RESEARCH ARTICLE

Unique clusters of *Archaea* in Salar de Huasco, an athalassohaline evaporitic basin of the Chilean Altiplano

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Abstract

Analyses of clone libraries from water and sediments of different sites from Salar de Huasco, a high-altitude athalassohaline wetland in the Chilean Altiplano, revealed the presence of five unique clusters of uncultured *Archaea* that have not been previously reported or specifically assigned. These sequences were distantly related (83–96% sequence identity) to a limited number of other clone sequences and revealed no identity to cultured *Archaea*. The abundance of *Archaea* and *Bacteria* was estimated using qPCR and community composition was examined through the construction of clone libraries of archaeal 16S rRNA gene. *Archaea* were found to be dominant over *Bacteria* in sediments from two saline sites (sites H4: 6.31×10^4 and site H6: $1.37 \times 10^4\ \mu S\ cm^{-1}$) and in one of the water samples (freshwater from site H0: $607\ \mu S\ cm^{-1}$). Euryarchaeotal sequences were more abundant than crenarchaeotal sequences. Many of the clone sequences (52%) were similar to uncultured archaeal groups found in marine ecosystems having identity values between 99% and 97%. A major fraction of the sequences (40%) were members of *Methanobacteria*, while others were included in the Marine Benthic Groups B and D, the Miscellaneous Crenarchaeotic Group, the Terrestrial Miscellaneous Euryarchaeotal Group, Marine Group I and *Halobacteria*. The presence of uncultured archaeal groups in Salar de Huasco extends their known distribution in inland waters, providing new clues about their possible function in the environment.

Introduction

Archaea are widely distributed in both extreme (e.g. hot springs, hydrothermal vents, solfataras, salt lakes, soda lakes, sewage digesters, rumen) and nonextreme (e.g. ocean waters, lakes, soil) environments (Chaban *et al.*, 2006). The domain *Archaea* consists of two major phyla: *Crenarchaeota* and *Euryarchaeota*. With the advent of molecular techniques, an immense number of 16S rRNA gene sequences of 'uncultured *Archaea*' have been retrieved in clone libraries from different environments (Schleper *et al.*, 2005). Some of these uncultured archaeal groups are known from marine environments (DeLong, 1998; Vetriani *et al.*, 1999; Takai *et al.*, 2001; Schleper *et al.*, 2005; Teske & Sørensen, 2008) and represent quantitatively important members of the pelagic deep-ocean picoplankton (Karner *et al.*, 2001). Among the *Euryarchaeota*, Marine Group II (marine plankton, anaerobic digestor), Marine Group III (marine sediments, marine plankton) (DeLong, 1998), Marine Benthic Group D (MBGD, deep sea and salt marsh sediments) (Vetriani *et al.*, 1999) and the South Africa gold mine euryarchaeotic group [SAGME-1, SAGME-2, sequences also included in the Terrestrial Miscellaneous Euryarchaeotal Group (TMEG); Takai *et al.*, 2001] have been reported frequently from terrestrial and marine environments (e.g. Inagaki *et al.*, 2003; Shao *et al.*, 2004; Sørensen *et al.*, 2005; Sørensen & Teske, 2006; Kendall *et al.*, 2007). Among the *Crenarchaeota*, the Marine Group I (MG-I), Marine Benthic Group A, B and Marine Benthic Group C (MBGC, deep sea sediments) were first reported from seawater (DeLong,

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1992; Fuhrman *et al.*, 1992; Vetriani *et al.*, 1999), and more recently also from subsurface marine sediments (Sørensen *et al.*, 2004; Teske, 2006). The Miscellaneous Crenarchaeotic Group (MCG) have a wider habitat range, which includes terrestrial and marine, hot and cold, surface and subsurface environments (Teske, 2006). Functional gene surveys and pure culture studies have indicated that at least some members of MG-I are aerobic and autotrophic ammonia oxidizers (Francis *et al.*, 2005; Könneke *et al.*, 2005). Studies using reverse transcription have demonstrated that several of these uncultured marine archaeal groups are metabolically active in deep subsurface sediments (Biddle *et al.*, 2006). Intensive studies of uncultured *Archaea* have been conducted especially in marine environments, including metagenomic analyses (e.g. Martin-Cuadrado *et al.*, 2008). However, little is known about their function in the environment. Various authors have described the occurrence and abundance of various archaeal groups in inland waters (e.g. Sørensen *et al.*, 2005; Galand *et al.*, 2006; Briée *et al.*, 2007; Auguet & Casamayor, 2008; Jiang *et al.*, 2008).

