

Microbial Colonization of the Salt Deposits in the Driest Place of the Atacama Desert (Chile)

Nunzia Stivaletta · Roberto Barbieri · Daniela Billi

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Abstract The Atacama Desert (Chile), one of the most arid places on Earth, shows hostile conditions for the development of epilithic microbial communities. In this study, we report the association of cyanobacteria (*Chroococidiopsis* sp.) and bacteria belonging to Actinobacteria and Beta-Gammaproteobacteria and Firmicutes phyla inhabiting the near surface of salt (halite) deposits of the Salar Grande Basin, Atacama Desert (Chile). The halite deposits were investigated by using optical, confocal and field emission scanning electron microscopes, whereas culture-independent molecular techniques, 16S rDNA clone library, alongside RFLP analysis and 16S rRNA gene sequencing were applied to investigate the bacterial diversity. These microbial communities are an example of life that has adapted to extreme environmental conditions caused by dryness, high irradiation, and metal concentrations. Their adaptation is, therefore, important in the investigation of the environmental conditions that might be expected for life outside of Earth.

Keywords Atacama Desert · Halite · Endoliths · 16S rDNA clone library · Extremophiles

Introduction

The hyperarid Atacama Desert in northern Chile is inhospitable to most living species. The extreme dryness, daily thermic excursion, salinity, hydrothermalism, oxidizing conditions, and toxic metal (e.g. arsenic) concentrations make the Atacama region a unique complex of extreme environmental settings. Despite such characteristics of extreme habitats, diverse

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N. Stivaletta (✉) · R. Barbieri

Dipartimento di Scienze della Terra e Geologico-Ambientali, Università di Bologna, Via Zamboni 67,
40126 Bologna, Italy

e-mail: nunzia.stivaletta@unibo.it

D. Billi

Dipartimento di Biologia, Università degli Studi di Roma Tor Vergata, Via della Ricerca Scientifica 1,
00133 Rome, Italy

endolithic communities of photosynthetic cyanobacteria associated with heterotrophic microorganisms have been recovered (Barbieri et al. 2009; De los Ríos et al. 2010; Kuhlman et al. 2008; Wierzchos et al. 2006, 2011). The survival of microorganisms in the Atacama Desert is in fact made possible thanks to the colonization of salt rocks (mostly halite) which allows the phenomenon of deliquescence (Davila et al. 2008). The halite absorbs very low amounts of water, which is present in the form of dew, and is then retained as a solution in the pores of the salt rock. Water is therefore made available in a liquid form when necessary. The microbial colonization of halite rocks, however, is in sharp contrast to the virtual absence of photosynthetic microorganisms in the surrounding soils (Navarro-González et al. 2003). Due to soil similarities between the Atacama Desert and sectors of the Martian surface, this region has been regarded as an effective Mars environmental analog (McKay et al. 1992; Navarro-González et al. 2003).

Several studies have demonstrated that sulfate and chloride mineral deposits and crusts are a convenient refuge for microorganisms in certain terrestrial stressful environments (Dong et al. 2007; Stivaletta and Barbieri 2009; Stivaletta et al. 2011; Wierzchos et al. 2006, 2011). The discovery of hydrated sulfates and chlorides having a likely origin from continental (lacustrine) evaporites on the Martian surface (e.g. Osterloo et al. 2008, 2010; Squyres et al. 2004) has considerably expanded the astrobiological potential of evaporite deposits (Stivaletta et al. 2009). Comprehensive studies on the microbial communities of hypersaline lakes from arid settings, which typically include continental sabkha/playa systems, may therefore have implications for the search of Martian life, if any. Deposits interpreted as a product of the sedimentation from lacustrine environments are actually considered a primary target for the selection of the landing site in the next planetary missions. Gale Crater on Mars surface contains likely evidences of early lacustrine phase (evaporite deposits) and was selected as a landing site for Mars Science Laboratory (MSL) (Anderson and Bell 2010 and references therein), in which the assessment of any evidence of life (past and present) or organic activity is the main scientific objective.

The present study focuses on the characterization of the microbial endolithic communities in the halite rocks that are found in the driest sector of the Atacama region (21°12' 53"S; 69° 51' 48" W) in order to increase our understanding of life-form diversity in terrestrial environments where the survival of microorganisms may be seriously conditioned by limiting environmental factors. For this purpose, a polyphasic approach based on a morphological study of the microbial communities in halite deposits by optical, confocal, and field emission scanning electron (FESEM) microscopes, combined to the genetic analyses of environmental 16S rRNA gene, has been performed. The molecular analysis was conducted through the construction of a library of environmental gene sequences of 16S rDNA and identification of different clones by RFLP analyses (restriction fragment length polymorphism) and sequencing. Elemental and mineralogical analyses by energy dispersive X-ray spectroscopy (EDX) and powder X-ray Diffraction (XRD) were, moreover, performed to investigate the minerals occurring in the halite deposits.

