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UV-Resistant Actinobacteria from High-Altitude Andean Lakes: Isolation, Characterization and Antagonistic Activities†

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ABSTRACT

Polyextremophiles are present in a wide variety of extreme environments in which they must overcome various hostile conditions simultaneously such as high UVB radiation, extreme pHs and temperatures, elevated salt and heavy-metal concentration, low-oxygen pressure and scarce nutrients. High-altitude Andean lakes (HAALs; between 2000 and 4000 m) are one example of these kinds of ecosystems suffering from the highest total solar and UVB radiation on Earth where an abundant and diverse polyextremophilic microbiota was reported. In this work, we performed the first extensive isolation of UV-resistant actinobacteria from soils, water, sediments and modern stromatolites at HAALs. Based on the 16S rRNA sequence, the strains were identified as members of the genera *Streptomyces*, *Micrococcus*, *Nesterenkonia*, *Rhodococcus*, *Microbacterium*, *Kocuria*, *Arthrobacter*, *Micromonospora*, *Blastococcus*, *Citrococcus* and *Brevibacterium*. Most isolates displayed resistance to multiple environmental stress factors confirming their polyextremophilic nature and were able to produce effective antimicrobial compounds. HAALs constitute a largely unexplored repository of UV-resistant actinobacteria, with high potential for the biodiscovery of novel natural products.

INTRODUCTION

High-altitude Andean lakes (HAALs; between 2000 and 6000 masl), located at the Dry Central Andes, are a group of sparse salt flats and shallow lakes suffering from the highest total solar and UVB radiation on Earth (1,2). Spanning from the Atacama Desert in Chile, through the Argentinean and Bolivian Puna up to the Peruvian Andes, these lakes are quite distant from crowded human cities, which experience a wide daily range in temperatures, high salinity (up to 30%), low-nutrient availability and high concentration of heavy metals and metalloids, specially arsenic (1,2).

In spite of the impairing hard conditions, a diverse microbial biodiversity was found flourishing in all niches: plankton, benthos, soil surface and subsurface microbiota, fumaroles, microbial mats and even modern stromatolites (3–9). HAALs' extremophiles are microbes adapted to live on a variety of hostile physicochemical conditions (1–3,10) which must often tolerate several extremes in combination at their natural habitat. Thus, they are better termed "polyextremophiles" to describe their resilience to several stressors simultaneously (4). Most reports on HAALs' polyextremophiles drove the efforts for isolation of microbial strains from the plankton, being Firmicutes and Gammaproteobacteria the most abundant groups. Less attention was given to the phylum Actinobacteria, a high G+C% Gram-positive group, with high biotechnological potential, being them the largest group producing bioactive compounds, and *Streptomyces* the main genus known as source of these compounds (11–13). Thus, a more intensive program for isolation and characterization of the actinobacterial population from the HAALs is needed as the major impetus driving research on extremophiles is their associated-biotechnological potential.

Among extremophiles, radio-resistant microbes were highlighted as important source of bioactive compounds (14,15). The application of extremophiles in industrial processes has opened a new era in biotechnology. The possible commercial applications of extremolytes and extremozymes include anticancer drugs, antioxidants, cell-cycle-blocking agents, bioremediation, UV blocking and new antibiotics, among others (3,15–18). Nevertheless, potentially beneficial biomolecules remain to be discovered from unexplored extreme environments where radio-resistant microbes are abundant, that is the HAALs.

Exposed to high solar irradiation, Andean ecosystems including hyperarid/arid soil, bofedales, salterns and lakes are a natural source for UV-resistance microbes (1–4,19,20). Specifically, HAAL strains belonging to the genus *Acinetobacter* turned out to be even more resistant than their closest taxonomical neighbors: *Acinetobacter baumannii* DSM 30007, *Acinetobacter johnsonii* DSM 6963 and *Acinetobacter lwoffii* DSM 2403. Dib *et al.* (19) isolated strains from HAALs (including members of Actinobacteria), which together with a high UVB resistance profile, showed an extraordinary antibiotic resistance. In a follow-up work, Dib *et al.* (2010) reported UV-resistant *Micrococcus* sp. carrying linear megaplasmids. These kinds of extrachromosomal elements are known to confer several valuable qualities, such as heavy-metal resistance (21), catabolic traits (22), hydrogen autotrophy (23) or the production of secondary metabolites (24).

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The ability of radio-resistant organisms to survive high levels of radiation has been linked to their efficient DNA repair mechanisms and ability to produce protective primary and secondary metabolic products (9,10,25,26). Also resistant organisms tend to withstand a high level of DNA and protein damage than normal or sensitive ones. The most extensively studied resistant microbe is, so far, *Deinococcus radiodurans*, which is the best known for its supreme resistance to ionizing radiation, but it is also resistant toward UVR, hydrogen peroxide and numerous other agents that damage DNA. Additionally, *D. radiodurans* is highly resistant to desiccation (2).

In the last three decades, the strategy for successful biodiscovery campaigns has been based on the premise that extreme environments, such as deep seas, desert and polar soils, are likely to contain novel microorganisms that, in turn, have the capacity to produce novel metabolites (13,15,16,27,28). This view is extended in the present work to the HAALs' ecosystems. We hypothesize that HAALs are ideal candidates for biodiscovery of novel cytotoxic/antibiotic products because (i) these lakes are pristine, isolated and little explored displaying unique microecosystems such as modern stromatolites (6); (ii) extreme conditions (alkalinity, hypersalinity, UVB irradiation) may be triggering the synthesis of secondary metabolites for protection and defense; (iii) the low-nutrient availability together with high environmental pressures preclude an extensive competition among taxa where cytotoxic compounds may be mandatory for survival.

In this work, we report the first extensive screening and characterization program of UV-resistant actinobacteria isolated from a plethora of niches, that is soils, benthos, biofilms and modern stromatolites in the Argentinean HAALs. The potential of the newly isolated microbes for the production of secondary metabolites for antagonistic activities is preliminarily explored and discussed herein.

MATERIALS AND METHODS

Site description and sampling. Table 1 indicates all the sampling sites where the isolates were obtained. They included the following lakes: Laguna Diamante (Catamarca, Argentina), Antofalla (Catamarca, Argentina), Laguna Santa María (Salta, Argentina), Laguna Socompa (Salta, Argentina), Tolar Grande (Salta, Argentina) and Salina Grande (Jujuy, Argentina). All of them belong to the HAALs, spreading out at up to 6000 m altitude, comprising ecosystems along the Altiplano–Puna Plateau up to the High Andes. As the ozone column is naturally thinner over the tropics and the UV intensity increases with the altitude, the Puna–High Andes region is consequently exposed to an exceptionally high solar irradiation including harmful UV levels; climatologists and biologists exploring the Puna–High Andes region reported astonishing solar levels in different sampling points or climatological stations for ground measurements (29,30) reaching 10 W m^{-2} in some lakes (1–4). Among these lakes, Laguna Socompa is placed at the base of the still active volcano Socompa. It is located at an altitude of 3570 m, and Farías *et al.* (2013) reported for the first time in Argentina stromatolites forming at the shore of this lake. These authors also studied the physicochemical characteristics of the water surrounding stromatolites and found that during the summer field campaign (February), the lake water at the stromatolites site was relatively warm (20–24°C), alkaline, rich in dissolved ions such as Na^+, K^+, Mg^{2+}, Ca^{2+}, Cl_2 and SO_4^{-2} (total conductivity up to 130 mS cm^{-1}), rich in organic carbon, nitrate, phosphate, silicate and iron, and contained a strikingly high amount of arsenic. Other extreme lake included in this study was Laguna Diamante, located inside the crater of Galan Volcano and placed at 4589 m (Catamarca, Argentina), which presents a unique set of conditions, that is high pH (9–11), high arsenic concentrations (up to 230 mg L^{-1}), high salinity (270 g L^{-1}, 217 mS cm^{-1}), high UV radiation (84 Wm^{-2} of UVA–UVB at noon). Gaylussite crystals covered by a red biofilm were collected from this lake and used for our isolation procedures (31).

Three HAAL samples were collected in February 2010 and October 2011, during austral summer and spring, respectively. The samples were obtained from soil, sediment, modern stromatolites and water from multiple lakes (Table 1, Fig. 1). All the samples were collected in sterile flasks and stored between 5 and 10°C until further processing at the laboratory (6).

Some strains previously isolated from Lagunas Azul, Vilama, Aparejos, Chaxas, Huaca-Huasi and Salina Grande (identified with A, V, Ap, Ch, H and LC in the strain code, respectively) and now belonging to the LIMLA-PROIMI Extremophilic Strain Collection were also used in this study. The isolates from Lagunas Socompa, Antofalla Verde and Rojo, Grande, Diamante, Tolar, Negra, Verde, Cabe and Pocitos (Act for all lakes and St for the ones isolated from stromatolites in the strain code) are reported in this study (Tables 2 and 3). Permission for sample collection in the Salta's lakes was granted by the Ministerio de Ambiente y Producción Sustentable, Salta, Argentina (number 000388; 17–09–2010).

Culture media and isolation procedure. Soil, sediment, modern stromatolites, biofilm, gaylussite crystals (associated with red biofilm (31), flamingo feces and water samples (1 g and 1 mL, respectively)) from lakes located in the HAALs were aseptically diluted in sterile 0.9% NaCl solution (9 mL) and incubated in an orbital shaker at 30°C at 150 rpm for 30 min. The suspensions were allowed to settle, and serial dilutions up to 10^{-6} were prepared. One hundred microliters of serial dilutions was spread over the surface of isolation agar media on Petri dishes. The following selective media were used to isolate Gram-positive bacteria (32): (i) GYM agar (10 g of malt extract, 4 g of yeast extract, 4 g of glucose, 2 g of Ca_2CO_3, 1000 mL of distilled water, 15 g agar, pH 7.5); (ii) ISP-2 (32) agar (10 g of malt extract, 4 g of yeast extract, 4 g of glucose, 1000 mL of distilled water, 15 g agar, pH 7.2); (iii) stromatolite agar (STA, 100 g dry stromatolite, 6 g yeast extract, 15 g agar, 1000 mL of distilled water). The pH of all media was adjusted to 7.0–7.5. All media were supplemented with nalidixic acid (10 μg mL^{-1}) and cycloheximide (10 μg mL^{-1}) to inhibit the growth of Gram-negative bacteria and fungi, respectively. The isolation plates were incubated at 30°C for 2 weeks. Colonies of different size, color and morphology were streaked individually several times until single colonies of a single type were observed. Stock cultures were prepared for each strain by transferring biomass from each of the purified isolates into vials containing 1.5 mL 20% (w/v) sterile glycerol solution and stored at −20°C.