Salar de Huasco is located in the Chilean Altiplano at an altitude of 3800 m and exhibits salinity conditions from freshwater to saturated salt waters (Dorador *et al.*, 2008a); it is considered an athalassohaline system because its salt composition is markedly different from that of seawater (e.g. Oren, 2006). Abiotic conditions in the Altiplano, including low temperatures (mean annual temperature $< 5\,^{\circ}C$), low atmospheric pressure (40% lower than that at sea level), high solar radiation ($< 1100\,W\,m^{-2}$), strong variation in different environmental properties at daily, annual and interannual time scales and a negative water balance, shape the biological communities in these water bodies (Vila & Mühlhauser, 1987). Considering the environmental conditions of this inland aquatic system, we expected to encounter a highly adapted archaeal community, including taxa adapted to high salt concentrations. In the present study, we describe the composition and abundance of archaeal assemblages in water and sediment samples at, in terms of salt concentration, four contrasting sites from Salar de Huasco using denaturing gradient gel electrophoresis (DGGE), clone libraries of the 16S rRNA gene and quantitative PCR (qPCR). In addition, the presence of ammonia-oxidizing *Crenarchaeota* was examined by cloning the ammonia monooxygenase gene *amoA*, reflecting the possible role of *Archaea* in the biogeochemical nitrogen cycle.

Materials and methods

Site description and sampling

During January 2005 (summer), two samples were collected per site at four different sites from the Salar de Huasco

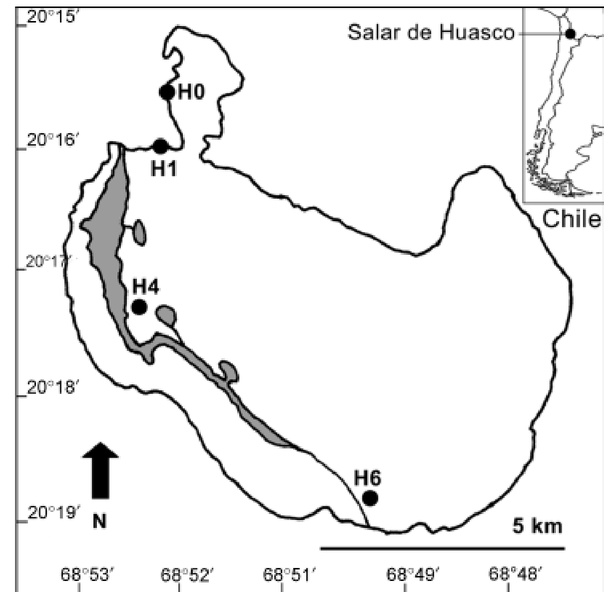

Fig. 1. Map indicating the location of Salar de Huasco and four study sites (H0, H1, H4 and H6). Gray areas indicate the presence of permanent lagoons.

(20°18′S, 68°50′W) (Fig. 1). Water samples were collected in plastic sterile bottles of 1 L from the surface and sediment samples were taken with a polycarbonate hand corer (30 cm length and 3 cm diameter). The surface area of the Salar extends to *c.* 50 km^2, with open water representing only 2.5 km^2, and the water level can vary seasonally (Risacher *et al.*, 2003). The ponds are shallow and located along a N–S aspect through the Salar, forming a natural salinity gradient with freshwater sites H0 (stream) and H1 (shallow lagoon) and the saline site H4 (shallow lagoon). Site H6 (shallow lagoon) exhibited lower salinity than site H4 because the water comes from a different freshwater stream (Risacher *et al.*, 1999). A summary of some key morphometric, physical and chemical characteristics of the sampling sites is presented in Table 1. A more detailed description of their properties was described in Dorador *et al.* (2008a).