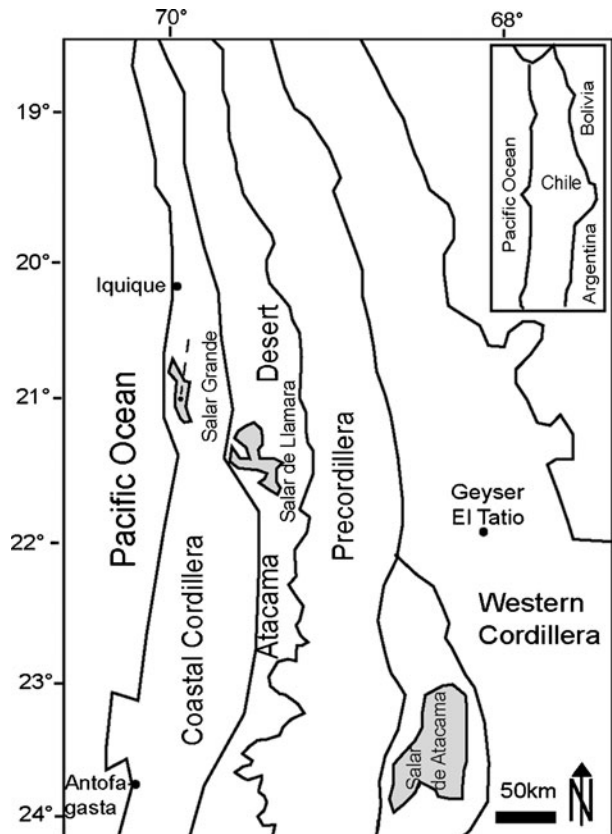
This approach allowed the identification in the halite rocks of members of Actinobacteria, Beta-Gammaproteobacteria and Firmicutes together with representatives of the genus *Chroococciopsis*, which is able to withstand extreme desiccation by entering in an ametabolic state known as anhydrobiosis (Billi and Potts 2002). Thus their finding in Chilean evaporites further contributes to the characterization of phototrophs thriving in the Atacama Desert. The finding of *Chroococciopsis*-like cells, supported by morphological analysis, were reported in halite deposits in the Salar Grande (De los Ríos et al. 2010) as well as in the Yungay area (Wierzchos et al. 2006).

Description of the Study Area

Atacama Desert

The Atacama Desert covers more than 1,000 Km along the Pacific Coast of northern Chile and is located between the Coastal Cordillera to the west and the Western Andean Cordillera to the east (Fig. 1). The aridity that characterizes this region since 10–15 Ma ago (Houston and Hartley 2003) is a result of the unique physiographic features that block the eastern and western access to humid air. The region is characterized by an ocean-desert climate and high thermic excursion, as a result of the confluence between the sub-tropical high zone and the cold Humboldt Current, which prevents the humid air from reaching northern Chile (Parrish and Curtis 1982). In the Coastal Cordillera and the Central Depression, which also include the Atacama Desert, the average annual rainfall is less than 1 mm (Clarke 2006). In the past, this system received its water from the High Andes, located to the east, and also through a fluvial system coming from the south. Changes in the climate, due to the systematic uplifting of the High Andes (Hollingworth 1964), started the development of a rain shadow area, which gave rise to the development of numerous dry saline lakes, locally called *salares*, and salt deposits, such as perchlorate, iodine and nitrate in the soil, together with halite, gypsum and anhydrite (Bohlke et al. 1997; Ericksen 1981). The wide halite deposits of the region are largely attributed to the weathering of Neogene or earlier continental evaporites (Risacher et

Fig. 1 Map of northern Chile showing the main morphostructural units (after Risacher et al., 2003). The dashed line shows the location of the sampled area in the Salar Grande



al. 2003). Arsenic and metallic sulfides occur in the Atacama Desert. Because of the river drainage of hydrothermally altered Andean regions, arsenic is abundant in the rivers, ground water, and sediments of the Atacama region (Smedley & Kinniburgh, 2002).

