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ORIGINAL ARTICLE

Analysis and characterization of cultivable extremophilic hydrolytic bacterial community in heavy-metal-contaminated soils from the Atacama Desert and their biotechnological potentials

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Abstract

Aims: To isolate and characterize the cultivable community of hydrolase producers (amylase, protease, lipase, DNase, xylanase and pullulanase) inhabiting heavy-metal-contaminated soils in extreme conditions from the Atacama Desert.
Methods and Results: A total of 25 bacterial strains showing hydrolytic activities have been selected including halotolerants, extremely halotolerants and moderate halophiles. Most hydrolase producers were assigned to the family *Bacillaceae*, belonging to the genera *Bacillus* (nine strains), *Halobacillus* (seven strains) and *Thalassobacillus* (five strains) and four isolates were related to members of the families *Pseudomonadaceae*, *Halomonadaceae* and *Staphylococcaceae*. The selected strains were then characterized for their tolerance pattern to six heavy metals, measured as minimal inhibitory concentrations (MICs).
Conclusions: The diversity found in the cultivable bacterial community analysed is more limited than that detected in other ecological studies owing to the restrictive conditions used in the screening. The dominant bacteria were Firmicutes and particularly, species related to the genus *Bacillus*.
Significance and Impact of the Study: This study is focused on the characterization of extremophilic hydrolytic bacteria, providing candidates as a source of novel enzymes with biotechnological applications.

Introduction

The Atacama Desert in northern Chile is a coastal desert usually described as one of the driest deserts on Earth, with a surface that has been minimally disturbed by natural erosion for millions of years (McKay *et al.* 2003; Hartley *et al.* 2005). This region includes arid and semi arid environments with many different saline deposits. In these saline deposits, evaporative basins called saltflats can be found (Chong 1984) containing a high percentage of salts from the leaching of volcanic rocks.

Until recently, regions of the Atacama Desert were considered the dry limit of photosynthetic activity and primary production (Warren-Rhodes *et al.* 2006), with extremely low levels of cultivable organisms and oxidized organic species detected in the soils (Navarro-González *et al.* 2003). In spite of the extreme conditions of this desert habitat, a broad spectrum of halotolerant and halophilic bacteria and archaeas have been found in salt-flats and hypersaline lakes in the area (Demergasso *et al.* 2004; Wierzchos *et al.* 2006; Connon *et al.* 2007; De los Ríos *et al.* 2010).

Different classification schemes have been designed to define the relationships of micro-organisms with salt. The most widely accepted classification is based on the optimal salt concentration for their growth (Kushner and Kamekura 1988).

The halotolerance of many enzymes derived from halotolerant and halophilic micro-organisms can be exploited wherever enzymatic transformations are

required to function under extreme physical and chemical conditions (Oren 2002a; Setati 2010). Hydrolases produced by halophiles have quite diverse potential usage in different areas (food industry, feed additive, biomedical sciences and chemical industries) (Rao *et al.* 1998; Kulkarni *et al.* 1999; Niehaus *et al.* 1999) and several halophilic enzymes have been tested for their potential biotechnological applications, including amylases, nucleases and proteases (Oren 2002b).

The Atacama Desert has increased its levels of heavy metals owing to natural and industrial processes (Salamanca *et al.* 2000). Cadmium, copper and zinc are among those heavy metals that are being released in the environment. Although some heavy metals constitute essential trace elements, most can be, at high concentrations, toxic to organisms from all branches of life, including bacteria (Nies 1999). However, some bacteria have adapted their physiology to tolerate the presence of metals or can even use them to grow. Owing to their exceptional characteristics, salt tolerant bacteria adapted to live in hostile environments may exhibit the potential to remove heavy metals from contaminated environments (Mishra *et al.* 2009; Zhuang *et al.* 2010). However, very little information is available on the heavy-metal tolerance of bacteria in hypersaline environments, with the exception of a few studies of metal-resistant halophilic bacteria isolated from different habitats such as the Maruit Lake, Egypt (Osman *et al.* 2010) or the Dead Sea Shore, Jordan (Amoozegar *et al.* 2005; Massadeh *et al.* 2005).

Therefore, in the present work, we studied the cultivable diversity of hydrolytic enzyme producers in a bacterial community from saline soils of the Atacama Desert. Furthermore, these strains were evaluated for their biotechnological potentials in terms of hydrolytic enzyme activity and heavy-metal tolerance.