DNA extraction, 16S rDNA amplification and electrophoresis analysis. The 51 selected isolates were subjected to 16S rDNA sequence analysis for precise genera identification. To prepare cultures for isolation of genomic DNA from the strains, a single colony was transferred to a sterile flask with 10 mL of LB. The flasks were incubated at 30°C under stirring at 150 rpm until dense growth was observed (2–5 days). Cell material from 1.5 mL of these cultures was harvested by centrifugation, and genomic DNA was extracted using the DNAeasy Kit (Qiagen) according to the supplied protocol and stored at −20°C prior to use. The 16S rRNA gene was amplified from genomic DNA by PCR with the bacterial universal primers F27 and R1492. PCR products were run in 0.8% agarose gel, stained with SYBR Safe (Invitrogen) and visualized using a Gel DocTM XR+ with Image LabTM software (Bio-Rad). Purification of DNA from gel slabs was performed using QIAquick Gel Extraction Kit (Qiagen), and DNA sequencing was performed by the dideoxy chain termination method with an ABI Prism 3730XL DNA analyzer, using the ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit (PE Biosystems) according to the manufacturer's protocol.

The obtained DNA sequences were compared with the data accessible through NCBI (National Centre for Biotechnology Information) using BLASTN. Sequences were aligned with most closely homologous *Actinobacteria* 16S rDNA sequences from the GenBank, and a phylogenetic tree was constructed using the Molecular Evolutionary Genetics Analysis (MEGA) software version 3.1 (33). The bootstrap values (1000 replicates) were obtained using the neighbor-joining method provided by the software. DNA sequences were deposited to GenBank under accession numbers KJ187409 through KJ187466.

Phylogenetic analysis. The isolates were identified using the EzTaxon server (http://www.ezbiocloud.net/eztaxon), on the basis of 16S rRNA sequence data. The most closely related type strains were also obtained from this server. GenBank accession numbers are shown in Table 2.

Sequences from 16S rDNA were aligned online with Silva Incremental Aligner (SINA) to the rRNA gene databases provided by the SILVA

Table 1. Geographical location and physicochemical conditions of the water surrounding the sampling sites.

Geographical position	Catamarca			Salta		Jujuy
Lake	Laguna Diamante	Antofalla Ojos de Campo	Laguna Santa María	Laguna Socompa	Tolar Ojos de Mar	Salina Grande Laguna Cabe
Global position	26° 0′49.75″S 67° 2′10.08″W	25°39′48.96″S 67°42′53.91″W	24° 5′31.73″S 67°21′16.71″W	24°32′10.60″S 68°12′32.64″W	24°37′36.21″S 67°22′25.45″W	23°37′22.88″S 65°53′4.08″W
Depth (cm)	20	10	5	10	10	ND
Altitude (masl)	4589	3350	4250	3570	3510	3400
pH	11	8.5	11	9	6.5	ND
Arsenic (mg L^{-1})	230	ND	ND	35	0.59	ND
Salinity (ppt)	194	250	57	170	175	ND
T (°C)	14	18	21	14	14	ND
O_2 (mg L^{-1})	1.02	5.18	1.27	6.92	2.87	ND
Conductivity (mS cm^{-1})	174	220	75.7	115	169	ND

ribosomal RNA project. Based on this alignment, phylogenetic trees were constructed with Fasttree 2.1.7 with the maximum-likelihood method using Jukes–Cantor evolution model. Trees were drawn in MEGA6.

Amplification of the 16S–23S rDNA spacer region (ITS) fingerprinting. PCR amplification of 16S–23S ribosomal DNA (rDNA) ITS fingerprinting was performed as previously described (34,35). For DNA isolation directly from colony material, a single colony was picked from a fresh culture on GYM medium and resuspended in 100 µl of sterile distilled water in a 0.5 mL tube. The mixture was vortexed for 15 s and then incubated at 99°C for 10 min. After centrifugation (5000 g, 4°C, 5 min), the supernatant containing the released DNA was used as the source of target DNA for PCR amplification. The internal transcribed spacer (ITS) region was amplified using primers S-D-Bact-1494-a-S-20 (5′-GTCGTAACAAGGTAGCCGTA-3′) and L-D-Bact-0035-a-A-15 (5′-CAAGGCATCCACCGT-3′). The PCR protocol used consisted of an initial denaturation step of 4 min at 94°C, followed by 30 cycles of 1 min denaturation at 94°C, 7 min annealing at 55°C and 2 min extension at 72°C, and a final extension of 7 min at 72°C and was maintained at 4°C until tested. Ramps between the denaturation step and hybridization is 50% compared to the default and from the hybridization step, and the extension is 95% regarding default. PCR products were run in 2% agarose gel, stained with SYBR Safe (Invitrogen) and visualized using a Gel DocTM XR+ with Image LabTM software (Bio-Rad).

Phenotypic trait analysis: morphology. After purification, fresh colonies incubated at 30°C on LB agar for 48 h were prepared for phenotypic trait analysis. Morphological characteristics of the 183 isolates were examined by light microscopy (400–1000X NIKON 80i eclipse, Japan) with Nikon Digital Sight camera DS-5M-L1. Gram stain status was determined with standard Gram stain according to the supplied protocol (Britania). Colony aspect was described as Bergey's Manual of Determinative Bacteriology (36) on ISP-2 agar media. For scanning electron microscopy, selected strains were grown on ISP-2 agar. The agar blocks were cut from the growth medium and fixed (glutaraldehyde 4.25% in phosphate buffer 0.1 M pH 7.4) for 3 h at 4°C followed by dehydration in a graded acetone series. The critic drying point was obtained by exchanging the acetone through liquid CO_2. The samples were covered by gold and visualized using a Zeiss Supra 55VP (Carl Zeiss NTS GmbH, Germany) scanning electron microscope (CIME-CCT-CONICET).

Phenotypic trait analysis: UV resistance and polyextremophilic profile. Polyextremophilic assays were carried out with 38 new isolated strains and 13 selected strains from the LIMLA collection (all Gram-positive strains). The drop method (10) was used for the screening of all parameters using LB agar media. Cell cultures were grown overnight, after measuring optical density at 600 nm (OD_{600}) to achieve a dilution of 0.5 at OD_{600}. In the case of strains with mycelial growth (*Streptomyces*), spore suspensions were produced according to Albarracin *et al.* (2010). Subsequently, the cell/spore suspensions were subjected to serial dilutions. Petri dishes were divided in 24 squares, so 24 strains (10 µL aliquots of 10^{-1} dilution) were then loaded onto LB agar plates.

To evaluate UVB resistance (280–320 nm), LB agar plates were exposed to 2.0–3.0 W m^{-2} UVB radiation at different times (0, 30, 60, 90, 120, 180 and 240 min) using UVB lamps (09815-06, Cole Parmer Instruments Company), with an emission maximum at 312 nm. The radiation intensity reaching agar surface in disposable Petri plates was measured under the plastic lid using a UVB/UVA radiometer (09811-56, Cole Parmer Instrument Company) to 312 nm with an amplitude band of 300–325 nm, and the plated were then incubated in the dark to prevent photoreactivation for 72 h at 30°C. *Escherichia coli* DH5α and *Streptomyces coelicolor* and *Exiguobacterium* sp. S17 and *Acinetobacter* sp. Ver3 strains were used as negative and positives controls, respectively, for *in situ* UVB resistance assay. Controls of unexposed samples were run simultaneously in darkness. Microbial growth was recorded with three positive signs (+++) when it was similar to the growth in the control, with two positive signs (++) when it was slightly different from the growth in the control, with one positive sign (+) when the growth was too low to allow colony counting, with a negative sign (−) when it was no growth at all. For plotting the data in a graphic, three, two or one sign was taken as 3, 2 or 1 unit of growth for each dilution and then summed up together to reach a unique value. Negative signs were counted as null unit of growth.

As the environmental origin of the strains was typically saline (Table 4), we tested halotolerance and halo-alkalotolerance by growing the strains in salt-supplemented media (NaCl and Na_2CO_3). The bacteria were grown in liquid medium until OD_{600}: 0.6 and then diluted 10^{-1} with sterile physiological saline solution. In the case of strains with mycelial growth (*Streptomyces*), spore suspensions were produced according to Albarracin *et al.* (2010) and then diluted 10^{-1} with sterile physiological saline solution. Ten microliters of these dilutions was inoculated to LB agar media supplemented with different concentrations of Na_2CO_3 2.5 and 5.0% and NaCl 1 to 30% w/v. Plates were incubated at 30°C for 72 h. To determine the tolerance of arsenic [As(V) and As(III)], LB agar media containing different concentrations of arsenic, As(III), 2.5, 5, 7.5 and 10 mM and As(V), 50, 100, 150 and 200 mM, were spread with 10 µL aliquots of 10^{-1} dilution of each strain. The plates were incubated at 30°C during 72 h. For further characterization of the strains, the temperature range at 4, 8, 15, 20, 25, 30, 37, 45, 50 and 65°C and pH range from 4 to 12 for growth were studied in LB agar media. All polyextremophilic assays were conducted in triplicate. Control LB agar plates were inoculated with the selected strains without the stress factor (salts, pH, temperature and arsenic). Microbial growth was recorded and plotted in graphics as indicated for UV assays (13).