Salar Grande

The Salar Grande, located approx. 80 Km south of Iquique, at an average altitude of 700 m a.s.l. (Fig. 1), is an intramontane basin of the Coastal Cordillera contiguous to the Pacific coastal cliff. It is a roughly N-S elongated dry salar, approx. 45 Km long and 5 Km wide, with an estimated thickness of approx. 100 m of almost pure halite (Garcia-Veigas et al. 1996). During the late Cenozoic, this evaporitic basin had a physical and hydrological connection with another lacustrine evaporitic system in the Llamara-Quillagua area, which is located to the east in the present Central Depression (Salar de Llamara) (Chong et al. 1999). The considerable halite accumulations in the Salar Grande might be explained as a consequence of this previous connection (Mortimer 1973).

The fossil salt accumulation of the Salar Grande dates back to the Late Miocene-Pliocene, when the area became hydrologically inactive (Chong 1988). No significant water recharge presently exists, although some scarce inputs are still provided from the east as groundwater flow through an E-W fracture system (Chong et al. 1999). The remarkable purity of the halite deposits of the Salar Grande—halite is 95–99 %, with very scarce amounts of sulfate minerals and terrigenous sediments (see the results section)—implies evolved parental brines from the east through the Llamara-Quillagua area.

The surface of the Salar Grande is morphologically irregular, covered by cream-colored dust, sand, and coarse rock fragments of a different origin. Locally, white spots reveal the underlying fossil salt deposits (Fig. 2). Near-surface, mm- to cm-thick green-colored bands are visible in the halite deposits when the rock is mechanically broken. These bands indicate microbial colonization just beneath the rock surface and, locally, between the pores created by deliquescence phenomena (Fig. 3a).

Materials and Methods

Optical and Scanning Electron Microscopy

After sampling, small halite rock fragments with green coloration were scraped in laboratory to obtain a powder, and then dissolved in distilled water and placed on microscope slides, sealed with a cover slip and finally observed with a transmitted light microscope. Freshly cut surfaces were also investigated with a ZEISS field emission scanning electron microscope (FESEM) with excitation energy and a working distance of 15 kV and 7–9 mm, respectively. The samples were surface metalized by coating with chrome for FESEM examination.

Elemental and Mineralogical Composition

The mineral composition of the sediments was obtained with a Philips PW 1710 X-ray diffractometer (XRD) by using $\text{CuK}\alpha$ radiation, and a 40 kV and 30 mA power supply. XRD patterns were collected from 3° to 65° (2θ). The elemental compositions were determined by X-ray spectroscopy (EDX) analyses with an Oxford INCA Energy 350 system attached to the FESEM.

Fig. 2 (a) Panoramic view of the Salar Grande. White spots reveal that fossil salt deposits (b) are present beneath a thin dusty layer