Materials and methods

Description of sampling sites and collection

Samples were collected in September 2010 from two different points in the Atacama Desert region (Chile), designated as follows: Atacama Salar (AS) (in the Pre-Andean Depression) (latitude 23°300′S, longitude 68°150′W) and Death Valley (DV), (latitude 22°555′S, longitude 68°120′ W), both closely located to copper and lead–zinc mine tailings. The AS is the largest saltflat in Chile and is located in the east region of the Antofagasta district of Chile. The Salar is 100 km long and 80 km wide, and the salts have an athalassohaline origin. The DV, also known as the Mars Valley, located in the Salt Mountain range, is very close to San Pedro de Atacama, standing out owing to its sand dunes of up to 150 m in height. The average annual precipitation in AS and DV is <1 and 4 mm, respectively, with a high UV index. At each site, we collected four samples, including pieces of halite crusts broken off AS and surface soils (0–20 mm) from DV. Samples were taken in sterile plastic containers and stored in an icebox until further processed. Concentrations of Na, Ca, K, Fe, Mg, B, As, Mn, Cu and Zn were determined via ICP-OES (Inductively coupled plasma-optical emission spectrometry) using an Iris Advantage spectrometer (Thermo Jarrel Ash Corporation, MA, Franklin, USA). Calcium carbonate ($CaCO_3$) was determined using an automated continuous flow analyser (Bran + Luebbe III, Germany). Soil pH and electrical conductivity (EC) were determined in a 1 : 5 soil/water extract. An electronic thermometer (Orion model 290) was used to measure temperature. Soil samples were air-dried, crushed and sieved through a 2-mm sieve. Total organic carbon (TOC) was analysed by dichromate oxidation and titration with ferrous ammonium sulphate (Walkley and Black 1934).

Isolation of hydrolytic producers

The isolation of micro-organisms producing hydrolytic enzymes was performed by diluting collected samples from the Atacama Desert (from 10^{-1} to 10^{-3}) in saline solution (0·9% NaCl, w/v) and inoculating 100 μl of the dilution into saline medium (BS-10 medium) with 10% (w/v) total salts and 1% (w/v) yeast extract and substrates to assay for amylase, protease, lipase, DNase, xylanase and pullulanase activities. The salts solution (SW) composition contained in the BS medium was as follows (g l^{-1}): NaCl (234), $MgSO_4{\cdot}7H_2O$ (61), $MgCl_2{\cdot}6H_2O$ (39), KCl (6), $CaCl_2$ (1), NaBr (0·7) and $NaCO_3H$ (0·2). When necessary, different concentrations of salts solution were added to BS medium as indicated. Solid media were prepared by adding 2% (w/v) bacteriological-agar (Difco Laboratories, Detroit, MI). The plates were incubated at 37°C in aerobic conditions.

Amylase activity was determined using solid BS-10 medium supplemented with 0·5% (w/v) soluble starch. After incubation for 15 days, the plates were flooded with 0·3% (w/v) I_2 −0·6% (v/v) KI solution: a clear zone around the colonies indicated hydrolysis of starch (Cowan and Steel 1982). Protease activity was screened qualitatively in the saline BS-10 medium amended with 2% (w/v) skim milk. Zones of precipitation of paracasein around the colonies appearing over the next 15 days were taken as evidence of proteolytic activity (Cowan and Steel 1982). Lipase activity of the strains was detected by screening for zones of hydrolysis around colonies growing on solid BS-10 medium, containing 1% (w/v) tributyrin after incubation for 15 days (Mourey and Kilbertus 1976). The presence of

DNase activity on plates was determined on DNase test agar (BBL) containing 10% (w/v) total salts. After incubation for 15 days, the plates were flooded with 1 N HCl solution. Clear halos around the colonies indicated DNase activity (Jeffries *et al.* 1957). Xylanase and pullulanase activities were tested in BS-10 medium with the chromogenic substrates (0·1%, w/v) AZCL-xylan and AZCL-pullulan (Megazyme, Bray, Ireland). After this time, xylanolytic and pullulolytic activities were detected showing clearing zones around the colonies.

The positive hydrolytic isolates (64 strains) were replicated in the solid BS-10 medium containing the corresponding substrates and were tested again for the hydrolytic activities. A Gram stain test of the colonies was performed before proceeding to further studies.