Antagonistic assays: secondary metabolites production screening. The capacity of the active isolates to produce secondary metabolites was tested. Isolates were suspended in distilled water and inoculated in the surface of modified Bennett's agar media (1 g of beef extract, 1 g of yeast extract, 10 g of glucose, 2 g of peptone, 10 g of NaCl, 15 g of agar, 1000 mL of distilled water) by streaking the producer across the middle of the plate. This was performed to allow the culture to be established on the agar surface and let secondary metabolites to spread out prior to inoculation of the plates with other strains. After 7 days of incubation at 30°C, six species of test bacteria were streaked perpendicular to the producing organism; this includes the same 51 chosen strains and another 21 strains (from the 183 isolates) of *Bacillus. Staphylococcus aureus*, *Enterococcus faecalis*, *Acinetobacter* sp. *Ver3*, *Exiguobacterium* sp. *S17*, *Escherichia coli* and *Rhodotorula* representatives were additionally used as controls. The strains were incubated at 30°C for 3 days (37). The production of cytotoxic compounds is expressed by the inhibition zones between the different

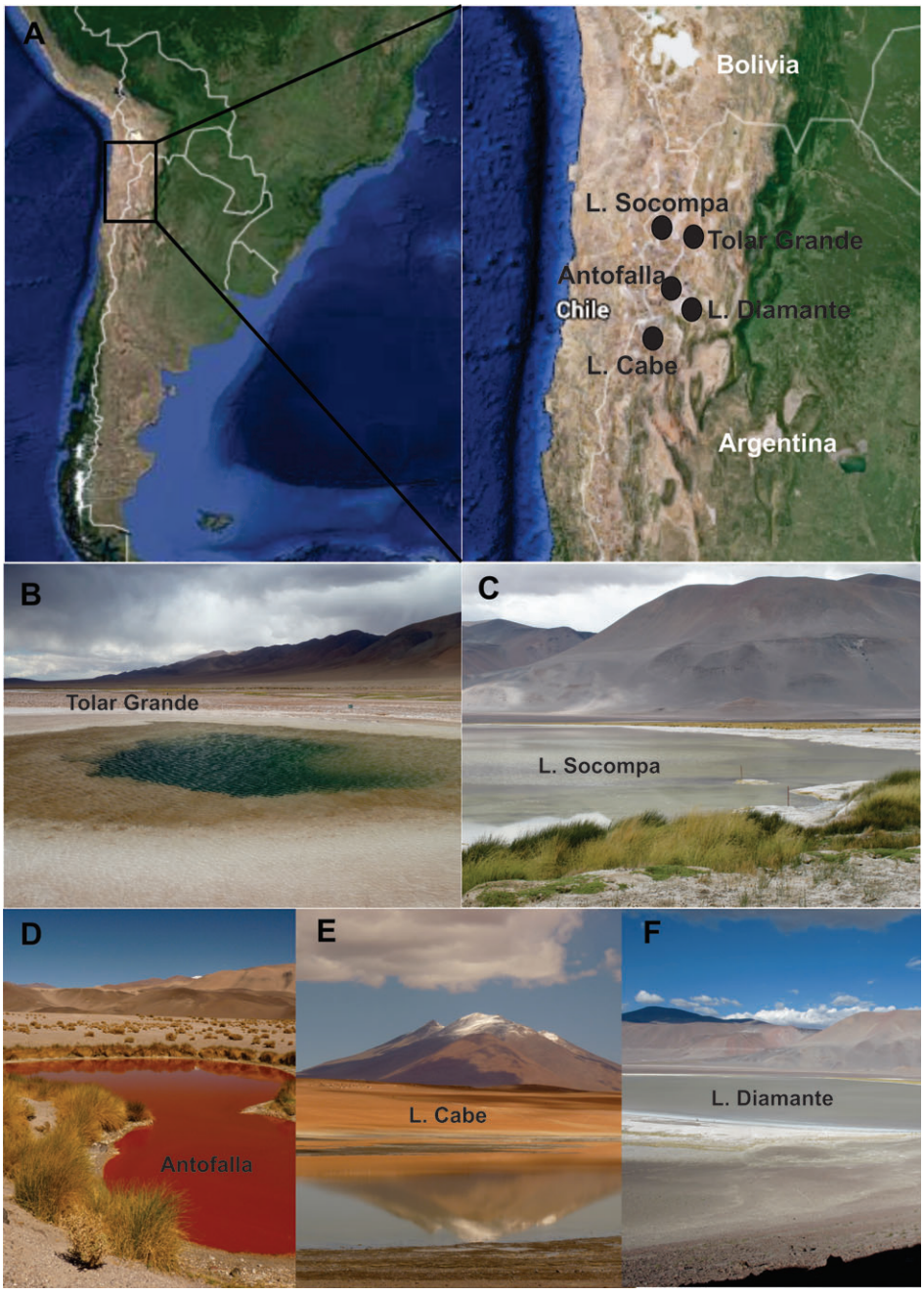

Figure 1. Sampling points. (A) Map showing location of sampling locations. (B) Tolar Grande (Salta, Argentina), (C) Laguna Socompa (Salta, Argentina), (D) Laguna de Antofalla (Catamarca, Argentina), (E) Laguna Cabe (Jujuy, Argentina) and (F) Laguna Diamante (Catamarca, Argentina).

isolates. Microbial inhibition score was recorded with three positive signs (+++) when growth was totally inhibited, with two positive signs (++) when it was partially inhibited, with one positive sign (+) when the growth was little inhibited, with a negative sign (−) when there was no inhibition at all. For plotting the data in a graphic, we followed the same procedure described for polyextremophilic assays (37).

RESULTS

Isolation of Gram-positive strains from diverse HAAL's niches

Selective isolation techniques were employed to specifically obtain actinobacteria from different niches of the HAALs (Fig. 1 and Table 1). A total of 183 Gram-positive isolates were acquired from all samples with shapes varying from rods to hyphae. Putative 38 actinobacterial strains plus 13 actinobacteria from the LIMLA collection were selected for further studies (Fig. 2). Tables 2 and 3 summarize the source and type of sample, and the phenotype of the selected isolates.

Molecular taxonomical and morphological characterization of selected HAAL actinobacteria

Sequencing of the 16S rRNA gene produced partial sequences of ca. 1400 bp in length, which allowed identify all isolates as

Table 2. List of the studied strains, isolation source, ITS fingerprinting and accession number of the sequences. Bold typeface identifies strains that were isolated from stromatolites.

			Phylogenetic affiliation				
Isolated strains	Isolation source	Type of sample	% Similarity based on 16S rRNA	Closest representative (type strains)	ITS fingerprinting group	Accession number	Reference
Act 1	LS	Soil	98.63	*Microbacterium terricola KV-448*	G1	KJ187409	Current paper
Act 4	LS	Soil	99.56	*Streptomyces pratensis ch24*	G2	KJ187410	Current paper
Act 5	LS	Soil	100	*Streptomyces pratensis ch24*	G3	KJ187411	Current paper
Act 6	LS	Soil	100	*Streptomyces pratensis ch24*	G4	KJ187412	Current paper
Act 7	LS	Soil	99.93	*Streptomyces pratensis ch24*	G5	KJ187413	Current paper
Act 8	LS	Soil	100	*Streptomyces pratensis ch24*	G4	KJ187414	Current paper
Act 11	LS	Soil	99.63	*Streptomyces anulatus NRRL B-2000*	G6	KJ187416	Current paper
Act 12	LS	Soil	99.88	*Streptomyces luridiscabiei NRRL B-24455*	G3	KJ187417	Current paper
Act 13	LS	Soil	99.85	*Streptomyces luridiscabiei NRRL B-24455*	G6	KJ187418	Current paper
Act 14	LS	Soil	100	*Streptomyces pratensis ch24*	G3	KJ187419	Current paper
Act 15	LS	Soil	95.69	*Streptomyces pratensis ch24*	G7	KJ187420	Current paper
Act 16	LS	Soil	99.63	*Streptomyces pratensis ch24*	G3	KJ187421	Current paper
Act 17	LS	Soil	99.63	*Streptomyces pratensis ch24*	G6	KJ187422	Current paper
Act 18	LS	Soil	99.92	*Nesterenkonia halotolerans YIM 70084*	G8	KJ187423	Current paper
Act 19	LS	Soil	99.42	*Streptomyces pratensis ch24*	G3	KJ187424	Current paper
Act 20	LS	Soil	99.79	*Nesterenkonia halotolerans YIM 70084*	G9	KJ187425	Current paper
Act 21	LS	Soil	99.42	*Streptomyces pratensis ch24*	G3	KJ187426	Current paper
Act 23	LS	Soil	100	*Streptomyces pratensis ch24*	G3	KJ187427	Current paper
Act 24	LS	Soil	99.28	*Kocuria polaris CMS 76or*	G10	KJ187428	Current paper
Act 25	LS	Soil	100	*Streptomyces pratensis ch24*	G11	KJ187429	Current paper
Act 26	LS	Soil	99.93	*Streptomyces pratensis ch24*	G6	KJ187430	Current paper
Act 27	LS	Soil	99.93	*Nesterenkonia halotolerans YIM 70084*	G12	KJ187431	Current paper
Act 46	LS	Soil	99.78	*Arthrobacter subterraneus CH7*	G13	KJ187439	Current paper
Act 48	LS	Soil	98.74	*Kocuria polaris CMS 76or*	G14	KJ187440	Current paper
Act 53	LS	Soil	100	*Streptomyces pratensis ch24*	G3	KJ187441	Current paper
Act 55	LS	Soil	100	*Streptomyces luridiscabiei NRRL B-24455*	G3	KJ187442	Current paper
Act 76	LS	Soil	100	*Streptomyces pratensis ch24*	G3	KJ187447	Current paper
Act 77	LS	Soil	100	*Streptomyces pratensis ch24*	G3	KJ187448	Current paper
Act 79	LS	Soil	99.85	*Streptomyces pratensis ch24*	G15	KJ187449	Current paper
St 80	**LS**	**Stromatolite**	**100**	***Micromonospora maritima D10-9-5***	**G16**	**KJ187450**	**Current paper**
St 82	**LS**	**Stromatolite**	**98.46**	***Blastococcus jejuensis KST3-10***	**G17**	**KJ187452**	**Current paper**
St 84	**LS**	**Stromatolite**	**99.92**	***Micrococcus yunnanensis YIM 65004***	**G18**	**KJ187454**	**Current paper**
St 85	**LS**	**Stromatolite**	**99.85**	***Streptomyces pratensis ch24***	**G2**	**KJ187455**	**Current paper**
Act 39	LD	Sediment	99.92	*Streptomyces sparsus YIM 90018*	G19	KJ187435	Current paper
Act 44	LD	Soil	99.12	*Streptomyces sparsus YIM 90018*	G19	KJ187437	Current paper
Act 69	LD	Gaylussite Crystals	99.76	*Micrococcus aloeverae AE-6*	G2	KJ187445	Current paper
Act 70	LD	Gaylussite Crystals	99.87	*Micrococcus aloeverae AE-6*	G18	KJ187446	Current paper
Act 134	LCb	Sediment	99.55	*Kocuria rosea DSM 20447*	G10	KJ187456	Current paper
A1	LA	Water	99.93	*Micrococcus yunnanensis YIM 65004*	G20	AM403127	Dib *et al.* (4)
A5	LA	Water	99.93	*Rhodococcus enclensis NIO-1009*	G21	DQ112024	Dib *et al.* (4)
A7	LA	Water	99.64	*Micrococcus yunnanensis YIM 65004*	G22	AM235879	Dib *et al.* (2010)
AP13	LAp	Flamingo Feces	98.99	*Brevibacterium ammoniilyticum A1*	G23	AM711595	Ordoñez *et al.* (3)
CH5	LCh	Flamingo Feces	99.93	*Microbacterium arborescens DSM 20754*	G1	AM711565	Dib *et al.* (2010)
CH9bis	LCh	Flamingo Feces	99.71	*Micrococcus aloeverae AE-6*	G18	KJ187463	Current paper
CH12	LCh	Flamingo Feces	99.78	*Microbacterium esteraromaticum DSM 8609*	G1	AM711570	Dib *et al.* (2010)
CH13	LCh	Flamingo Feces	99.86	*Rhodococcus pyridinivorans PDB9*	G24	AM711571	Dib *et al.* (2010)
CH14	LCh	Flamingo Feces	99.58	*Microbacterium esteraromaticum DSM 8609*	G25	AM711572	Dib *et al.* (2010)
H5	LH	Water	99.78	*Micrococcus yunnanensis YIM 65004*	G20	KJ187464	Dib *et al.* (2010)
LC5	LC	Water	98.72	*Citricoccus zhacaiensis FS24*	G26	KJ187466	Ordoñez *et al.* (3)
V5bis	LV	Water	99.78	*Micrococcus aloeverae AE-6*	G27	KJ187465	Current paper
V7	LV	Water	99.64	*Micrococcus yunnanensis YIM 65004*	G27	AM403126	Dib *et al.* (4)