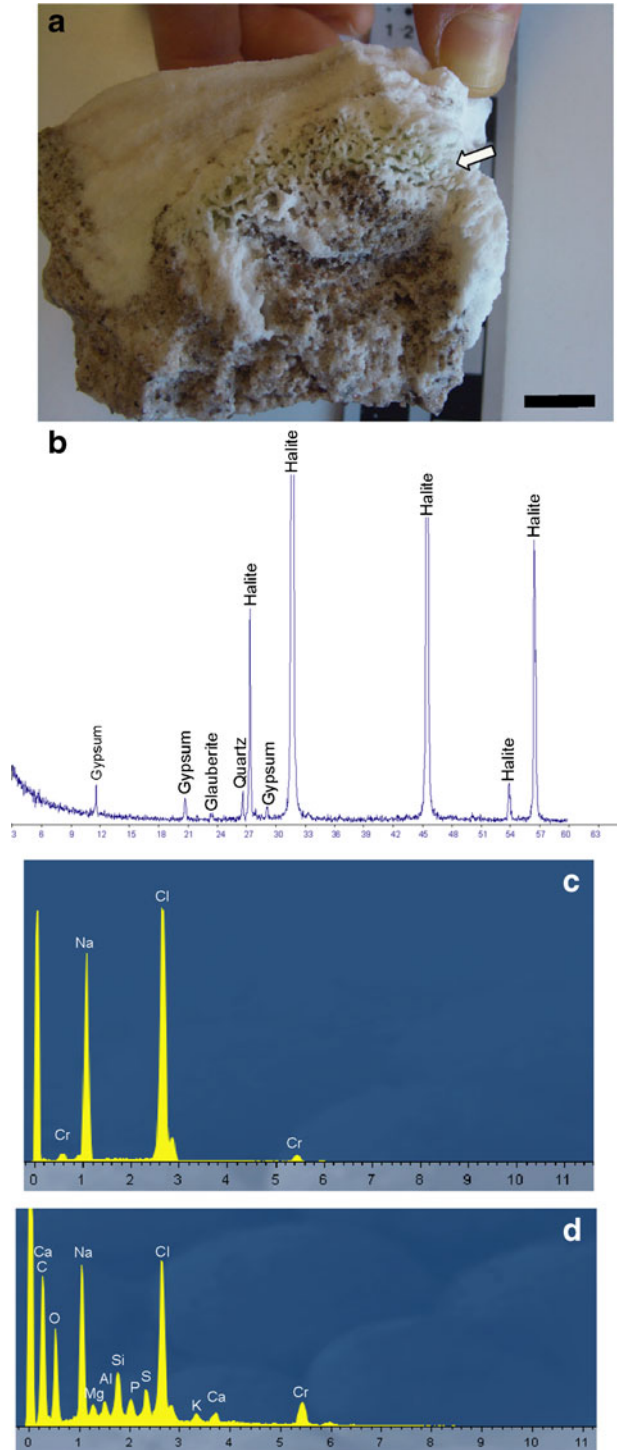
Confocal Laser-Scanning Microscopy

Fragments of halite rock with phototrophic growth were stained with the cell-impermeant nucleic acid dye SYTOX-Green (Molecular Probes, S-7020) at a final concentration of 50 μM for 5 min in the dark, at RT. This DNA dye selectively penetrates cells with damaged plasma membranes (SYTOX-Green positive), whereas intact cells are impermeable to the staining (SYTOX-Green negative); after washing, the cell-permeant nucleic acid stain DAPI (Molecular Probes, D1306) was added at a final concentration of 5 $\mu\text{g}/\text{ml}$ for 10 min, in the dark at RT. Live and dead cyanobacterial cells were identified by combining SYTOX-Green staining and the photosynthetic pigment autofluorescence as previously reported (Billi 2009). After staining, the specimens were transferred to a microscope slide and examined using an Olympus FV1000 confocal laser-scanning microscope (CLSM). SYTOX-Green and DAPI were excited using a 488 nm and 405 laser respectively, while a 543 laser was used to reveal the photosynthetic pigment autofluorescence.

DNA Extraction from Environmental Samples

Total genomic DNA was extracted from fragments of halite rock with phototrophic growth by using a method previously developed to achieve the lysis of cyanobacteria surrounded by

Fig. 3 (a) Hand sample of halite from the Salar Grande. The pale green colour indicates the presence of phototrophic microorganisms. Pore cavities (arrow) are due to the phenomenon of deliquescence. Scale bar: 1 cm (b) Example of XRD spectrum showing the typical composition of the halite deposits. (c, d) Representative EDX spectra of the halite deposits and their microbial colonizers



thick envelopes (Billi et al. 1998) and modified as follows. After washing with sterile water, pellets were resuspended in 300 μ l of phenol saturated with 0.1 M Tris hydrochloride (pH 7.4) containing glass beads (20 % vol/vol) and subjected to four 2-min cycles of heating at 65 °C and vortexing for 30s. Cell debris and glass beads were eliminated by centrifugation and the aqueous phase was extracted once with tris-phenol/chloroform/isoamyl alcohol (25:24:1). 1/5 volume of TE buffer (1 mM EDTA [pH 8.0], 10 mM Tris hydrochloride [pH 7.4]) was then added to the mixture and a second phenol extraction was performed. The pooled aqueous phases were extracted with chloroform/isoamyl alcohol (24:1) and nucleic acids precipitated with cold ethanol and sodium acetate (pH 4.5, 0.3 M final concentration), by overnight incubation at 20 °C.

Amplification of 16S Ribosomal RNA Genes and Clone Library Construction

The entire genomic DNA obtained from the environmental sample was used as a PCR template to amplify prokaryotic 16S rRNA genes by using a pair of universal primers corresponding to *Escherichia coli* 16S rRNA gene positions 522–536 (5'-CAGCCGCGGTAATAC-3') and 1405 to 1391 (5'-ACGGGCGGTGTGTAC-3') (Lane et al., 1985). A 50- μ l volume PCR reaction was incubated for 35 cycles of 94 °C for 1 min, 55 °C for 1 min, and 72 for 1 min followed by an extension at 72 °C for 10 min with a GeneAmp PCR system 2700 (Applied Biosystems). PCR product of the expected size were purified from agarose gel by using the SV Gel and PCR DNA Extraction System (Promega), cloned into pGEM-T Easy Vector (Promega), and electroporated into ElectroMAX DH5 α -E cells (Invitrogen). Positive recombinants were screened on X-Gal (5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside)—IPTG (isopropyl-b-D-thiogalactopyranoside)—ampicillin indicator plates. Positive clones were identified by PCR amplification with pGEM-T Easy vector primer pairs pUC/M13F and pUC/M13R. Positive clones were used for the library construction that was used for restriction fragment length polymorphism analysis (see below).