Salt requirement

The growth response to NaCl of the isolates was examined in BS medium containing 0, 1, 3, 5, 7·5, 10, 12·5, 15, 17·5, 20, 25 and 30% (w/v) NaCl and incubated at 37°C on a rotary shaker (New Brunswick Scientific Co, NJ, USA) operating at 200 rev min^{-1}. Growth curves of the isolates were obtained by monitoring the culture absorbance at 600 nm using a Perkin-Elmer spectrophotometer at different incubation times. For this purpose, 250-ml flasks containing 50 ml of saline medium were each inoculated with 100 μl of a stationary-phase culture. Samples (500 μl) were taken at 6, 12, 18, 24, 40 and 48 h to monitor the culture absorbance.

Heavy-metal tolerance testing

For the determination of tolerance to heavy metals, solid BS medium containing the optimal concentration of NaCl for each isolate was used. The range of concentrations for the heavy metals tested (cadmium, zinc, nickel, iron, copper and cobalt) ranged from 0·5 to 4 mmol l^{-1} (0·5, 1, 2, 3 and 4 mmol l^{-1}). Controls consisting of media without metals and inoculated with the test microorganisms were carried out in all experiments. After incubation at 37°C for 2–3 days, the results were interpreted. The lowest concentration of metal that completely prevented growth was named the minimum inhibitory concentration (MIC). The MICs for all strains were determined by triplicate.

Isolation of genomic DNA and amplification of 16S rRNA gene

DNA of isolated strains was extracted and precipitated following the CTAB protocol for bacterial genomic DNA preparations (Wilson 1987). The 16S ribosomal RNA gene was amplified by polymerase chain reaction (PCR) using total DNA as the template and universal primers designed for Bacteria. The 16S rRNA PCR amplification was performed using the forward primer 16F27 (5′-AGAGTTTGATCMTGGCTCAG-3′) and the reverse primer 16R1488 (5′-CGGTTACCTTGTTAGGACTTCACC-3′) (Lane 1991). The following programme was used for amplification: one cycle of 95°C for 5 min, 25 cycles of 94°C for 1 min, 50°C for 1 min, and 72°C for 2 min, and then an extension step for 10 min at 72°C. The amplified DNA fragments were analysed on 1% (w/v) agarose gels stained with ethidium bromide and photographed with UV illumination.

Amplified rDNA restriction analysis (ARDRA) and 16S rRNA sequence analysis

The PCR products of the 16S rRNA gene were digested with 1 U of the restriction endonucleases *Alu*I and *Hha*I (Fermentas MBI, Vilnius, Lithuania) at 37°C for 2 h. The digestion products were detected by gel electrophoresis in 2% agarose gels and visualized after staining with ethidium bromide. ARDRA profiles were analysed, and representative unique clones were selected for nucleotide sequence determination. The amplified DNA fragments were sequenced using an automated DNA sequencer model 9700 (Applied Biosystems, Foster City, CA). The 16S rRNA gene sequences obtained were compared to the sequences in the GenBank data bases and aligned with the most similar sequences available to construct the phylogenetic trees using the ARB software package (Ludwig and Strunk 1996). Evolutionary distance matrices were calculated using the neighbour-joining algorithm (Ludwig *et al.* 1998). Phylogenetic trees using different methods (distance matrix, maximum parsimony and maximum likelihood) were constructed and compared to elucidate the confidence of local topologies.

Nucleotide sequence accession numbers

The nucleotide sequences reported in this work have been deposited in the EMBL Nucleotide Sequence Database under accession numbers HE586566 to HE586590.

Results

Physicochemical analysis of the collected samples

Both sampling areas selected in the Atacama Desert are characterized by a high solar radiation and large temperature fluctuations between day and night.

Table 1 shows the physicochemical characterization of the collected samples. The main ionic composition in

Table 1 Chemical and physical properties of collected samples from the Atacama Desert region

	Isolation site	
Parameter	Atacama Salar (AS)	Death Valley (DV)
Na^+ (mg l^{-1})*	1648	222
Ca^{2+} (mg l^{-1})*	184	233
K^+ (mg l^{-1})*	163	214
Fe (mg l^{-1})*	29·6	195
Mg^{2+} (mg l^{-1})*	196	59·43
B (mg l^{-1})*	51·91	0·311
As (mg l^{-1})*	1·194	0·276
Mn (mg l^{-1})*	35·84	0·05
Cu (mg l^{-1})*	3·13	0·017
Zn (mg l^{-1})*	3·972	<0·001
pH	8·68 ± 0·064	9·54 ± 0·11
EC (mS cm^{-1})	127 ± 11·3	0·633 ± 0·0133
TOC (mg kg^{-1})	<0·1	0·91 ± 0·01
Moisture (%)	7·30	3·50

