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Arsenic Resistant Bacteria Isolated from Arsenic Contaminated River in the Atacama Desert (Chile)

G. Escalante · V. L. Campos · C. Valenzuela · J. Yañez · C. Zaror · M. A. Mondaca

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Abstract In this study, arsenic resistant bacteria were isolated from sediments of an arsenic contaminated river. Arsenic tolerance of bacteria isolated was carried out by serial dilution on agar plate. Redox abilities were investigated using KMnO4. arsC and aox genes were detected by PCR and RT-PCR, respectively. Bacterial populations were identified by RapID system. Forty nine bacterial strains were isolated, of these, 55 % corresponded to the reducing bacteria, 4% to oxidizing bacteria, 8% presented both activities and in 33% of the bacteria none activity was detected. arsC gene was detected in 11 strains and aox genes were not detected. The activity of arsenic transforming microorganisms in river sediment has significant implications for the behavior of the metalloid.

Keywords ArsC and aox genes · Arsenic-resistant bacteria · Arsenic-contaminated · Reducing and oxidizing bacteria

Arsenic is a toxic metalloid naturally found as inorganic oxyanion arsenate As(V) and arsenite As(III) species. Arsenate is the predominant specie in oxygenated aqueous environment, whereas arsenite species predominate under anoxic or reduced conditions, being 100 times more toxic than As(V) (Al-Abed et al. 2007; Taerakul et al. 2007; Neff 1997). Arsenite has the ability to bind to sulfhydryl groups of proteins and dithiols such as glutaredoxin. On the other hand, arsenate is a chemical analog of phosphate and can inhibit oxidative phosphorylation (Ordoñez et al. 2005).

Microorganisms are known to play an important role in the biochemical cycle of arsenic, through its conversion to species with different solubility, mobility, bioavailability and toxicity (Silver and Phung 2005). Several bacteria involved in arsenic transformation processes-featuring reduction, oxidation and methylation mechanisms-have been reported (Ellis et al. 2001). Arsenate reduction could occur as a respiratory process or as part of arsenic resistance, through the gene *arsC* encoded arsenate reductase, arsenite oxidation or methylation as well as active extrusion of arsenite from the cell. Arsenite oxidation can be coupled to chemoautotrophic growth, or occurs as a detoxification mechanism (Silver and Phung 2005).

There are regions in the world where arsenic is present at very high levels due to natural geological conditions, as in the Bengal Basin and Northern Chile (Smedley and Kinniburgh 2002). Indeed, in the case of the Atacama Dessert in Northern Chile, arsenic is leached out from volcanic materials naturally present in watershed areas in the Andes, adding to antropogenic pollution due to mining

G. Escalante · V. L. Campos · C. Valenzuela · M. A. Mondaca (✉)
Microbiology Department, University of Concepción, P.O. Box 160-C, Correo 3, Concepción, Chile
e-mail: mmondaca@udec.cl

V. L. Campos
e-mail: vcampos@udec.cl

J. Yañez
Chemical Inorganic and Analytical Department, University of Concepción, Concepción, Chile

C. Zaror
Chemical Engeneering Department, University of Concepción, Concepción, Chile

V. L. Campos
Environmental Science Center EULA-CHILE, University of Concepción, Concepción, Chile

activities. As a result, some rivers contain arsenic concentration levels in the range 0.1–1.5 mgL^{-1}, becoming a serious health risks to the local population (Queirolo et al. 2000; Yañez et al. 2005).

Since the local microbial biota is expected to be adapted to those conditions, this work aims to characterize arsenic-resistant bacteria isolated from sediments obtained from a highly polluted river in Northern Chile.

Materials and Methods

Sediment samples (upper 2 cm) were collected from Camarones River (18°57′S, 69°30′O) of Northern Chile, a narrow and shallow river featuring arsenic concentrations up to 1 mgL^{-1} (Yañez et al. 2005). Sediment samples were maintained at 4°C during transportation to laboratory for further processing.