actinobacteria. Accordingly, they were assigned to the genera *Streptomyces*, *Micrococcus*, *Microbacterium*, *Nesterenkonia*, *Kocuria*, *Rhodococcus*, *Arthrobacter*, *Micromonospora*, *Blastococcus*, *Brevibacterium* and *Citrococcus*, with sequence identity above 98% to type strains. A tree was constructed using the isolates and the most similar type strain to each (Fig. 3). Some genera presented more variation; for example, the two *Rhodococcus* strains were more similar to distinct type strains than to each other. The same situation arises in the *Microbacterium* isolates. However, the three *Nesterenkonia* isolates were closely related and showed the highest similarity with the same type strain. For instance, several strains belonging to *Streptomyces* were also very closely related; in fact, the isolates Act 8 and Act 25 had exactly the same sequence but depicted clear phenotypic

Table 3. Micro- and macroscopic characteristics of the strains.

Strain	Phenotype			
	Microscopic Cell Shape	Macroscopic		
		Reverse Color	Surface & Texture	Spore
Microbacterium Act 1	Rods	Y	Smooth & Mucoid	–
Streptomyces Act 4	Hyphae	Br	Rough & Dry	G
Streptomyces Act 5	Hyphae	Br	Rough & Dry	W
Streptomyces Act 6	Hyphae	Y	Rough & Dry	Be
Streptomyces Act 7	Hyphae	Br	Rough & Dry	G
Streptomyces Act 8	Hyphae	Y	Rough & Dry	G
Streptomyces Act 11	Hyphae	Be	Rough & Dry	Be
Streptomyces Act 12	Hyphae	Br	Rough & Dry	W
Streptomyces Act 13	Hyphae	Br	Rough & Dry	W
Streptomyces Act 14	Hyphae	Br	Rough & Dry	G
Streptomyces Act 15	Hyphae	Br	Rough & Dry	G
Streptomyces Act 16	Hyphae	Br	Rough & Dry	G
Streptomyces Act 17	Hyphae	Br	Rough & Dry	G
Nesterenkonia Act 18	Coccus	S	Smooth & Mucoid	–
Streptomyces Act 19	Hyphae	Br	Rough & Dry	G
Nesterenkonia Act 20	Coccus	Y	Smooth & Mucoid	–
Streptomyces Act 21	Hyphae	Br	Rough & Dry	Br
Streptomyces Act 23	Hyphae	Be	Rough & Dry	W
Kocuria Act 24	Coccus in tetrads	S	Brittle & Viscous	–
Streptomyces Act 25	Hyphae	Br	Rough & Dry	W
Streptomyces Act 26	Hyphae	Br	Rough & Dry	G
Nesterenkonia Act 27	Coccus	S	Smooth & Mucoid	–
Arthrobacter Act 46	Cocci	Y	Smooth & Mucoid	–
Kocuria Act 48	Coccus in tetrads	S	Brittle & Viscous	W
Streptomyces Act 53	Hyphae	Br	Rough & Dry	W
Streptomyces Act 55	Hyphae	Br	Rough & Dry	W
Streptomyces Act 76	Hyphae	Be	Rough & Dry	Be
Streptomyces Act 77	Hyphae	Br	Rough & Dry	Br
Streptomyces Act 79	Hyphae	Br	Rough & Dry	W
Micromonospora St 80	Branched mycelium	S	Rough & Moist	Bl
Blastococcus St 82	Coccus	S	Rough & Moist	–
Micrococcus St 84	Coccus in tetrads	Y	Dull & Moist	–
Streptomyces St 85	Hyphae	Br	Rough & Dry	W
Streptomyces Act 39	Hyphae	Br	Rough & Dry	W
Streptomyces Act 44	Hyphae	Br	Rough & Dry	W
Micrococcus Act 69	Coccus in tetrads	Y	Dull & Moist	–
Micrococcus Act 70	Coccus in tetrads	Y	Dull & Moist	–
Kocuria Act 134	Coccus in tetrads	S	Smooth & Mucoid	–
Micrococcus A1	Coccus in tetrads	Y	Smooth & Mucoid	–
Rhodococcus A5	Rods	S	Dull & Moist	–
Micrococcus A7	Coccus	Y	Smooth & Mucoid	
Brevibacterium Ap13	Rod coccus	S	Brittle & Viscous	–
Microbacterium CH5	Rods	Y	Smooth & Mucoid	–
Micrococcus CH9bis	Coccus in tetrads	Y	Smooth & Mucoid	–
Microbacterium CH12	Rods	Y	Smooth & Mucoid	–
Rhodococcus CH13	Rod coccus	S	Smooth & Mucoid	–
Microbacterium CH14	Rods	Y	Smooth & Mucoid	–
Micrococcus H5	Coccus in tetrads	Y	Dull & Moist	–
Citrococcus LC5	Coccus in tetrads	Y	Brittle & Viscous	–
Micrococcus V5bis	Coccus	Y	Smooth & Mucoid	–
Micrococcus V7	Coccus in tetrads	Y	Smooth & Mucoid	–

differences. This could be also a limitation of the databases regarding certain genera, but it is not the case for *Streptomyces*. A "rare" strain is found between the isolates: Act 15 is the strain with less similarity to a type strain, being 95.69% similar to *Streptomyces pratensis* ch24T (JQ806215). For details, see Figure S1.

ITS fingerprinting method is a molecular tool based on the sequence and length heterogeneity of the bacterial rRNA operon 16S–23S intergenic spacer and provides a high phylogenetic resolution. It can discriminate bacterial isolates up to the subspecies level. To manage the large set of isolates in our collection, ITS-PCR fingerprinting was applied as a complementary screening method. Among the 183 isolates, the 51 chosen strains proved to have 27 distinct groups (G1–G27) (Fig. 3). Each group was composed by strains belonging to the same genus, although some genera were represented by more than one group. The most common pattern was ITS-G3 constituted by 11 *Streptomyces* strains from Laguna Socompa. The second most frequent pattern was ITS-G18, formed by six *Micrococcus* strains isolated from Laguna Socompa and

Table 4. Physiological features measured in the strains, including range of growth at different temperatures, pHs, NaCl, Na_2CO_3, As (III) and As (V).