Mean values ± standard deviation (n = 3) of Moisture content, Electrical Conductivity (EC), pH and Total Organic Carbon (TOC) in samples. *Average values of the collected samples from two sampling sites.

both sampling sites was different. Samples from AS presented higher salt concentrations, particularly Na^+ followed by Mg^+, Ca^+, K^+ and Mn^+ ions. The analysis of samples from DV revealed a similar concentration of Ca^+ and K^+ ions but significantly higher concentrations of Fe. The pH values differed in AS and DV although in both cases alkaline values were obtained. The TOC content was lower in AS (<0·1 mg kg^{-1}) than DV (0·91 mg kg^{-1}). Sodium concentration and electrical conductivity displayed the largest difference along our study samples.

Screening and selection of extremophilic micro-organisms producing hydrolytic enzymes

A total of 64 colonies showing hydrolytic activity were chosen for analysis. The 16S rRNA genes of these isolated strains were amplified. Restriction analysis of the amplified 16S rRNA genes using the restriction endonucleases *Alu*I and *Hha*I indicated the presence of different groups of restriction patterns. Twenty-five unique isolates representing the major cluster groups were selected for further analysis.

The most frequent hydrolytic activity detected in our study was DNase (20% of the hydrolytic population), followed by amylase and lipase activities. From the 25 selected isolates by full 16S rRNA gene sequencing, 21 of them showed DNase activity (16 isolates from AS and five isolates from DV), 20 showed amylase activity (16 isolates from AS and four isolates from DV) and 19 showed lipase activity (14 isolates from AS and five isolates from DV). Concerning the pullulanase producers (17 strains), this group represents the 17% of the hydrolytic population (15 isolates from AS and two isolates from DV). In the analysed community, the proteolytic producers constituted 15% of the isolates showing hydrolytic activities (10 isolates from AS and five isolates from DV). Xylanases were the least hydrolases represented (eight isolates from AS and three isolates from DV) (Table 2). Combined activities were detected in most of the isolates. Eleven strains presented five hydrolytic activities, nine strains presented four hydrolytic activities, two strains presented three hydrolytic activities and three strains presented two hydrolytic activities. No strains with all of the hydrolytic activities tested have been found in this study.

Determination of the saline requirement

The assay performed to determine the salt growth profile for the 25 isolates (Table S1, supporting information) revealed that most of them grew in the NaCl range of 2–25% (w/v) and showed optimal growth at 7·5% (w/v) NaCl. Seven strains showed optimal growth without NaCl, although they were able to grow in presence of NaCl and other salts. Only two strains showed optimal growth without NaCl but were able to grow at high concentrations of salts (up to 17·5% NaCl).

Phylogenetic analysis of the isolates

Comparative sequence analyses of the 16S rRNA genes from the 25 isolates were performed (Table 2). The majority of the isolates were Gram-positive bacteria assigned to the family *Bacillaceae* and included in the genera *Bacillus* (nine strains), *Halobacillus* (seven strains) and *Thalassobacillus* (five strains). Two Gram-negative isolates were assigned as members of the *Pseudomonadaceae* and *Halomonadaceae* families, included in the genera *Pseudomonas* and *Halomonas*, respectively. Only two species were detected in the study belonging to the genus *Salinicoccus* within the *Staphylococcaceae* family. Essentially, similar results were obtained using different methods to construct the phylogenetic trees. Therefore, we only include the neighbour-joining tree (Fig. 1).

Among the hydrolytic community analysed, the strains isolated from the AS samples, containing higher salt concentrations were assigned to five different genera. However, the strains isolated from DV samples, with moderate salinity and organic matter content, but presenting alkaline pH, were all identified as species of the genus *Bacillus*.