PCR-Restriction Fragment Length Polymorphism (RFLP) Analysis and Sequencing

Each PCR product obtained from the 16S rDNA clone library after amplification with pUC/M13F and pUC/M13R was purified by using QIAquick PCR Purification Kit (Qiagen), digested with the restriction enzyme *AluI* (BioLabs) and analyzed in a 1.5 % ethidium bromide-stained agarose gel. Clones with different banding patterns were chosen for 16S rRNA gene sequencing (BMR-Genomics, Padova, Italy). The obtained sequences of about 900 bp 16S rDNA were aligned using the CLUSTALX program and then compared to sequences retrieved from database by using the Basic Local Alignment Search Tool (BLAST) algorithm (Altschul et al. 1990). The sequences determined in this study were submitted to the GenBank with the accession numbers JN106369–JN106378.

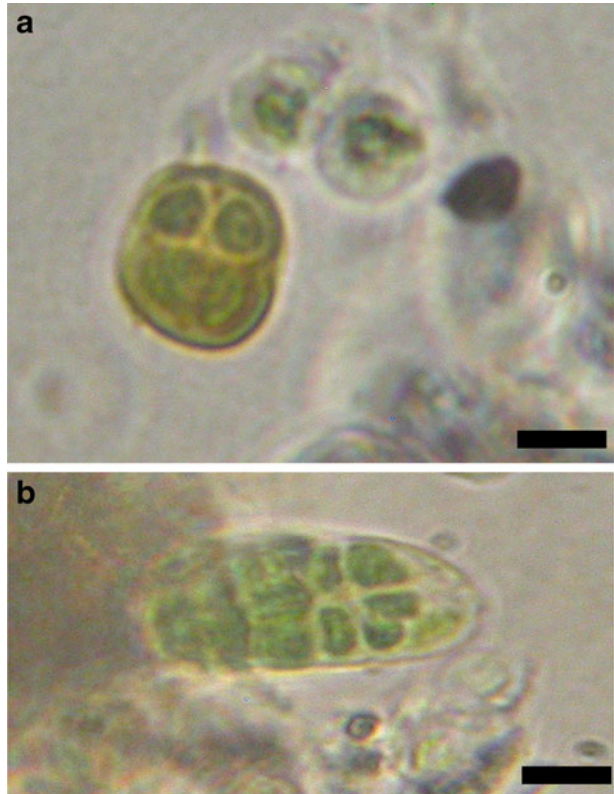
Results and Discussion

Characteristics of the Fossil Deposits of Halite

The rock nature in desert areas is of particular importance for lithobiontic microorganisms because rocks are the physical environments on which they can establish and, as a consequence, the microbial-mineral interactions largely determine their success. X-ray diffraction (XRD) and X-ray Spectroscopy (EDX) (Fig. 3b–d) have revealed the almost pure halite

(NaCl) composition of the Salar Grande salt, with accessory amounts of gypsum ($\text{CaSO}_4 \cdot 2\text{H}_2\text{O}$), glauberite ($\text{Na}_2\text{Ca}(\text{SO}_4)_2$), and quartz (SiO_2). In addition, EDX analyses (Fig. 3d) have shown peaks of Al that are related to terrigenous material, and Mg and K, that are associated with small amounts of potassium magnesium sulfates in rock salts. The halite deposits represent decanted brines from the Salar de Llamará (Fig. 1), whereas the small amounts of sulfates are interpreted as new cycles of salt precipitation (Mortimer 1973). Sulfates and quartz occur together in the brown 1–2 cm thick surficial horizons throughout the halite deposits (Fig. 3a). The hygroscopic nature of halite, which enables the retention of water when the relative humidity of the air is greater than 70–75 % (Davila et al. 2008), is an important factor to support the microbial colonization of the Salar Grande. In addition, cavities on crystal surfaces created by the condensation of vapor water into an aqueous solution (Fig. 3a) are suitable microniches for the development of microbial life. Endolithic environments may generally keep a greater amount of water than the surface: the pore water from soil, humidity, and the water vapor, that condenses at night even in the hottest deserts, represent water reservoirs that are then absorbed by some specific rocks, especially when they consist of hygroscopic minerals (Davila et al., 2008). In addition, the millimetric thickness of rocks colonized by endoliths is sufficient to provide protection from high UV radiation and, at the same time, the translucence of the halite mineral enables the process of photosynthesis by microorganisms. The translucence is a typically characteristic of the rocks colonized by endoliths in the Dry Valley of Antarctica (i.e. Friedmann 1982). The microbial colonization of the surface and near-