Microcosm experiments were carried in two flask, each one containing 5 g of sediment and 400 mL of chemically definided medium (CDM) (Macur et al. 2004), enriched with As(III) (75 μM) and As(V) (250 μM) respectively. Then, samples were incubated at 20°C for 14 days.

Bacteria were isolated by adding 1 g of homogenized sediment to 10 mL of NaCl (0.85%), and shaking at 100 rpm for 5 min. The mixture was serially diluted and 0.1 mL aliquots were plated onto R2A agar and incubated at 25°C for 48 h (Battaglia-Brunet et al. 2002). After growth, a number of differents colonies were purified. Bacterial isolates were identified by RapID ONE System and RapID NF Plus System (Remel) according to the supplier's instructions.

The Minimum Inhibitory Concentration (MIC) of isolates was determined by the agar dilution technique,on LB agar plates amended with variable concentrations of sodium arsenite, between 0.5–100 mM and sodium arsenate between 0.5–1,000 mM. Each plate was inoculated with cell suspensions from fresh precultures to a final density of approximately 3×10^7 CFU mL^{-1} and incubated for 24 h at 25°C. An agar plate with bacteria and without the metalloid was used as a control. The MIC is defined as the lowest concentration that causes no visible growth (Valenzuela et al. 2008).

Redox transformation of arsenic was screened using the $KMnO_4$ method described by Salmassi et al. (2002). Chemically defined medium (CDM) with As(III) (500 μM) or As(V) (1,000 μM) were inoculated with isolates, incubated at 25°C for 48 h and 60 μL of 0.01 M $KMnO_4$ were added to 1 mL of the culture. A pink color indicated that arsenate was present, whereas a clear yellow or orange colored solution indicated that arsenite was present.

For *arsC* gene detection, genomic DNA was extracted from each isolate by freezing. The extracted DNA was used as template for the amplification by PCR. The primers sets utilized were arsC-1-F (5′ GTAATACGCTGGAGATG ATCCG 3′) and arsC-1-R (5′ TTTTCCTGCTTCATCAAC GAC 3′) corresponding to the ars operon from *Escherichia coli* according to the description of Saltikov y Olson (2002); and arsC-1-F (5′ AGTCCTGTTCATGTGYAC 3′) and arsC-1-R (5′ TGGCGTSGAAYGCCG 3′) corresponding to the *ars* operon of *Pseudomonas aeruginosa* and *Pseudomonas putida* according to the description of Saltikov and Olson (2002) and Macur et al. (2004). PCR products were separated by electrophoresis in 1.2% agarose gel and visualized by staining with ethidium bromide in UV transilluminator (Muller et al. 2003).

The *aox* gene detection was performed incubating the bacterial strains in R2A broth during 24 h; cultures were induced by addition of 250 mgL^{-1} of As(III) 12 h before extraction. The RT-PCR was carried out as described by Muller et al. (2003).

Results and Discussion

A total of 49 bacterial strains were isolated from sediments based on their abilities to grow in the presence of arsenic. Resistance to arsenic was defined as the ability to grow on agar containing either 7 mM As(III) or 20 mM As(V) at 25°C (Rokbani et al. 2007). Fourty five of 49 isolates were resistant to arsenite and all isolates were resistant to arsenate. The strains UC-9, UC-29, UC-90, UC-31, UC-54, UC-58, UC-101 and UC-72 were capable of tolerating arsenate above 1,000 mM. The strains UC-4, UC-34, UC-66, UC-5, UC-7, UC-39, UC-31, UC-45, UC-60 and UC-68 were capable of tolerating arsenite concentrations greater than 40 mM (Table 1a, b).

Arsenate and arsenite levels tested in this study are higher compared to those used in previous studies. Jackson et al. (2005) reported arsenic resistant levels of 400 and 10 mM of arsenate and arsenite, respectively. Studies conducted by Macur et al. (2004) reported arsenate resistance levels of 0.25 mM, from estuarine samples. Anderson and Cook (2004) obtained one isolate from arsenic-contaminated gold mine tailings that was able to grow at 100 mM arsenate (the maximum concentration tested), but the majority of their isolates showed growth inhibition at lower concentrations.