Table 2 summarizes the hydrolytic activities of the isolates. The DNase producers represented the group with

Table 2 Bacterial diversity based on the 16S rRNA gene and hydrolytic activities of the strains isolated from the desert soils samples

				Hydrolytic activity					
Strain	Closest relative	% Similarity	Accession no.	Protease	Lipase	Amylase	DNase	Pullulanase	Xylanase
DV1	*Bacillus seohaeanensis*	99	AY667495	+	–	–	+	–	–
DV2				+	+	–	+	–	–
DV3				–	+	–	+	–	–
DV4	*Bacillus subtilis* subsp. *inaquosorum*	99	EU138467	+	+	+	–	+	+
AS1				+	+	–	+	–	+
DV5	*Bacillus stratosphericus*	99	AJ831841	–	–	+	+	+	–
DV6	*Bacillus oceanisediminis*	99	GQ292772	+	+	+	+	–	+
AS2	*Bacillus mojavensis*	99	EU138460	–	+	–	+	–	–
DV7	*Bacillus licheniformis*	97	AB301007	+	+	+	–	–	+
AS3	*Pseudomonas halophila*	99	AB021383	+	+	+	+	–	–
AS4	*Halobacillus hunanensis*	98	FJ425898	+	+	+	+	+	–
AS5				+	+	+	+	+	–
AS6	*Halobacillus profundi*	98–99	AB189298	–	–	+	+	+	+
AS7				–	–	+	+	+	+
AS8				–	+	+	+	+	+
AS9				–	–	+	+	+	+
AS10				–	–	+	+	+	+
AS11	*Thalassobacillus devorans*	98–99	AJ717299	–	+	+	+	+	–
AS12				–	+	+	+	+	–
AS13				+	+	+	+	+	–
AS14				+	+	+	+	+	–
AS15				+	+	+	+	+	–
AS16	*Halomonas organivorans*	98	NR_029029	+	+	+	+	+	–
AS17	*Salinicoccus roseus*	98	NR_026311	+	+	+	–	+	+
AS18				+	+	+	–	+	+

the highest number of strains (21 strains), including isolates with high affinity to the genera *Bacillus*, *Halobacillus*, *Thalassobacillus* and closely related to the species *Pseudomonas halophila* and *Halomonas organivorans*. Among the amylolytic strains (20 strains), most of them were related to moderate halophiles included in the genera *Halobacillus*, *Thalassobacillus*, *Salinicoccus*, *Halomonas* and *Pseudomonas*. Only four strains were closely related to halotolerant members of the genus *Bacillus*. Isolates producing lipolytic enzymes (19 strains) showed high affinity to species related to the genera Bacillus, *Pseudomonas*, *Halobacillus*, *Thalassobacillus*, *Halomonas* and *Salinicoccus*. The presence of lipolytic activity was an important characteristic to differentiate isolates assigned to the same species (*Bacillus seohaeanensis*, *Halobacillus hunanensis* and *Halobacillus profundi*). A total of 17 strains showed pullulanase activity. In comparison with other activities, an absence of pullulanase activity was found among the *Bacillus* strains. Concerning the isolates producing proteolytic enzymes (15 strains), we detected strains related to the genera *Bacillus*, *Pseudomonas* and *Halomonas* and closely related to *Thalassobacillus devorans*, *H. hunanensis* and *Salinicoccus roseus*. Only 11 strains showed xylanase activity and were included in the genera *Halobacillus*, *Bacillus* and *Salinicoccus*.

Response to heavy metals

The heavy-metal tolerance levels of the isolates from the Atacama Desert to six heavy metals, expressed as MICs, are shown in Table 3. There is not a specific and accepted metal concentration that could be used as a standard to define universally metal resistance. Moreover, metal salts and microbial media components can interact in ways which make data interpretation difficult. Thus, in general, for the metal tested, those strains which were not inhibited by 4·0 mmol l^{-1} of the metal ions could be considered tolerant to these metals. The strains that were not inhibited by 3·0 mmol l^{-1} could be considered moderately tolerant to these metals.

On the basis of the MICs, the highest toxicities were found with cadmium and zinc. Only one strain related to *Thalasobacillus devorans* (AS14) was cadmium tolerant. Three strains, related to the genera *Bacillus* (DV4 and DV5) and *Thalassobacillus* (AS12) could be considered as moderately tolerant to cadmium. Only two strains related to the genus *Bacillus* (DV1 and DV6) were considered tolerant to zinc and three strains (DV3, DV4 and DV5) as moderately zinc tolerant. In contrast, Ni was shown to be the least toxic metal for the isolates, with 56% of the tested isolates demonstrating tolerance to this metal. The