Fig. 4 (a, b) Transmitted light micrographs of cyanobacterial cells colonizing the halite deposits of the Salar Grande, that are morphologically consistent with the genus *Chroococcidiopsis*. Scale bars: 10 μm



surface rock in the Atacama Desert is, therefore, selective and seems to be largely dependent on the mineral composition.

The nature of the green horizons was investigated by optical and field emission scanning electron microscopy (FESEM). Green horizons revealed the presence of cyanobacterial cells arranged in clusters with a thick sheath (Figs. 4, 5) that have been assigned, after a preliminary morphology-based analytical approach, to the *Chroococcidiopsis* genus (see the next section). Colonies occur between single halite crystals (Fig. 5a–d), as well as in microcavities created by the dissolution of halite crystals where they adhere to the cavity

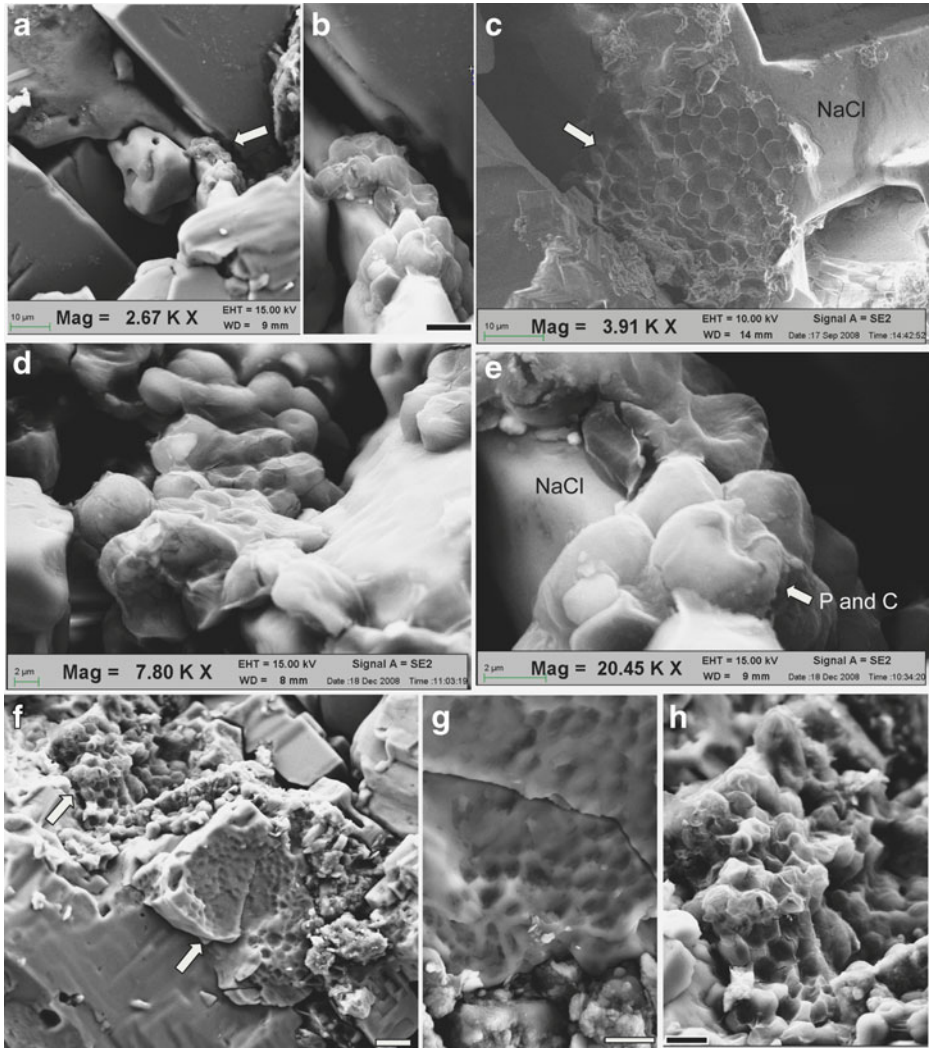


Fig. 5 FESEM micrographs from the pale green horizons of the halite in the Salar Grande. (a, b) Collapsed cells among cubic halite crystals embedded in extracellular substances. Scale bar in B: 5 μ m. (c) Cells in the cavities of dissolved halite crystals. (d, e) Close-up of cyanobacterial cells belonging to *Chroococcales*. The cellular collapse is presumably due to the vacuum conditions during the FESEM observations. (f–h) Pattern of circular pits impressed on halite crystals (arrows) likely after cells degradation. A combination of pits and remnant cells occur together in the same area (h). Scale bars: 10 μ m