A qualitative $KMnO_4$ screening technique was used to detect the oxidation of As(III) to As(V) or the reduction of As(V) to As(III). Twenty-seven out of the 49 selected isolates where able to reduce arsenic (55%); two strains was able to oxidize arsenic (4%); four show arsenic oxidizing as well as reducing capabilities (8%), and 16 bacteria had not detectable activity (33%) (data not shown).

Table 1 Tolerance to arsenic of isolates from Camarones river sediment

Strain	Species	MIC (mM)		*ars*C	*aox*
		As(III)	As(V)		
(a)					
Γ-proteobacteria					
UC-5	*Pantoea agglomerans*	40	200	–	–
UC-7	*Pantoea agglomerans*	40	200	–	–
UC-11	*Pantoea agglomerans*	20	400	–	–
UC-14	*Erwinia americana*	4	400	–	–
UC-18	*Erwinia americana*	8	400	–	–
UC-20	*Escherichia hermanii*	10	100	–	–
UC-39	*Serratia odorifera*	40	400	+	–
UC-42	*Serratia odorifera*	2	800	–	–
UC-31	*Serratia odorifera*	40	1,000	–	–
UC-45	*Serratia odorifera*	40	800	–	–
UC-50	*Yersinia intermedia*	20	800	–	–
UC-52	*Hafnia alvei*	20	100	–	–
UC-53	*Serratia marcescens*	20	800	–	–
UC-54	*Pantoea agglomerans*	20	1,000	–	–
UC-58	*Enterobacter aerogenes*	20	1,000	+	–
UC-60	*Serratia marcescens*	40	800	+	–
UC-62	*Serratia plymuthica*	20	800	+	–
UC-71	*Serratia odorifera*	20	800	+	–
UC-80	*Enterobacter aerogenes*	20	200	–	–
UC-87	*Pseudomonas stutzeri*	20	400	–	–
UC-93	*Pseudomonas fluorescens*	20	800	–	–
UC-96	*Enterobacter aerogenes*	20	200	+	–
UC-98	*Enterobacter cloacae*	20	800	–	–
UC-99	*Enterobacter cloacae*	2	200	+	–
UC-101	*Serratia odorifera*	20	1,000	–	–
UC-103	*Chryseomonas meningosepticum*	20	200	+	–
A-proteobacteria					
UC-1	*Sphingomonas paucimoibilis*	20	800	–	–
UC-16	*Sphingomonas paucimobilis*	8	400	–	–
UC-35	*Sphingomonas paucimoibilis*	20	100	–	–
UC-37	*Sphingomonas paucimoibilis*	20	800	+	–
UC-66	*Sphingomonas paucimobilis*	40	400	–	–
(b)					
B-Proteobacteria					
UC-4	*Moraxella nonliquefaciens*	40	400	–	–
UC-9	*Alcaligenes faecalis*	20	1,000	–	–
UC-12	*Moraxella osloensis*	8	400	–	–
UC-22	*Moraxella osloensis*	6	400	–	–
UC-23	*Moraxella osloensis*	20	400	–	–
UC-26	*Alcaligenes xylosoxidans*	20	100	–	–
UC-29	*Comamonas testosteroni*	20	1,000	–	–
UC-30	*Comamonas testosteroni*	20	400	–	–
UC-34	*Moraxella nonliquefaciens*	40	400	–	–
UC-86	*Burkordelia cepacia*	20	800	–	–
UC-90	*Burkordelia cepacia*	20	1,000	+	–
UC-91	*Burkordelia cepacia*	20	800	–	–
UC-94	*Moraxella osloensis*	20	800	–	–
Not identified					
UC-72	Not identified	20	1,000	–	–
UC-77	Not identified	20	800	–	–
UC-78	Not identified	20	800	–	–
UC-21	Not identified	4	50	–	–
UC-68	Not identified	40	800	+	–

These results suggest that bacteria can influence the arsenic speciation in the environment.

Eleven out of the 49 (22,4%) arsenic-resistant isolates presented a PCR product of approximately 370 bp, expected size related to arsenate reductase (Saltikov and Olson 2002) (Fig. 1). The *ars* system is a widely studied arsenic resistance mechanism, whose *arsC* gene codifies a soluble enzyme, arsenate reductase, that catalyzes arsenate reduction to arsenite (Jackson and Dugas 2003).