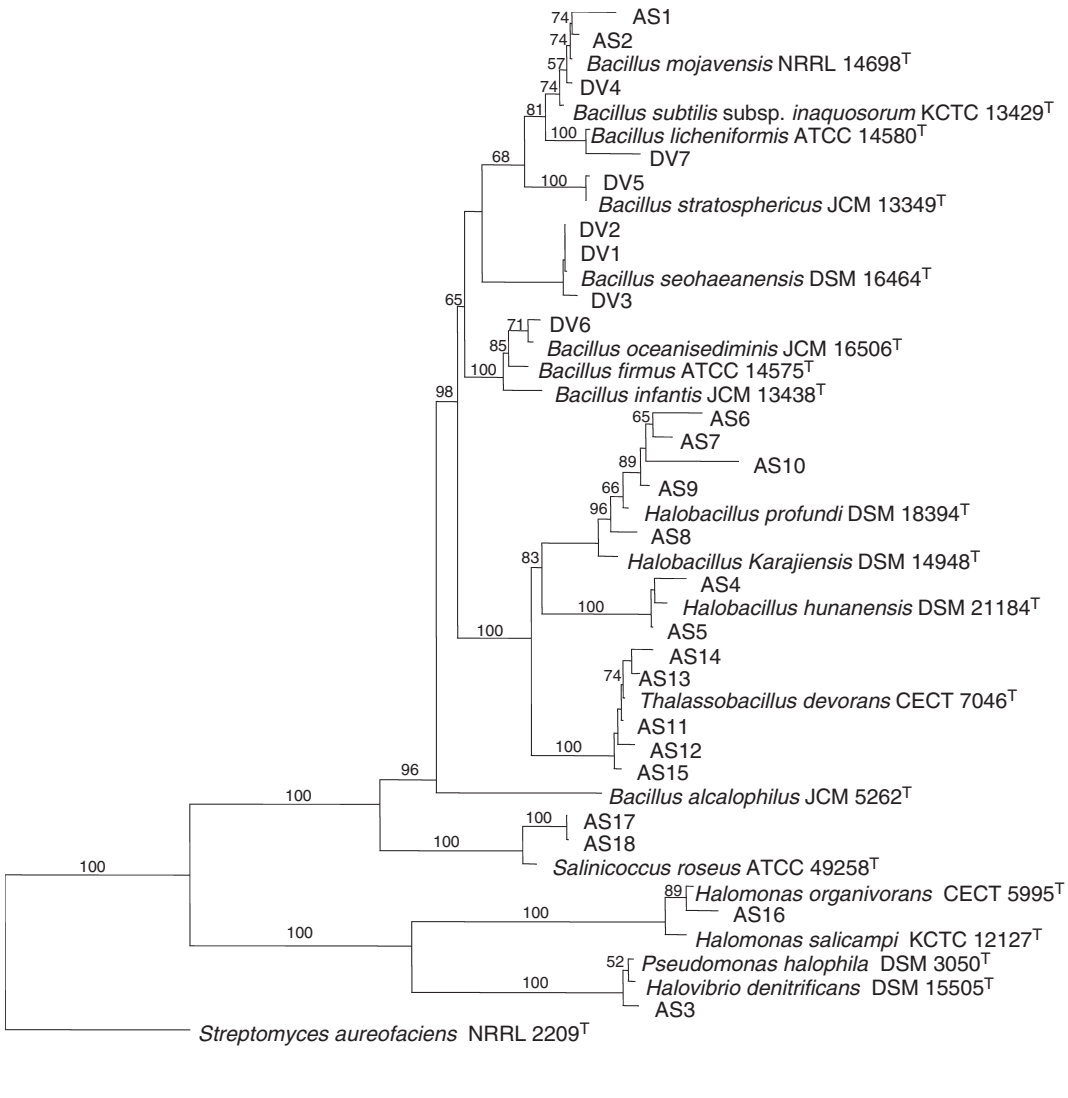

Figure 1 Phylogenetic neighbour-joining tree based on the analysis of 16S rRNA gene sequences, showing the diversity of micro-organisms able to produce hydrolytic enzymes. The isolates described in the present study are shown in bold. Isolates from Atacama Salar are indicated as AS, and isolates from Death Valley are indicated as DV. 16S rRNA gene sequences from the isolates correspond to sequences of 1400 bp. Bootstrap values (>50%, based on 1000 replications) are shown at branch points. Bar represents a 2% sequence difference.

susceptibility levels of the isolated strains to Fe and Cu was more heterogeneous, nine strains (36% of the total isolates) were tolerant to iron and one strain was moderately tolerant. Seven strains (DV2, DV4, DV5, AS7, AS9, AS13 and AS15) were tolerant to copper and two strains (AS11 and AS14) were moderately tolerant to this metal. Species related to *H. hunanensis* (AS4 and AS5) were the most sensitive to all the metal tested. In contrast, the strains related to *Bacillus subtilis* (DV4) and *Bacillus stratosphericus* (DV5) were the most tolerant to the heavy metals tested, showing higher tolerance to four different metal ions (Fe, Co, Ni and Cu) and moderate tolerance to Cd and Zn.