	Physiology						
Strain	Temperature range	pH range	NaCl tolerance	Na_2CO_3 tolerance	UVB resistance	As (V) resistance	As(III) resistance
Microbacterium Act 1	8–45°C	6–12	5%	0 mM	240 min	200 mM	7.5 mM
Streptomyces Act 4	4–65°C	5–12	20%	0 mM	240 min	200 mM	7.5 mM
Streptomyces Act 5	4–65°C	5–12	10%	0 mM	240 min	200 mM	7.5 mM
Streptomyces Act 6	4–65°C	4–12	15%	0 mM	240 min	200 mM	7.5 mM
Streptomyces Act 7	8–65°C	5–12	5%	5 mM	240 min	200 mM	7.5 mM
Streptomyces Act 8	8–65°C	5–12	5%	0 mM	240 min	200 mM	7.5 mM
Streptomyces Act 11	8–65°C	5–12	25%	0 mM	180 min	150 mM	7.5 mM
Streptomyces Act 12	4–65°C	5–12	15%	0 mM	240 min	200 mM	7.5 mM
Streptomyces Act 13	4–65°C	5–12	25%	0 mM	240 min	200 mM	7.5 mM
Streptomyces Act 14	15–65°C	5–12	5%	0 mM	240 min	200 mM	7.5 mM
Streptomyces Act 15	4–65°C	5–12	25%	0 mM	240 min	200 mM	7.5 mM
Streptomyces Act 16	15–55°C	5–12	25%	0 mM	240 min	200 mM	7.5 mM
Streptomyces Act 17	4–65°C	5–12	25%	0 mM	240 min	200 mM	5 mM
Nesterenkonia Act 18	8–37°C	6–12	25%	5 mM	240 min	200 mM	5 mM
Streptomyces Act 19	15–65°C	5–12	5%	0 mM	240 min	150 mM	2.5 mM
Nesterenkonia Act 20	4–37°C	5–12	25%	5 mM	240 min	200 mM	0 mM
Streptomyces Act 21	20–65°C	5–12	5%	0 mM	240 min	200 mM	7.5 mM
Streptomyces Act 23	15–65°C	6–12	5%	0 mM	120 min	200 mM	2.5 mM
Kocuria Act 24	4–37°C	4–12	15%	5 mM	240 min	200 mM	0 mM
Streptomyces Act 25	4–65°C	5–12	25%	0 mM	240 min	200 mM	5 mM
Streptomyces Act 26	4–65°C	5–12	25%	0 mM	240 min	200 mM	2.5 mM
Nesterenkonia Act 27	4–37°C	5–12	25%	5 mM	240 min	200 mM	7.5 mM
Arthrobacter Act 46	8–65°C	5–12	25%	0 mM	240 min	200 mM	7.5 mM
Kocuria Act 48	8–37°C	7–10	10%	0 mM	240 min	200 mM	7.5 mM
Streptomyces Act 53	4–65°C	5–12	20%	0 mM	240 min	200 mM	7.5 mM
Streptomyces Act 55	8–65°C	5–12	25%	0 mM	240 min	200 mM	5 mM
Streptomyces Act 76	4–65°C	5–12	25%	5 mM	240 min	200 mM	5 mM
Streptomyces Act 77	15–65°C	5–12	25%	0 mM	240 min	200 mM	5 mM
Streptomyces Act 79	8–45°C	5–12	10%	0 mM	240 min	200 mM	2.5 mM
Micromonospora St 80	15–65°C	4–12	25%	0 mM	240 min	200 mM	10 mM
Blastococcus St 82	8–65°C	5–12	25%	0 mM	240 min	200 mM	10 mM
Micrococcus St 84	4–65°C	4–12	25%	0 mM	240 min	200 mM	10 mM
Streptomyces St 85	8–55°C	5–12	25%	0 mM	240 min	200 mM	10 mM
Streptomyces Act 39	4–65°C	5–12	5%	0 mM	240 min	200 mM	7.5 mM
Streptomyces Act 44	8–55°C	5–12	5%	0 mM	240 min	200 mM	5 mM
Micrococcus Act 69	8–65°C	4–12	25%	5 mM	240 min	200 mM	7.5 mM
Micrococcus Act 70	8–65°C	4–12	25%	0 mM	240 min	50 mM	0 mM
Kocuria Act 134	8–55°C	4–12	25%	0 mM	240 min	200 mM	7.5 mM
Micrococcus A1	8–65°C	5–12	15%	0 mM	240 min	200 mM	10 mM
Rhodococcus A5	8–37°C	4–12	5%	0 mM	240 min	200 mM	7.5 mM
Micrococcus A7	15–65°C	5–12	10%	0 mM	240 min	200 mM	10 mM
Brevibacterium Ap13	4–65°C	5–12	10%	0 mM	240 min	200 mM	10 mM
Microbacterium CH5	8–45°C	5–12	5%	0 mM	240 min	200 mM	10 mM
Micrococcus CH9bis	8–65°C	4–12	7.5%	0 mM	240 min	200 mM	10 mM
Microbacterium CH12	15–45°C	5–12	5%	0 mM	240 min	50 mM	7.5 mM
Rhodococcus CH13	8–65°C	5–12	5%	0 mM	240 min	200 mM	10 mM
Microbacterium CH14	8–37°C	5–12	5%	0 mM	240 min	0 mM	7.5 mM
Micrococcus H5	8–55°C	5–12	7.5%	0 mM	240 min	200 mM	10 mM
Citrococcus LC5	8–55°C	4–12	5%	0 mM	240 min	200 mM	10 mM
Micrococcus V5bis	15–55°C	5–12	10%	0 mM	240 min	200 mM	10 mM
Micrococcus V7	8–45°C	5–12	5%	0 mM	240 min	200 mM	7.5 mM

Chaxas. Supporting Information Table S1 and Fig. 3 summarize the ITS groups.

The distribution of the isolated actinobacteria with respect to the sampled lake (Fig. 4A), the niche (Fig. 4B) and the genera (Fig. 4C) was likewise analyzed. Laguna Socompa was the lake in which the major quantity of strains was isolated from, followed by L. Chaxas, L. Diamante and L. Azul. In contrast, L. Antofalla, Tolar Grande, Santa María, Salar Pocitos, L. Verde and L. Negra did not yield any actinobacterium in spite of the different media and procedures employed. The niche of isolation was also important for obtaining actinobacteria: Soil samples were by far the ones producing the major quantity of isolates which is known to be a common habitat for actinobacteria (Albarracin *et al.*, 2010), followed by water, feces and stromatolites. Most isolates were identified as belonging to the *Streptomyces* genus, followed by *Micrococcus* and *Microbacterium*. Strains belonging to *Nesterenkonia*, *Kocuria*, *Rhodococcus*, *Arthrobacter*, *Micromonospora*, *Blastococcus*, *Micromonospora* and *Citrococcus* were less abundant in our samples.

The morphology of representatives of each genus was observed using SEM (Fig. 4D). For *Streptomyces*, it was observed the typical hyphal network and for *Microbacterium* the

short rods occurring singly or in irregular clusters. The *Nesterenkonia* representative showed their coccoid cells grouped in pairs, tetrads and irregular clusters, while *Kocuria* cells grouped in tetrads. The *Arthrobacter* strain presented coccoid shape, a morphotype commonly found in this genus when cells are in the stationary phase or when they are exposed to desiccation and starvation (38).

UV resistance and polyextremophilic profile

To determinate the ability of the selected 51 actinobacterial strains to tolerate high doses of UV radiation and other extreme conditions common at their original environment, we carried out physiological assays against different extreme conditions as explained in the above-method section. The extreme conditions tested cover the range of original conditions assumed to be present by previous analyses of our group (1–8) and also those considered opposite, performed as controls and to assess complete physiological ranges. For instance, high pH is common at the lake samples, but we also tested low pH. The polyextremophilic profiles of each strain are then summarized (Table 4).

Data obtained from the physiological assays are shown in Fig. 5 as percentages of the strains belonging to each genus that had a good growth after exposed to a given stress factor. Note that the percentages were based on the number of strains per genera, that is 25 strains for *Streptomyces*, nine for *Micrococcus*, four for *Microbacterium*, three for *Nesterenkonia* and *Kocuria*, two for *Rhodococcus* and only one strain from the rest of genera. For the graphic, two temperature ranges were considered as low (4–30°C) and high (37–65°C). In this case, just few strains were able to grow in the low range of temperature. Although representatives of *Streptomyces, Micrococcus, Nesterenkonia, Kocuria and Brevibacterium* were able to grow at 4°C, this was not optimum. In turn, a good number of strains grown in the range of high temperature and belonged to *Micrococcus*, *Streptomyces*, *Micromonospora*, *Arthrobacter*, *Brevibacterium*, *Rhodococcus* and *Blastococcus* genera. In comparison, control strains including *Streptomyces coelicolor* showed poor growth at higher temperatures (data not shown).

Regarding UVB resistance, all 51 actinobacterial isolates showed a high tolerance to the exposure from 2.0 to 3.0 W m^{-2} UVB radiation at different times corresponding to 100% of strains per genera. Only two *Streptomyces* strains (Act 11 and Act 23), both isolated from the soil of Laguna Socompa, did not tolerate the maximum time of irradiation, being able to tolerate only 180 and 120 min of exposure to UVB radiation, respectively, resulting in this case to a value of 92% for *Streptomyces*. *Streptomyces coelicolor*, a common laboratory horse strain in actinobacteria research, could develop well after 90 min of exposure but was inhibited after 120 min of exposure. In turn, *Escherichia coli* was quite sensible to UVB; that is, the inhibition of growth was evident after 60 min, while after 240 min, the strain was not able to develop.

The same diluted drop protocol was performed to evaluate tolerance to Na_2CO_3 and NaCl, using culture media supplemented with different concentrations of Na_2CO_3 (2.5 and 5.0 mM) and NaCl (1–30% w/v). All isolates tolerate up to 5% NaCl (w/v), being moderately halophiles (Table 4). Moreover, 21 strains belonging to *Streptomyces*, *Micrococcus, Nesterenkonia, Kocuria, Arthrobacter, Micromonospora* and *Blastococcus* genera grew on NaCl up to 25% (Table 4). Although HAALs' salinities varied between 5 and 32%, we found no strain growing at NaCl 30%. Only seven strains were able to grow on LB agar media supplemented with Na_2CO_3 5.0 mM (Fig. 4), which included the three *Nesterenkonia* strains Act 18, Act 20 and Act 27 (100% of the strains), and considered facultative alkalophilic salt-tolerant bacteria. In contrast, control actinobacterial strain *S. coelicolor* was not able to develop well in salt-supplemented media. It was inhibited at concentrations of NaCl 10% to 30% and with concentrations of Na_2CO_3 2.5 and 5.0 mM.