Various researchers have already reported that amplification of the *ars* genes and unknown chromosomal *ars* areas during a PCR is influenced by many factors, such as the type of primer used, conformational variation in the extracted DNA, thermal cyclic conditions and the composition of buffers or agents (Chang et al. 2007). Ford et al. (2005) failed to correlate the level of arsenate resistance with the prevalence of *ars* genes in a set of aerobic and anaerobic bacteria.

Although the oxidation of As(III) to As(V) is known to be regulated by the presence of arsenite oxidase enzyme, the arsenite-oxidase gen was not detected in this study (see Table 1a, b). There was not amplification of any genes for arsenite oxidase in the isolates, despite the fact that arsenite oxidizing activity was experimentally shown. Such activity could be explained by other alternative mechanisms of arsenic resistance. Nevertheless, it is possible that the resistance arsenic genes in the investigated bacteria could be different to those described in the literature.

A total of 49 isolates were identified by RapID ONE System and RapID NF Plus System (Remel) and distributed between three mayor bacterial lineages (Table 1a, b). γ-Proteobacteria (26 isolates), β-Proteobacteria (13 isolates) and α-Proteobacteria (5 isolates). Most genera recovered from the sediments were: *Moraxella*, *Alcaligenes*, *Comamonas*, *Burkordelia*, *Sphingomonas*, *Pantoea*,

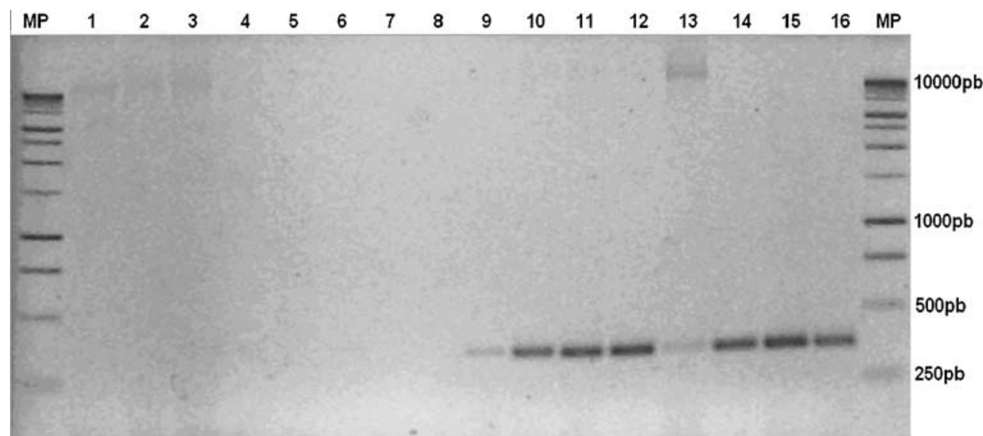

Fig. 1 Electrophoresis in gel of agarose from PCR products using primers *ars*C-1-F y *ars*C-1-R. *Line 1 S. paucimobilis*; *2 P. agglomerans*; *3 E. americana*; *4 E. hermanii*;*5 C. testosteroni*; *6 H. alvei*; *7 M. osloensis*; *8 S. odorifera*; *9 P. agglomerans*; *10 E. aerogenes*; *11 S. plymuthica*; *12 S. odorifera*; *13 P. stutzeri*; *14 B. cepacia*;*15 E. cloacae*; *16 C. Meningosepticum*; MP, DNA ladder

Erwinia, *Serratia*, *Enterobacter* and *Pseudomonas*. The genera identified were not previously reported in literature as arsenic resistant.

This is the first work that describe arsenic resistant bacteria, isolated from sediments from Camarones river, involved on the arsenic biogeochemical cycles. These arsenic-resistant bacteria could grow in the presence of arsenite and arsenate, and could play an important role in natural arsenic speciation. Futher studies are required to understand the physiological role of biological transformation of arsenic.

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