Discussion

Few environmental studies have focused on the study of the bacterial community inhabiting saline and hypersaline habitats in the Atacama Desert (Demergasso *et al.* 2004; Connon *et al.* 2007; Lester *et al.* 2007; Mishra *et al.* 2009; De los Ríos *et al.* 2010) and, in particular, to our knowledge, no screening programmes have been designed to select extremophilic micro-organisms with the ability to produce enzymes that hydrolyse polymeric compounds, although the hydrolases play a key role in the geochemical cycling of nutrients as the hydrolysis of high molecular weight biopolymers constitutes an initial step

Table 3 Minimal inhibitory concentrations (MICs) of the metal ions tested against the bacterial hydrolase producers

	MIC (mmol l^{-1})					
Strain	Cd	Zn	Ni	Fe	Cu	Co
DV1	0·5	4	4	2	0·5	0·5
DV2	0·5	1	4	4	4	0·5
DV3	0·5	3	4	2	1	0·5
DV4	3	3	4	4	4	4
AS1	0·5	0·5	4	2	2	1
DV5	3	3	4	4	4	4
DV6	0·5	4	4	4	1	1
AS2	0·5	0·5	4	2	1	1
DV7	0·5	0·5	4	3	1	4
AS3	0·5	0·5	1	0·5	0·5	0·5
AS4	0·5	0·5	0·5	0·5	0·5	0·5
AS5	0·5	0·5	0·5	0·5	0·5	0·5
AS6	0·5	0·5	1	0·5	0·5	4
AS7	0·5	0·5	3	0·5	4	4
AS8	0·5	0·5	1	4	0·5	0·5
AS9	0·5	0·5	1	0·5	4	2
AS10	0·5	0·5	3	1	0·5	2
AS11	0·5	0·5	4	4	3	1
AS12	3	0·5	1	4	0·5	2
AS13	0·5	0·5	1	0·5	4	2
AS14	4	0·5	4	4	3	2
AS15	0·5	2	2	0·5	4	3
AS16	0·5	0·5	4	4	2	4
AS17	0·5	1	2	0·5	2	1
AS18	0·5	0·5	2	0·5	2	1

in the metabolism of organic compounds in the different ecosystems.

Under the restrictive conditions used in our screening, a total of 64 fresh cultures showing hydrolytic activities were isolated and from them, 25 strains were selected and characterized. Most isolates (16 strains) grow optimally in media containing between 5 and 7% (w/v) total salts, being able to grow in most cases up to 25% (w/v) salts. Thus, they can be classified as moderate halophiles according to the classification proposed by Kushner and Kamekura (1988). All the moderate halophiles were isolated from the AS. The number of halotolerant isolates was remarkably lower compared with that of moderate halophiles. Representative members of the halotolerant group were detected in both sampling sites, and the two extremely halotolerant strains (DV2 and AS2) were isolated from the Death Valley.

The most frequent hydrolytic activity detected in our study was DNase, followed by amylase and lipase activities. In the analysed community, we also detected pullulanase and protease producers. Xylanases were the least represented hydrolases. Sánchez-Porro *et al.* (2003) showed the abundance of five hydrolytic enzymes including amylase, protease, lipase, DNase and pullulanase in a community of moderate halophiles isolated from salterns in Spain, and they described amylase producers as the most abundant isolates. Furthermore, Moreno *et al.* (2009) studied the diversity of extreme halophiles, producing lipase, protease, amylase and nuclease in hypersaline ecosystems in South Spain, concluding that 70% of total of the hydrolytic isolates were also amylase producers. However, Baati *et al.* (2010) in the screening performed in salt mines of Sfax (Tunisia) detected a similar percentage of proteases, amylases and DNases producers. Rohban *et al.* (2009) investigated the ability of halophilic strains isolated from a hypersaline lake in Iran to produce nine different extracellular hydrolases (inulinase, pectinase, cellulase and xylanase). In contrast to our results, the most frequent activity expressed by these isolates was lipase, and the least prevalent activity represented was DNase.