DNA extraction and PCR amplifications

Environmental DNA was extracted from water and sediment samples from each site. For DNA extraction, water samples were filtered on-site in a 0.22-μL pore-size filter without prefiltration, with the volume of filtered water ranging between 0.05 L for saline sites (sites H4 and H6) and 1 L for the freshwater site (site H0). For DNA extractions of sediment samples, we used 600 mg of homogenized sediment. All DNA extractions were carried out using the UltraClean Soil DNA isolation kit (MoBio Laboratories Inc.) according to the manufacturer's instructions.

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Table 1. Physical and chemical characteristics of water samples and microbial abundance at the four sites in Salar de Huasco

	Sites			
Characteristics	H0	H1	H4	H6
Location	20°15′32″, 68°52′25″	20°16′08″, 68°52′29″	20°17′41″, 68°53′00″	20°19′43″, 68°50′19″
Altitude (m)	3799	3795	3789	3789
Type	Stream	Lagoon	Lagoon	Lagoon
Conductivity (μS cm^{-1})	607	645	6.31×10^4	1.37×10^4
Total dissolved salts (g L^{-1})	0.42	0.46	64.93	9.38
Dissolved oxygen (mg L^{-1})	6.9	10.3	0	8.4
pH	7.7	8.7	8.2	8.8
Temperature (°C)	16.6	19	20.1	12
Hour	14:30	16:00	15:50	10:15
N-NO_3^- (μg L^{-1})	55.5	53.5	60	30
P-PO_4^{3-} (μg L^{-1})	40.3	20.3	3.91×10^3	807
S-SO_4^{2-} (mg L^{-1})	22.1	26.6	3990	141.2
Si (mg L^{-1})	19.9	12.4	25.4	18.1
Hardness (mg L^{-1})	162.5	130	3500	1000
Total alkalinity (mM)	2	1.52	12	7
Chl*a* (μg L^{-1})	2.2	8.5	39	114.3
Cations	$Ca^{2+}>Na^+>K^+>Mg^{2+}$	$Ca^{2+}>Na^+>K^+>Mg^{2+}$	$Mg^{2+}>Ca^{2+}>K^+>Na^+$	$Ca^{2+}>Mg^{2+}>Na^+>K^+$
Archaeal abundance water (cells mL^{-1})	69	174	1.68×10^4	7.8×10^4
Archaeal abundance sediment (cells g^{-1})	3.24×10^6	5.04×10^7	3.6×10^7	5.94×10^6
Bacterial abundance water (cells mL^{-1})	45	288	5.34×10^5	2.7×10^6
Bacterial abundance sediment (cells g^{-1})	2.16×10^8	5.94×10^8	1.08×10^7	2.52×10^6

Amplification of 16S rRNA archaeal gene was performed using a nested PCR approach. Fragments of 1500 bp were obtained with primers Ar4F (positions 8–25) and Un1492R, and then primers Ar3F (positions 7–26)–Ar9R (positions 906–927) (Jurgens *et al*., 2000) were used to amplify the 16S rRNA gene from *Archaea* with the first-round PCR products as templates in a nested PCR. The reverse primer used, Ar9R (Jurgens *et al*., 2000), exhibited mismatches with the uncultured groups SAGMEG, Marine Hydrothermal Vent Group (MHVG, Takai & Horikoshi, 1999), Ancient Archaeal Group (AAG, Takai & Horikoshi, 1999) and Deep-Sea Archaeal Group (DSAG, Inagaki *et al*., 2003) (Teske & Sørensen, 2008), which can possibly underestimate the presence of these groups in environmental samples. Each PCR reaction contained 10 × PCR buffer with 2 mM $MgCl_2$ (Roche), 200 μM dNTP mixture (Gibco), 1 pmol of each primer, 2.5 U *Taq* polymerase (Roche), 10–100 ng template DNA and water to a final volume of 50 μL. The PCR conditions used were initial denaturation at 94 °C for 5 min, 35 cycles of denaturation (30 s at 94 °C), annealing (45 s at 55 °C) and extension (1.5 min at 73 °C). Archaeal *amoA* gene fragments were amplified by PCR using primers CrenAmo1F and CrenAmo1R (Könneke *et al*., 2005) and the following PCR conditions: initial denaturation at 94 °C for 3 min; 30 cycles of denaturation (30 s at 94 °C), annealing (30 s at 56 °C) and extension (3 min at 72 °C); and a final extension at 72 °C for 1 min. For cloning, PCR reactions were carried out with Pfu polymerase (Promega) following the manufacturer's instructions.