Growth at different pH values was analyzed in the pH 4–12 range. It was obvious from Fig. 5 that HAALs' actinobacterial strains were able to tolerate better the high pH than the low pH. Thus, 100% of strains (50 isolates) belonging to the genera *Micrococcus, Streptomyces, Nesterenkonia, Micromonospora,*

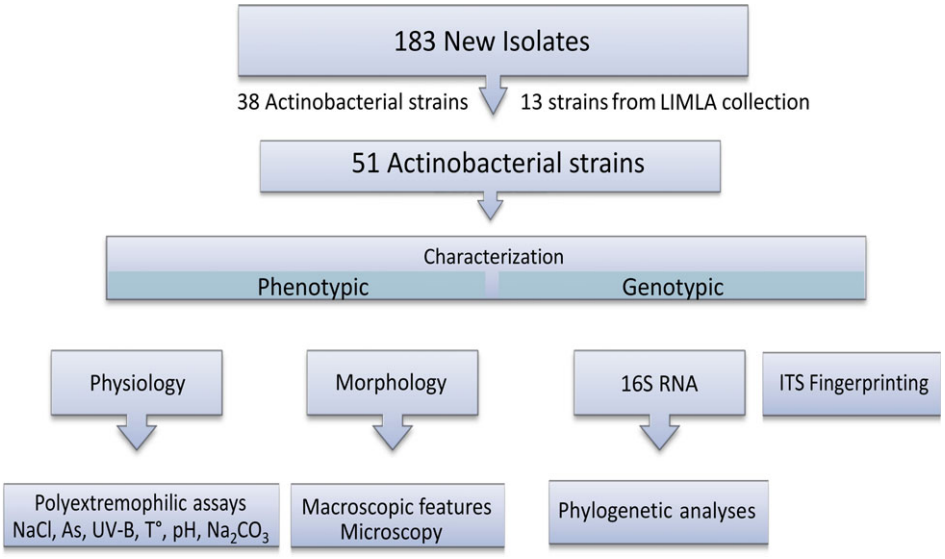

Figure 2. General outline of the procedures and methodology followed in this work. [Color figure can be viewed at wileyonlinelibrary.com]

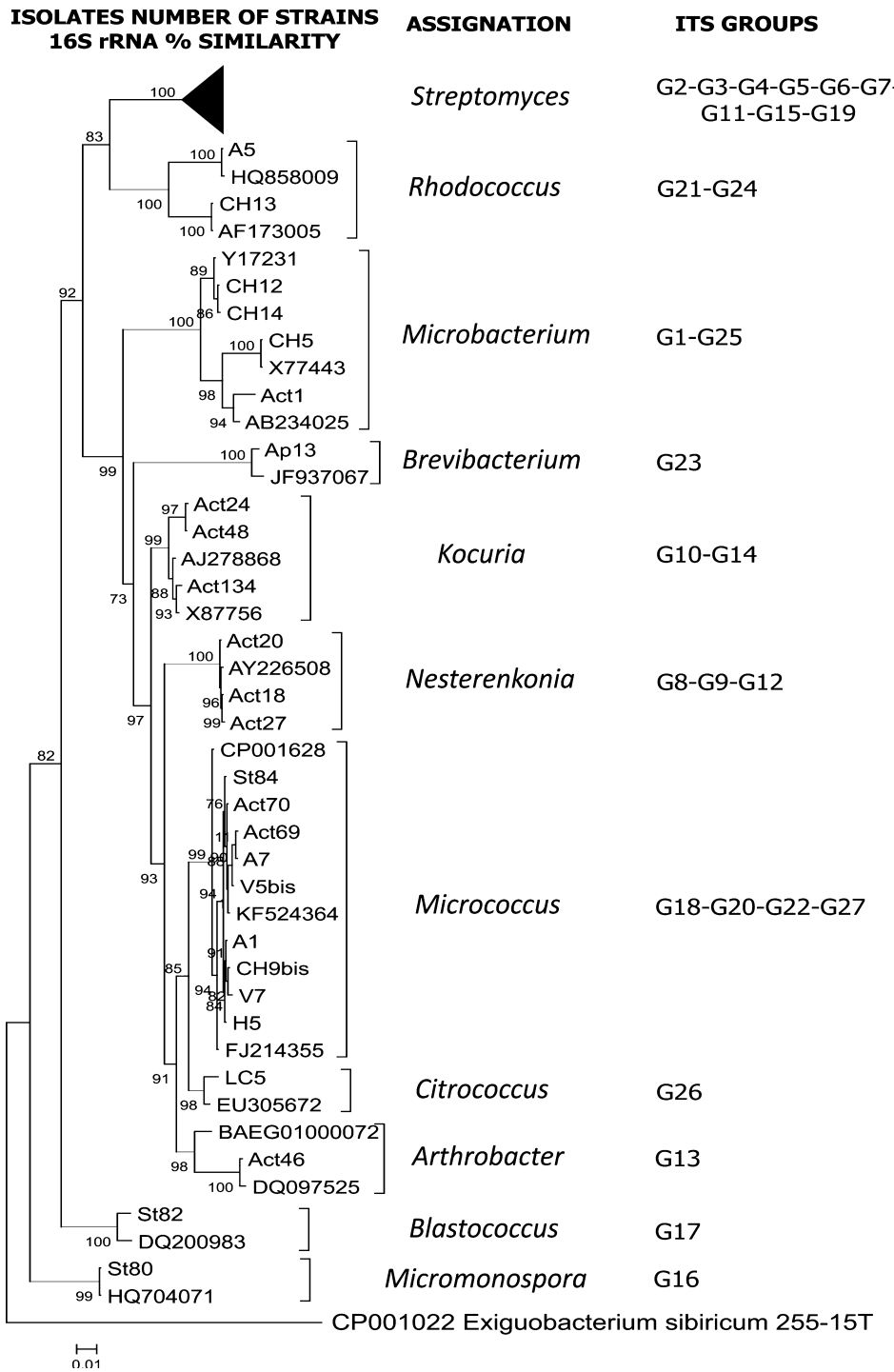

Figure 3. Phylogenetic tree based on the similarity at 97% of the 16S RNAr gene, its assignation and the ITS groups.

Arthrobacter, Brevibacterium, Rhodococcus, Microbacterium, Kocuria, Citricoccus and *Blastococcus* were able to develop at pH 12; the only exception was *Kocuria* Act 48 (Table 4). In turn, only 100% of the strains belonging to *Microcomonospora* and *Citrococcus* could grow well at low pH. Also, some isolates belonging to the genera *Micrococcus* (44%), *Streptomyces* (4%), *Rhodococcus* (50%) and *Kocuria* (66%) could grow at this low pH. For the control, *Streptomyces coelicolor,* the range of growth was between pH 6 and pH 10.

Thirty-seven strains tolerated 7.5 mM As (III), while only 13 (*Microbacterium*, *Micromonospora*, *Blastococcus*, *Micrococcus*, *Brevibacterium*, *Rhodococcus* and *Citrococcus*) were able to grow at 10 mM As (III). Regarding As (V), 46 grew at the maximum concentration tested (200 mM) including members of the eleven genera. In contrast, *Streptomyces coelicolor* could grew well in the range of As (III) 2.5 mM to 7.5 mM but was more sensitive for the As (V), showing inhibition of growth at concentrations higher than 100 mM.

Antagonistic activities of polyextremophilic actinobacteria

To preliminarily determine whether the isolates produced any antimicrobial compounds, an antagonistic assay was performed by confronting all isolated strains with each other on agar nutrient media yielding a 51 × 72 isolate array where 3672 antagonism assays were scored. The failure of several species to grow near the producing organism may indicate that it produced an active compound against these competitors.

From the results, it is obvious that all the isolates exhibited antagonistic activities against at least one of the tested bacteria; these also included *E. coli*, *Bacillus*, *Enterococcus faecalis*, *Staphylococcus aureus* and two yeasts (*Rhodotorula* sp.). The production of antagonistic compounds was measured and plotted in a graphic (Fig. 6), where three, two or one sign was taken as 3, 2 or 1 unit of inhibition and then summed up together to reach a unique value. Negative signs were counted as null unit as indicated in material and methods (37). All strains with a score above the threshold value 120 were considered as highly productive (Fig. 7A). The data obtained clearly showed that the representatives of *Streptomyces, Microbacterium* and *Micrococcus* were potentially the most prominent antimicrobial producers among the tested strains. The origin of isolation (niche) was also important with regard to bioactivity (Fig. 7B). Strains isolated from feces were much more productive than those isolated from soils and water, while strains isolated from the stromatolite were the less active strains.

DISCUSSION

Novel actinobacteria and their products derived from poorly explored areas of the world highlight the importance of investigating new habitats (39,40). In addition, the fact that they are extremophiles increases this importance. Thus, we carried out the first extensive isolation of actinobacteria from a wide range of niches of high-altitude lakes including modern stromatolites, the highest on Earth. According to Quintana *et al.* (39), the use of traditional selective isolation media for Actinobacteria still can yield novel groups of taxonomic variation within this vast assemblage of microorganisms. So, selective isolation techniques with different media and conditions and using as source different kind of samples including soil, sediment, modern stromatolites, flamingos feces and water, from Lagunas Socompa, Antofalla Verde and Rojo, Grande, Diamante, Tolar, Negra, Verde, Cabe and Pocitos were performed. In this way, we have completely characterized 51 actinobacterial strains belonging to 11 genera, that is *Streptomyces*, *Micrococcus, Microbacterium, Nesterenkonia, Kocuria, Rhodococcus, Arthrobacter, Micromonospora, Blastococcus, Brevibacterium* and *Citricoccus.* The results