surfaces (Fig. 5c). Cells exhibit a compressed/partially collapsed morphology likely related to the vacuum applied during the FESEM analysis (Fig. 5e). EDX analyses have shown carbon (C) and phosphorus (P) peaks as elements associated with the cells (Figs. 3d, 5e). The FESEM observations also revealed a pits framework (average diameter of each single pit: 4–5 μm) locally impressed on the surface of certain halite crystals (Fig. 5f–h). Because a combination of pits and cells occur together in the same areas (Fig. 5h), pits are interpreted as a product of cell degradation. This pattern impressed and preserved on halite crystals might represent useful evidence of microbial life in fossil deposits.

The presence of living and dead cells among cyanobacteria with a *Chroococciopsis*-like morphology was revealed by SYTOX-Green staining and observation with a CLSM. Figure 6 shows a CLSM image of dead cyanobacterial cells exhibiting SYTOX-Green stained nucleoids and red autofluorescence of the photosynthetic pigments (arrow). Whereas living cyanobacteria resulted SYTOX-Green negative with photosynthetic pigment autofluorescence (asterisk) and DAPI-stained nucleoids; a few dead and live non-phototrophic bacteria were visualized as SYTOX-Green positive or DAPI-stained cells, respectively (arrowheads).

Microbial Diversity

When the total genomic DNA obtained from the halite fragments was amplified by using universal 16S rRNA gene-targeted primers, a PCR amplicon of about 900 bp was yielded, which was used to obtain a 16S rDNA clone library. The RFLP analysis of 50 randomly selected clones with *A**lu*I enabled the identification of different banding patterns and the following clone selection for the 16S rDNA gene sequencing. The BLAST search identified representatives of Cyanobacteria, Actinobacteria, Beta- Gamma Proteobacteria, and Firmicutes (Table 1).

Among the sequenced clones, a cyanobacterial 16S rDNA gene was identified closely related (99 % similarity) to: i) *Chroococciopsis thermalis* (FJ805841), isolated from soil

Fig. 6 CLSM image showing: i) dead cyanobacteria with SYTOX-Green stained nucleoids and pigment autofluorescence (arrow); ii) live cyanobacteria lacking the SYTOX-Green staining with pigment autofluorescence and DAPI-stained nucleoids (asterisk); iii) dead and live non-phototrophic bacteria occurring as SYTOX-Green positive or DAPI-stained cells, respectively (arrowheads). Bar=5 μm

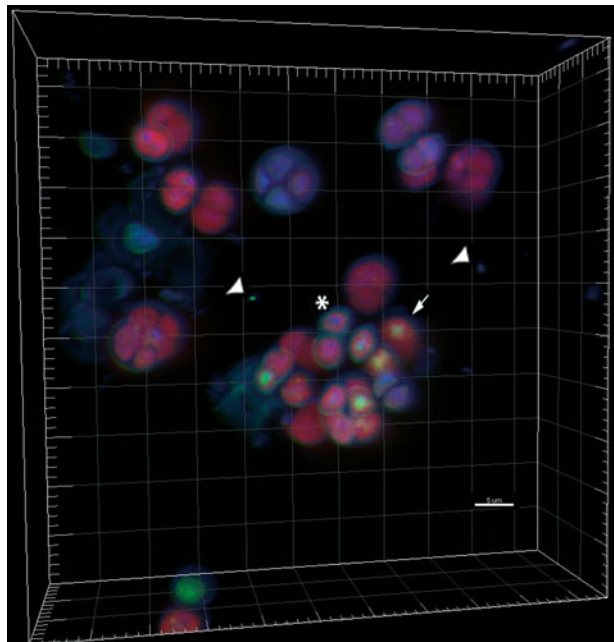


Table 1 Phylogenetic characterization of representative bacterial 16S rDNA clones from the Salar Grande as inferred after BLAST searches