DGGE analysis

DGGE was performed according to Casamayor *et al*. (2000) with PCR products of archaeal 16S rRNA gene generated in a nested approach. Primers 344F (Raskin *et al*., 1994) and 915R (Stahl & Amann, 1991) were used to amplify the archaeal 16S rRNA gene PCR product. The 344F primer contained an additional 40 nucleotide GC-rich sequence (GC clamp) at its 5′-end in order to maintain stable melting behavior during DGGE (Muyzer *et al*. 1993). The PCR conditions were as described above and PCR amplification was carried out using a touchdown protocol as follows: an initial denaturing step of 5 min at 94 °C, 20 cycles of 30 s at 94 °C, 45 s at 65–55 °C (decreased by 0.5 °C every cycle) and 1.5 min at 72 °C, and then 10 cycles of 30 s at 94 °C, 45 s at 55 °C and 1.5 min at 72 °C. PCR products were applied onto 7.5% polyacrylamide gels containing a linear gradient of 30–60% denaturant where 100% denaturant was defined as 7 M urea and 40% formamide. DGGE was carried out in the BioRad D Gene System (Bio-Rad Laboratories, Hercules, CA) at 60 °C, 200 V, for 6 h. Gels were stained with the SYBR Gold nucleic acid gel stain (Molecular Probes). In order to examine the relationships between communities in the different samples, a matrix was constructed from the

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distribution pattern of the bands in different samples, and cluster analyses (UPGMA), based on percent similarity between the samples, were conducted using the multivariate statistical package (MSVP, version 3.12d; Kovach Computing Services, Wales, UK).

Cloning and 16S rRNA gene sequence analysis

Archaeal clone libraries were generated from water and sediment samples collected from the four study sites. Samples for cloning (one sample per site) were selected according to their richness detected by DGGE (data not shown). Purified amplicons were cloned into pCR-Blunt vector (Invitrogen) according to the manufacturer's instructions. Analysis of the inserts, sequencing and sequence analysis were described previously (Dorador *et al*., 2008b). Briefly, sequences were checked for chimeras, rarefaction curves were constructed and nonparametric richness estimators S_{ACE} and S_{Chao1} and the Shannon–Weaver diversity index were determined manually and via the web interface available at http://www.aslo.org/lomethods/free/2004/0114a.html (Kemp & Aller, 2004).

Phylogenetic analysis

The phylogenetic affiliations of the archaeal sequences obtained here were estimated as described elsewhere (Dorador *et al*., 2008b). Sequences were aligned using the alignment tool of the ARB package (http://www.arb-home.de), a maximum likelihood analysis in the program PHYML (Guindon *et al*., 2005) with the GTR substitution model and 100 bootstrap resamplings. Trees were edited using MEGA4 (Tamura *et al*., 2007). Sequences with similarities $> 97\%$ were considered to represent the same phylotype (Stackebrandt & Goebel, 1994).

Nucleotide sequence accession numbers

The nucleotide sequences from this study are available in GenBank (http://www.ncbi.nlm.nih.gov) under accession numbers EU481526–EU481630 for 16S rRNA gene sequences and FJ839431–FJ839434 for archaeal *amoA* sequences.

qPCR

qPCR was conducted using SYBR-Green PCR Master Mix (Biotools) in an MJ Mini-Opticon thermocycler (Bio-Rad). Primers for *Archaea* (ARCH349F and ARCH806R) and *Bacteria* (UBactF and UBactR) (Takai & Horikoshi, 2000; Nadkarni *et al*., 2002) were used for the quantification of total archaeal and bacterial 16S rRNA genes, respectively. The reaction mixture contained 10 μL of SYBR-Green PCR Master Mix (Biotools), 1 μL of template DNA (∼10 ng), 1 μL of the corresponding oligonucleotide primers (final concentration of 0.25 μM) and nuclease-free H_2O added to a total of 20 μL. The *Archaea* amplification program consisted of an initial denaturing at 95 °C for 10 min, and then 40 cycles of 95 °C for 35 s, 56 °C for 40 s and 72 °C for 20 s. The *Bacteria* amplification program consisted of an initial denaturing at 95 °C for 10 min, and then 40 cycles of 95 °C for 30 s, 60 °C for 30 s and 72 °C for 20 s. For both amplification programs, the fluorescence measurements were recorded at the end of each extension step and the melting curves were measured with a ramp increasing the temperature from 50 to 95 °C by 1 °C every 5 s. All analyses were performed in triplicate including a negative control.

Standard curves and data analysis

To generate bacterial and archaeal standard curves, we extracted plasmid DNA from 16S rRNA gene clones containing *Escherichia coli* and clone Hua1-w87, respectively. The plasmid DNA was quantified using gel electrophoresis with a DNA low-mass ladder (Promega). Standard curves for bacterial and archaeal 16S rRNA genes were constructed using serial dilutions of these plasmids' DNA, ranging from 850 to 0.0085 pg, as a template. The DNA concentrations in ng μL^{-1} were transformed to 16S rRNA gene copy number μL^{-1}, by means of the following transformation: first, we obtained the molecular weight of DNA (plasmid + insert), assuming that 660 is the average molecular weight of a base pair. We divided Avogadro's number (6.02×10^{23}) by this molecular weight to estimate the abundance of molecules per 1 g. Finally, this value was divided by 1×10^9 to obtain the gene copy number per ng of DNA. The calibration curves were generated using the OPTICON MONITOR™ ver. 3.1.32 software (Bio-Rad Laboratories), and for each standard, the concentration was plotted against the cycle number, and the value at which the fluorescence signal increased above the threshold value was the cycle threshold (C_t value). The bacterial and archaeal standard curve showed an efficiency of 1.23 (R^2 value of 0.995) and 0.91 (R^2 value of 0.999), respectively.

Results

Microbial abundance in Salar de Huasco and quantification of 16S rRNA genes

The total numbers of microbial cells in water and sediment samples of sites H0 and H1 were calculated by qPCR results assuming one copy of 16S rRNA gene per cell and were 10^1 and 10^2 cells mL^{-1} and 10^6–10^8 cells g^{-1}, respectively. For sites H4 and H6, these values fluctuated between 10^4 and 10^6 cells mL^{-1} for water and 10^6 and 10^7 cells g^{-1} for sediment (Table 1). The estimated abundance of *Bacteria* and *Archaea* in waters and sediments from the four sites of Salar de Huasco is shown in Fig. 2. There was a clear

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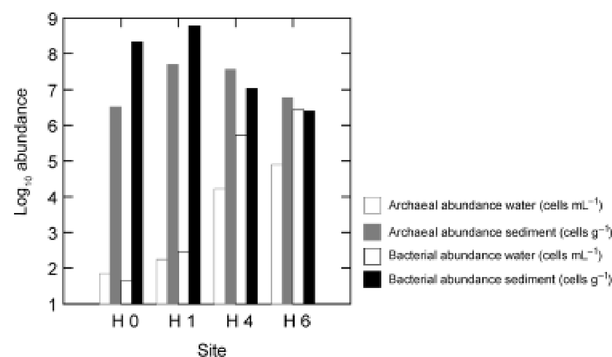

Fig. 2. Relative abundance of *Bacteria* and *Archaea* in Salar de Huasco at the sampling sites, determined by qPCR.

differentiation between water and sediment samples throughout the salinity gradient from site H0 (freshwater stream) to H4 (saline lagoon) (Fig. 2).