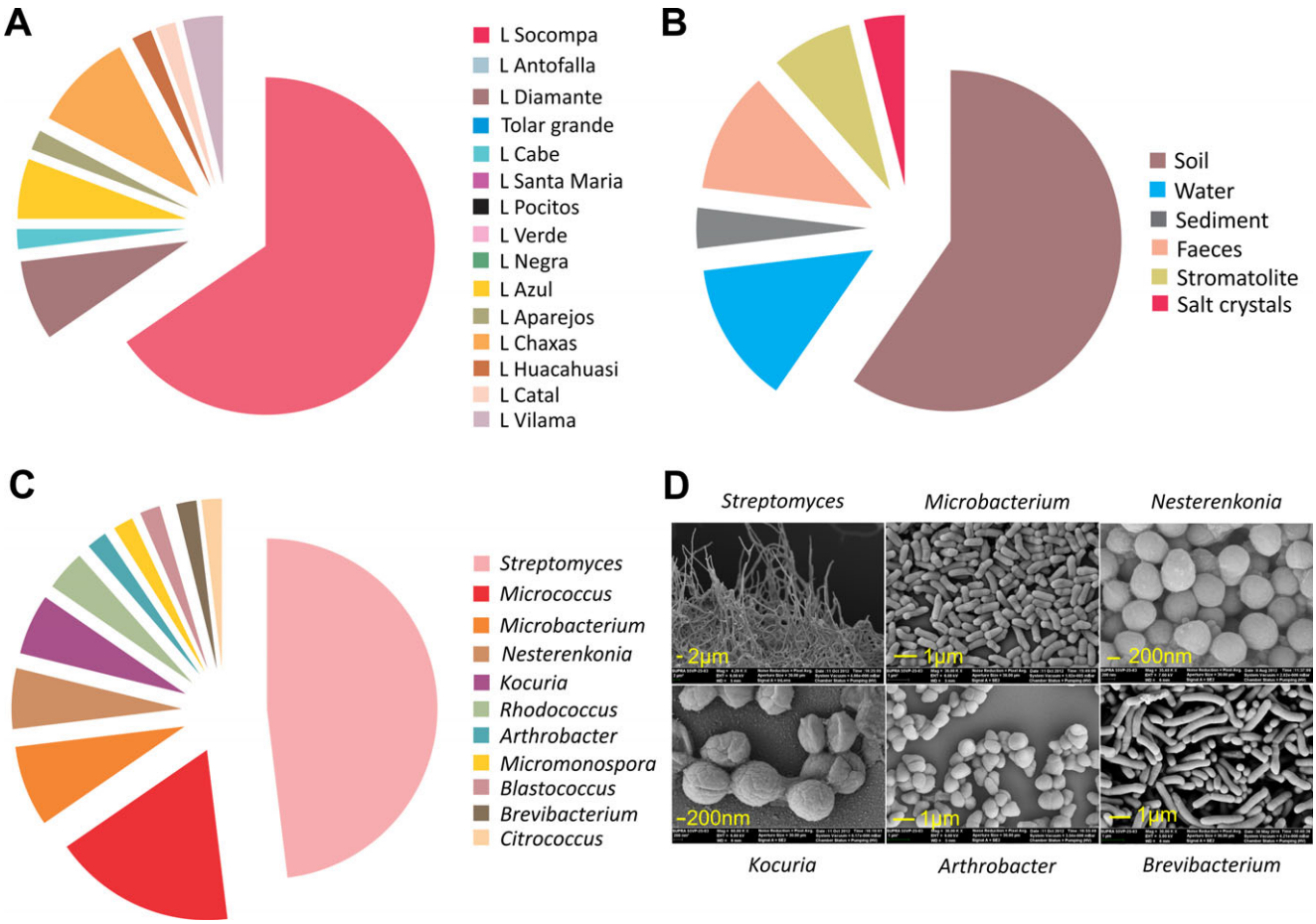

Figure 4. (A) Distribution of bacterial isolates based on the lakes where they were isolated from. (B) Distribution of bacterial isolates based on the niches. (C) Distribution of bacterial isolates based on their genus assignations. (D) Scanning electron microscopy of representative strains.

revealed a significant diversity of actinobacteria in the HAALs and suggested that this environment can be a source of novel "rare" actinobacteria.

As *Streptomyces* is a common genus isolated from the High Andes (41–43), we attempted to compare the phylogenetic affiliation of our isolates with that already described (41,43) from the Atacama Desert (Fig. S1). Three well-defined clusters were observed, that is Atacama, Socompa and Diamante, suggesting that although these locations share some characteristics, such as high salinity and high UV irradiation regime, the wide geographical distance forces them to behave as "islands" which select different species. The majority of the strains isolated in this work were from samples of L. Socompa, and among them, the *Streptomyces* genus was the most abundant group, including a novel strain Act 15 with low similarity to a type strain of *Streptomyces pratensis* ch24T (JQ806215), suggesting the possibility of describing a new species at Socompa.

Cultivated-independent molecular techniques indicated the occurrence of actinobacteria in various modern stromatolites—including Socompa and Shark Bay (Australia) stromatolites—with relative abundances in the range a few percent to tens of percent (6,44). In dry modern freshwater stromatolites from Ruidera Pools Natural Park, Spain libraries were dominated by sequences related to *Cyanobacteria*, although a diverse and abundant assemblage of noncyanobacterial sequences was also found, including members of Actinobacteria (*Knoellia* sp. and *Arthrobacter* sp.) (45). Here, for the first time, we reported the isolation of *Micromonospora*, *Blastococcus*, *Micrococcus* and *Streptomyces* strains from stromatolites. Something to highlight is that the genus *Blastococcus* comprises three species, isolated from sea (46) and soil and plant (47) samples but most emblematically from stones (48). The genus belongs to *Geodermatophilaceae*, which are unique model systems to study the ability to thrive on or within stones, and their proteogenomes (referring to the whole protein arsenal encoded by the genome) could provide important insight into their adaptation mechanisms. The species *Blastococcus saxobsidens*, comprising strains isolated from calcareous stones, was described in 2004 (49) and genome-sequenced together with *Modestobacter marinus* and *Geodermatophilus obscurus* (50). This genus also has the ability to resist adverse environmental conditions such as ultraviolet light, ionizing radiation, desiccation and heavy metals (51–53). This resistance to environmental hazards represents a trait of Terrabacteria, a well-supported phylogenetic group composed of Actinobacteria and four other major lineages of eubacteria (Firmicutes, Cyanobacteria, Chloroflexi and Deinococcus-Thermus) that colonized land 3.05–2.78 Ga (54).

Understanding the limits for life is of additional biotechnological interest due to the applications of extremophiles and their biomolecules in industrial processes. Microbial strains isolated from extreme environments are commonly able to tolerate multiple harsh conditions which was the case for most of the HAAL actinobacteria strains characterized in this work. As observed for the multiple extreme conditions tolerated by these actinobacteria, the herein described actinobacteria are considered polyextremophiles. According to Mesbah and Wiegel (55), polyextremophiles are of great interest, as their adaptive mechanisms give insight into the abilities of bacteria to survive in environments which were previously considered prohibitive to life. Our results showed that all 51 actinobacteria tested were resistant to UVB at different doses. Ordoñez *et al.* (3) also found highly resistant UVB bacteria on the HAALs, two of them belonging to *Nocardia* and *Micrococcus* genera. HAAL-isolated strains are then excellent models (1) for studying adaptive responses and mechanisms that underlie light sensing and UV resistance in environmental microbes (2). Available genomes of model HAAL polyextremophilic microbes (20,56) and several metagenomes of microbialites will allow to perform in-depth basic and applied research (6–8,31,55). In this work, we also pointed out the challenge to study mechanisms of UV resistance in coincidence with secondary metabolite production in polyextremophilic actinobacterial strains.

Regarding NaCl tolerance, our results indicated that all isolates could be considered moderately halophilic (17) which agreed with the average conditions found at the HAALs (1–8). Moreover, representatives of the 11 genera were facultative alkaliphiles (17), tolerating the highest pH values. Several of the genera described in this study have been previously reported as halotolerant, for example *Nesterenkonia* isolated from southern Tunisia, Brazilian Mangrove sediment and hypersaline Ekho Lake in East Antarctica (57,58) and *Arthrobacter* strains isolated from southern Tunisia (59). Alkaliphilic *Streptomyces* were likewise found in various types of soils of Georgia (60) and in saline soils and lake sediments from China (61). Zenova *et al.* (62) described alkaliphilic and halophilic *Streptomyces* and *Micromonospora* strains isolated from different types of Russian soils. In

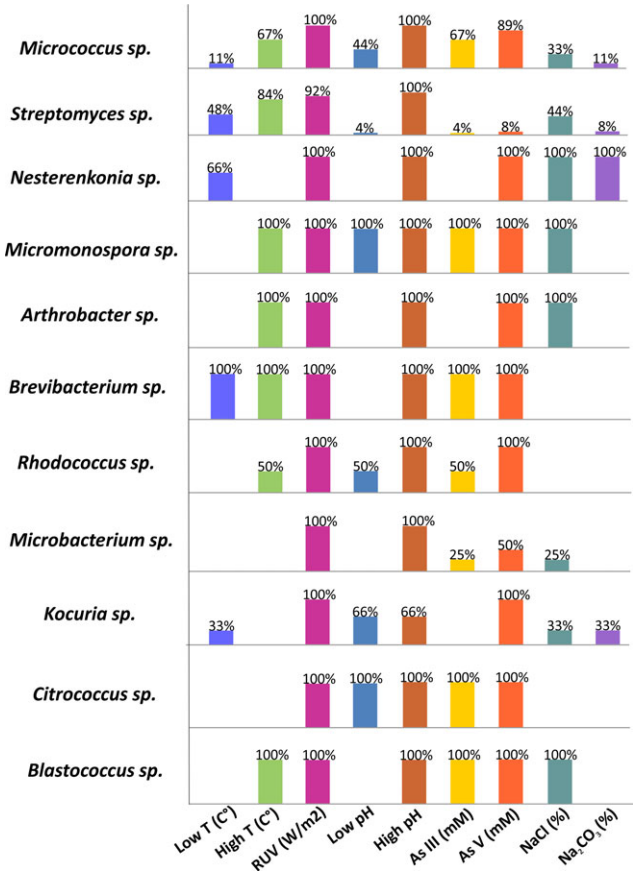

Figure 5. Polyextremophilic profile of the strains based on their genera. The percentage of the number of strains per genera that grew in each extreme condition is represented. [Color figure can be viewed at wileyonlinelibrary.com]

Figure 6. Activity profile depicting the results of the antagonistic assay confronting all isolated strains with each other. Production of antagonistic compounds where three, two or one sign was taken as 3, 2 or 1 unit of inhibition and then summed up together to reach a unique value. Negative signs counted as null units. [Color figure can be viewed at wileyonlinelibrary.com]

the saline soils of a Chinese province, Li *et al.* (63) described the alkaliphilic *Streptomyces sodiiphilus* sp. nov. actinomycete, indicating saline environments as likely source to new species bioprospection.

Considering the tolerance to high temperature, several actinobacteria strains fit into the facultative thermophiles group (17) which may indicate that hydrothermal conditions quite common in the lakes (6-31) and high sun irradiation regime of soils (1,2) selected strains able to grow under higher temperatures. Thermophilic and thermotolerant actinobacteria were found in diverse niches such as moldy hay, self-heating plant residues, cereal grains, sugar cane bagasse, decaying vegetable materials, soils, compost heaps, sea sediment and hot springs (for a review, see (64)), but this is the first time that thermophilic actinobacteria were described in stromatolites. In coincidence with our findings, the complex of thermotolerant actinomycetes described for the Mongolia desert steppe included representatives of *Streptomyces and Micromonospora* (62). Likewise, in the mountain meadow

soils of the central Caucasus, *Micromonospora* and *Saccharopolyspora* genera predominated among the thermotolerant actinomycetes (62). On the other hand, some of the genera described in our work were also found to be psychrophilic; Margesin *et al.* (65) found psychrophilic *Arthrobacter* and *Rhodococcus* in cryoconite glacier, tolerating 1°C.

About the arsenic assays, our results clearly showed that arsenic resistance is widespread in HAAL actinobacteria. In general, the tested actinobacterial strains are much more tolerant to higher concentration of As (V) than to As (III). These findings concur previous reports on the characterization of HAAL microbes (1–8,19,20) and also agree with the high concentration of arsenic found there, consequence of the geomorphology of the Puna–High Andes region. Some genera described in this work were reported before as As-resistant strains; *Kocuria arsenatis* sp. nov., an endophytic bacterium, was isolated from roots of two heavy-metal-resistant plants in mine tailings in Mexico (66), while *Rhodococcus* and *Arthrobacter* sp. strains with significant As-reductase activities were isolated from arsenic-contaminated groundwater in India (67). Nevertheless, arsenic resistance/adaptation mechanisms are still poorly studied in actinobacteria. In this sense, our work provided ca. 50 strains of 11 different genera that can be likely models for studying this toxic-metalloid metabolism aiming at biotechnological applications.

Bacteria in natural environments compete for space and resources (68). So, as surviving strategy, they produce substances that can inhibit the growth of other organisms (natural compounds that are toxic or inhibitory to their competitors) providing an advantage to colonize a niche. Toxic secretions create antagonist interactions between producers and nonproducers (69). The apparent diversity of actinobacteria obtained from the HAALs prompted us to investigate the potential production of potential antimicrobial compounds of the selected strains, mostly against each other which are considered the most appropriate models to study antagonistic "indigenous" activities. *Streptomyces, Microbacterium* and *Micrococcus* were the most prominent producers of cytotoxic compounds among the isolates tested, being CH5 and CH9bis isolated from flamingo feces, the ones with the highest score. However, all the isolates showed inhibition against other strain, indicating that all of them have some potential for antimicrobial production and suggesting that antagonistic interactions may be common in the HAALs' niches. Identification of the inhibitory compounds is currently in progress. HAAL actinobacteria already proved potential for biotechnological applications; Bequer Urbano *et al.* (2012) described *Rhodococcus* sp. strains were able to produce triacylglycerols (TAG) or wax esters (WS) under nitrogen-limiting culture conditions. On the other hand, Dib *et al.* (2010) described rare actinobacteria from HAALs and screened these novel isolates for the presence of megaplasmids. Ten of these strains contained one or more giant linear plasmids including *Brevibacterium*, *Kocuria* and *Micrococcus* genera. The importance of their isolation is

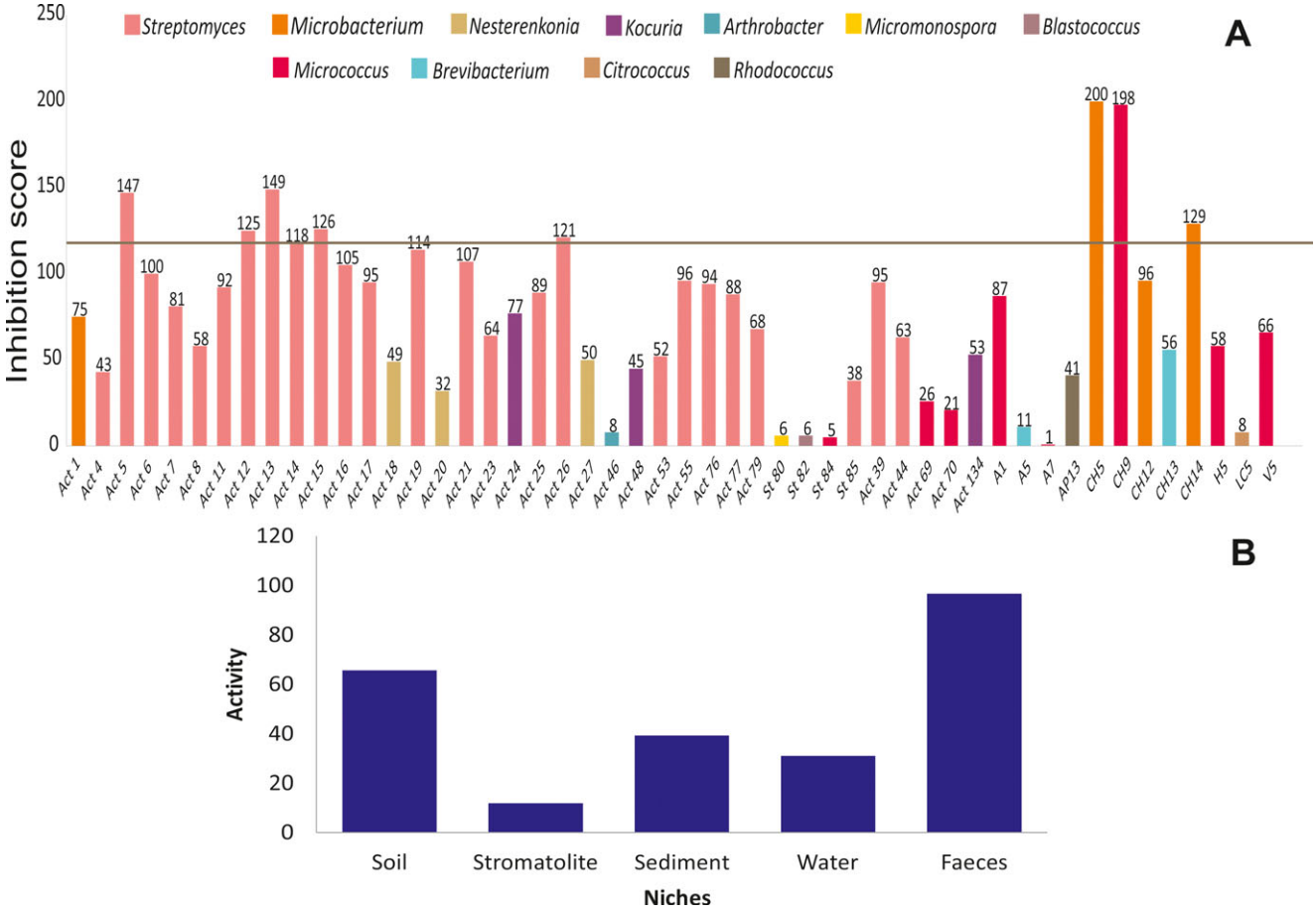

Figure 7. (A) Inhibition score for each strain. The horizontal line represents a threshold value of 120. (B) The scores were also plotted based on the niche where the strains were isolated from.

demonstrated by the fact that many of the successful anti-infective products on the market are produced by such microbes. Genes encoding biosynthetic enzymes, resistance proteins and regulatory systems associated with microbial natural products are typically clustered. *Streptomyces* produces the majority of the clinical antibiotics of natural origin, and antifungal, anticancer, antiparasitic and immunosuppressive agents (40,41,43,47). According to Nett *et al.* (70), orphan secondary metabolic gene clusters represent a large resource of new chemical entities and a novel source of drug candidates. Challis *et al.* (71) investigate the "cryptic" or "orphan" natural product biosynthetic gene clusters found within the genomes of *Streptomyces coelicolor* and other sequenced microbes that encode natural product biosynthesis-like proteins not associated with the production of known metabolites by mining their genomes. Current work is focused on assessing the secondary metabolite production of HAAL *Streptomyces* using a variety of media and extreme culturing conditions, including UV, for monitoring differential expression of PKS and NRPS genes by a transcriptomic approach.

CONCLUSIONS

In conclusion, a large number of UV-resistant actinobacteria were isolated from HAALs, including the so-called rare actinobacteria. Almost all of them were able to tolerate multiple extreme conditions, being polyextremophiles. To our knowledge, this is the first study to describe the isolation of actinobacteria from modern stromatolites, thus providing with a new niche for natural product prospection. The preliminary screening indicated the potential of most strains to produce bioactive compounds, and these behaviors suggested a likely role of cytotoxic compounds for competitive advantage of actinobacteria in their original environments. Our results provide further evidence of significant biodiversity of actinobacteria in extreme habitats and support high-altitude Andean lakes as a largely unexplored repository of novel and polyextremophilic bacteria, specially rare actinobacteria, with high potentiality as producers of new natural products and genes for their biosynthesis. Current research is focused on analyzing biosynthetic clusters in the genomes of HAAL actinobacteria and within available metagenomes.

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SUPPORTING INFORMATION

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

Figure S1. Phylogenetic tree based on the similarity at 97% of the 16S RNAr gene, corresponding to the *Streptomyces* fraction from the phylogenetic tree of the Fig. 3.

Table S1. Distribution of strains by ITS group.

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