Clone/accession number	Phylogenetic ascription	Closest sequence relative/ Accession number (GenBank)	%identity (BLAST)
ASG1 (JN106369)	Bacteria; Proteobacteria; Betaproteobacteria; Burkholderiales;	<i>Achromobacter</i> sp. UYSO2/JF262578	98–99 %
ASG5 (JN106370)	Alcaligenaceae; <i>Achromobacter</i>		
ASG8 (JN106371)			
ASG9 (JN106372)			
ASG10 (JN106373)			
ASG11 (JN106374)	Bacteria; Firmicutes; Lactobacillales; Streptococaceae; <i>Streptococcus</i>	<i>Streptococcus sanguinis</i> /AB596946.	99 %
ASG14 (JN106375)	Bacteria; Actinobacteria; Actinobacteridae; Actinomycetales; Propionibacterineae; Propionibacteriaceae; <i>Propionibacterium</i>	<i>Propionibacterium acnes</i> / EF558730	99 %
ASG16 (JN106376)	Bacteria; Proteobacteria; Gammaproteobacteria; Pseudomonadales; Moraxellaceae; <i>Acinetobacter</i>	<i>Acinetobacter</i> sp. R7-5A/HQ154563	99 %
ASG17 (JN106377)	Bacteria; Proteobacteria; Gammaproteobacteria; Pseudomonadales; Moraxellaceae; <i>Acinetobacter</i>	<i>Acinetobacter</i> sp. CmN A3/HM352317	99 %
ASG3 (JN106378)	Bacteria; Cyanobacteria; Pleurocapsales; <i>Chroococcidiopsis</i>	<i>Chroococcidiopsis</i> sp. CCMEE 584/AY301004	99 %

samples in Germany; ii) *Chroococcidiopsis* sp. SAG 2023 (AJ344552), a photobiont of the lichen *Thyrea pulvinata* in Austria (Fewer et al. 2002); and iii) *Chroococcidiopsis* sp. CCMEE 584, isolated from hypolithic growth in the Gobi Desert (AY301004, Billi, unpublished data). The environmental *Chroococcidiopsis*-like sequence here identified shared 91 % similarity with the Chilean strain *Chroococcidiopsis* sp. CCMEE 123 (AF279109) isolated from endolithic growth in a granite boulder from the coastal desert of the Guanaqueros Peninsula, Coquimbo, Chile (Billi et al. 2001). The identified *Chroococcidiopsis*-like sequence from the halite of the Salar Grande also differed from *Chroococcidiopsis* spp. strains isolated from hot and cold deserts currently under sequencing (Billi, unpublished).

In the halite fragments here investigated four bacterial lineages, Betaproteobacteria, Gammaproteobacteria, Actinobacteria, and Firmicutes were identified. The sequences were related (98–99 % similarity) to *Achromobacter* sp., *Propioniumbacterium* sp., *Acinetobacter* sp., and *Streptococcus* sp. (Table 1).

Numerous sequences of genera belonging to the Firmicutes, Actinobacteria and Proteobacteria phyla were identified in desiccated soils of Chile (Connon et al. 2007). These bacterial phyla are commonly found in soils around the world. Species of *Alcaligenes* and *Moraxella*, belonging to the Beta-Gammaproteobacteria phyla, were identified as arsenic resistant bacteria for the first time in 2009 from the sediments of arsenic contaminated rivers in the Atacama Desert (Campos et al. 2011; Escalante et al. 2009). Moreover, *Acinetobacter* sp. and *Achromobacter* sp. were identified from arsenic contaminated soils in China (Cai et al., 2009). The Betaproteobacteria clones (ASG1,5,8,9,10) reported in the present study were closely related to a new arsenite-oxidizing bacterium BEN (AY027504) isolated from Australian gold mining environment. Further investigations are required to understand the physiological role of these heterotrophic bacteria associated with *Chroococcidiopsis* sp.

Concluding Remarks

The present study provides a window into the diversity of microbial communities inhabiting halite deposits located in the driest sector of the Atacama region. These communities are adapted to the complex of extreme environmental settings centered on hyperaridity. The colonization of the halite deposits by *Chroococcidiopsis* shows that microbial life is largely possible in spite of the extreme hyperarid conditions. This colonization is thought possible by the physical properties of the halite, such as hygroscopy and a relatively transparent nature, together with the physiological adaptation of this cyanobacterium. The colonization of rock near-surfaces and hypersaline microhabitats by *Chroococcidiopsis* and associated bacteria deserves interest because it might represent a way for the microbial colonization of the salt lake environments that have been described from different Mars areas within the last decade.

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