Construction of 16S rRNA gene clone libraries and estimation of archaeal richness

Clone libraries of 16S rRNA genes were constructed from sites H0, H1, H4 and H6. In total, 137 clones from water samples and 197 from sediments were obtained. Rarefaction curves from the four sites showed saturation at low phylotype numbers, from 4 to 11 in water samples and from 10 to 32 phylotypes in sediment samples (Supporting Information, Fig. S1). The richness estimators S_{ACE} and S_{Chao1} (Chao, 1984; Chao, 1987) provided estimates of the total number of phylotypes ranging from 5 to 44 (S_{ACE}) and 4 to 26 (S_{Chao1}) in water samples and from 23 to 107 and 18 to 91 in sediment samples (Table 2). The Shannon–Weaver diversity index indicated a higher archaeal diversity in sediments (1.4–3.0) compared with the water samples (0.7–1.7). The number of DGGE bands ranged between 4 and 16 in water samples and between 9 and 10 in sediment samples, which are lower than the richness estimated by clone libraries, and did not show differences between water and sediment samples. Nevertheless, the composition of the DGGE bands shows one cluster that largely consists of sediment samples but also includes the water sample from site H6 (Fig. S2). The pattern of bands from water samples collected from different sites within the Salar de Huasco showed no clear grouping, indicating that they were only distantly related. For example, water samples from H4 and H1 were $< 60\%$, and the water sample from H0 was $< 50\%$ similar to water samples from all the other sites.

Phylogenetic analysis of archaeal communities in water and sediment

Crenarchaeota were absent from the water samples (the exception was site H0 with clones exclusively belonging to MG-I) and represented a minor fraction in the sediments. *Euryarchaeota* dominated in the sediments and were the exclusive archaeal representatives in water, except site H0, although different groups dominated in the different water samples. All sediments had a clearly different composition as compared with the corresponding water samples (Fig. 3 and Fig. S4).

Crenarchaeota identified by clone sequences represented 14% of all clones and belonged to different phylogenetic groups. All clones from H0-w (four phylotypes) and a single phylotype from H0-s were affiliated to MG-I (Fig. S4) and clustered in Group I.1b and Group I.1a (DeLong, 1998; Schleper *et al.*, 2005), respectively. This cluster includes sequences from soils, sediments, freshwater and subsurface sediments. Sequences from H0-w were 97% similar to an uncultured crenarchaeon retrieved from a radioactive thermal spring in the Austrian Alps (Weidler *et al.*, 2007) and with the moderately thermophilic crenarchaeote Candidatus *Nitrososphaera gargensis* (Hatzenpichler *et al.*, 2008). Representatives of the MCG were found in sediments from sites H0, H1 and H6 (four phylotypes). Clone QLW1200-A33 from Qinghai Lake (Jiang *et al.*, 2008) exhibited 97% sequence identity with the phylotypes Hua6-s39 and Hua6-s29. Also, the MBGB was found only in sediment samples (H0-s, H4-s, H6-s). Databases contain sequences of MGBG retrieved from subseafloor sediments, lake sediments and hydrothermal vents (Fig. 4 and Fig. S4).

Most of the clone sequences from water and sediment samples were affiliated to various groups of *Euryarchaeota* (Figs 3 and 4): *Halobacteria*, *Methanosarcinales*, *Methanomicrobiales*, MBGD, TMEG and a group of unidentified *Euryarchaeota* (7% of the clones). Methanogenic *Archaea* were most frequently detected in Salar de Huasco (40% of the clones) (Fig. 3).

Halobacteria were represented by a single phylotype Hua6-w15, the closest cultured relative (89% sequence identity) of which was *Halorubrum lipolyticum* (Cui *et al.*, 2006) isolated from Aibi salt lake in Xin-Jiang, China.

Methanosarcinales represented 25% of all clones and were found in samples H1-w, H0-s and H1-s (Fig. 3 and Fig. S4). The most frequent clone sequence from H1 (phylotype Hua1-s2) was 97% similar to *Methanosaeta concilii* (Eggen *et al.*, 1989), while the most abundant clone from H0 (phylotype Hua0-s82) was 98% similar to *Methanomethylovorans hollandica*. This methanogen was isolated previously from freshwater sediment and utilizes dimethyl sulfide as a carbon and energy source (Lomans *et al.*, 1999). Clone Hua0-s95 was 94% similar to *Methanolobus oregonensis*, isolated from anoxic, subsurface sediments of a saline, alkaline aquifer near Alkali Lake in Oregon, USA (Liu *et al.*, 1990).

Methanomicrobiales were represented by 16% of the clones. Members of this group were found in sediment samples from the sites H0, H1 and H4, but a single clone

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Table 2. Number of clones and phylotypes, richness estimators S_{ACE} and S_{Chao1}, number of bands in DGGE and Shannon diversity (H′) in the libraries of 16S rRNA gene from water and sediment

Site	Clone library	Number of clones in library	Number of phylotypes observed	Predicted value of S_{ACE}	Predicted value of S_{Chao1}	Shannon diversity index (H′)	Number of bands in DGGE
H0	Water	27	4	5	4	0.7	16
H1	Water	56	6	8	7	1.1	4
H4	Water	33	11	44	26	1.6	10
H6	Water	21	8	17	10	1.7	7
H0	Sediment	64	23	34	30	2.8	10
H1	Sediment	35	13	23	18	2.1	10
H4	Sediment	45	10	36	32	1.4	9
H6	Sediment	53	32	107	91	3	9

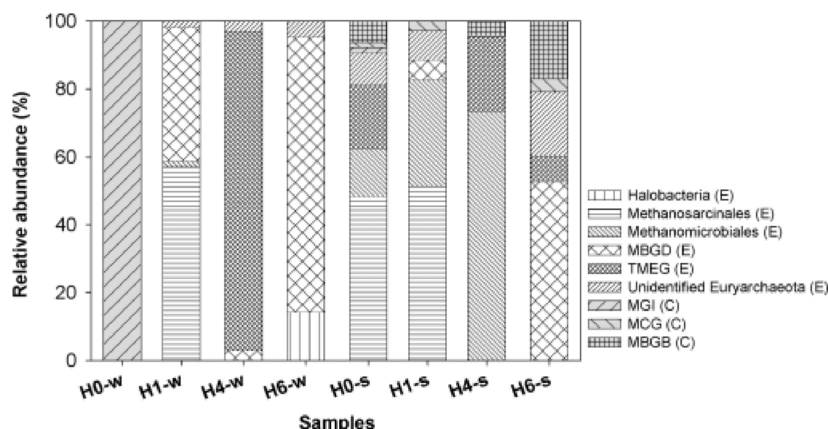

Fig. 3. Composition of clone libraries of 16S rRNA gene from water and sediment samples. Affiliation of the phylogenetic groups is indicated for Euryarchaeota (E) and Crenarchaeota (C). Group designations: MBGD, Marine Benthic Group D; MBGB, Marine Benthic Group B (Vetriani *et al.*, 1999); TMEG, Terrestrial Miscellaneous Euryarchaeotal Group (Takai *et al.*, 2001); MG-I, Marine Group I (DeLong, 1998); MCG, Miscellaneous Crenarchaeotic Group (Teske, 2006).

(Hua1-w46) was also retrieved from the water of site H1 (Fig. S4). Clone Hua4-s2 was the most abundant clone from the sediment sample of site H4 and exhibited 95% sequence identity to the clone MH1492_4F retrieved from a minerotrophic fen (Cadillo-Quiroz *et al.*, 2008). This clone also had 94% sequence identity to the Candidatus *Methanoregula boonei*, an acidiphilic methanogen isolated from an acidic peat bog (Bräuer *et al.*, 2006). Clone Hua1-s38 exhibited 96% sequence identity to *Methanolinea tarda* (Sakai *et al.*, 2007), a methanogen isolated from rice paddy fields that is a member of the Rice Cluster I.

The MBGD was represented by 21% of all clones and was found in sites H1, H4 and H6 (Fig. 3 and Fig. S4), with most clone sequences obtained from site H6 (81% of the clones from water and 53% from the sediment). