L13: DNA Ladder

Table 1 Arsenite oxidizing capacity of isolates from Camarones river sediments

Isolates	% Oxidized As(III)
FN-13	95.6
FN-15	97.3
FN-41	98.9
FN-48	96.0
FN-57	95.8
FN-58	100.0
FN-66	98.0
FN-70	100.0
FN-71	100.0

Initial As(III) initial concentration 0.5 mM

Table 2 Closest GenBank Neighbors, sequence similarities of isolates from Camarones river sediments

Isolates	Closest GenBank neighbor (% similaridad)
FN-13	
FN-15	*P. fluorescens* (AY973265)[a] (100)
FN-66	*P. putida* (DQ178233)[a] (100)
FN-70	
FN-71	
FN-41	*P. marginalis* (DQ232737)[a] (99)
FN-48	*P. vancouverensis* (AM293568)[a] (99)
FN-57	*P. putida* (AF094743)[a] (99)
	P. putida (AY391278)[a] (99)
FN-58	*P. putida* (D85995)[a] (98)
	P. putida (AF094742)[a] (98)

[a] GeneBank accession number

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