It is interesting to emphasize that combined hydrolytic activity was frequently detected in isolates from both sampled areas, AS and the Death Valley. These results support previous studies in other hypersaline habitats (Sánchez-Porro *et al.* 2003; Moreno *et al.* 2009), although the number of strains showing combined hydrolytic activity is higher in the present study.

Based on the comparison of partial sequences of 16S rRNA genes, most environmental isolates able to produce hydrolytic enzymes were Gram-positive bacteria, assigned to the family *Bacillaceae*, comprising species of the genera *Bacillus* (Cohn 1872), *Halobacillus* (Spring *et al.* 1996) and *Thalassobacillus* (García *et al.* 2005). *Bacillus* is well known as an extracellular enzymes producer and many industrial processes use species of this genus for commercial production of enzymes (Schallmey *et al.* 2004). Only two isolates were related to the Gram-negative bacteria *Ps. halophila* (Sorokin *et al.* 2006) and *H. organivorans* (García *et al.* 2004). The other characterized isolates were related to *S. roseus* (Ventosa *et al.* 1990).

Although no organic matter is detected in the samples obtained from the AS, a broader diversity is found among the isolates if compared to that obtained from the Dead Valley, identifying only hydrolytic producers from the genus *Bacillus*. Probably, the presence of hydrolases is more significant among the population found in the AS samples to obtain nutrients in an adverse environment.

The cultivable microbial diversity found in this study is more limited than that detected in other ecological studies performed in diverse areas of the Atacama Desert, selecting only strains of the phyla γ-Proteobacteria (families *Halomonadaceae* and *Pseudomonadaceae*) and Firmicutes (families *Bacillaceae* and *Staphylococcaceae*). However, this study was carried out under more restrictive conditions. Our results differ from others in the preponderant hydrolytic bacteria. While the overall

preponderant bacteria in our study were Firmicutes (92%), γ and β-Proteobacteria were reported as predominant in other studies (Rohban *et al.* 2009; Baati *et al.* 2010) in which Firmicutes did not appear or they were a minority.

Several studies have demonstrated that metal toxicity can be heavily influenced by environmental factors such as pH, temperature, soluble organic matter, clay minerals, inorganic anionic and cationic components (Nieto *et al.* 1989). Responses to the heavy metals tested in this study were very heterogeneous, with at least three different MICs being detected for the different metal ions in species belonging to the same genus (Table 3). In fact, four different MICs were found for zinc, copper and cobalt in *Bacillus* species. This suggests that halotolerant and halophilic bacteria express a very heterogeneous behaviour in connection with their individual natural susceptibility to the six heavy metals tested in the present study. Here, the highest toxicities were found with cadmium and zinc (80% of isolates were sensitive). This might be due to the fact that Cd is toxic per se and has not been found to be essential for biological functions in bacteria. Only one strain related to *T. devorans* showed high tolerance to cadmium (4·0 mmol l^{-1}). The majority of strains were tolerant to nickel. The prevalence of Ni-resistant isolates has been shown in other saline environments previously described (Gaballa *et al.* 2003). Species related to *H. hunanensis* were the most sensitive to the heavy metals tested and the strains related to *Bacillus subtilis* and *Bacillus stratosphericus* were the most tolerant, showing tolerance to four different metal ions (Fe, Co, Ni and Cu) and moderate tolerance to Cd and Zn. *Bacillus* species have been shown to have the ability to accumulate gold, cadmium, chromium, copper, iron and manganese (El-meleigy *et al.* 2010). In our study, Gram-positive bacteria presented higher tolerance (less growth inhibition) to heavy metals than their Gram-negative counterparts, although Gram-negative bacteria tend to be the predominant prokaryotes in metal-polluted environments, owing to the structure and composition of their cell walls (Duxbury 1986).

In conclusion, in this work, we analysed and characterized the cultivable extremophilic hydrolytic bacterial community inhabiting the saltflats of the Atacama Desert. The isolated strains were evaluated for their biotechnological potentials in terms of hydrolytic enzyme activity and heavy-metal tolerance. We identify two outstanding implications: (i) the discovering of the Atacama Desert as a source for isolation of novel enzymes with biotechnological potential and (ii) development of heavy-metal-tolerant micro-organisms as potential innovative technology for bioremediation operations to remove heavy metal from saline soils.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Salt growth range pattern of the isolates.

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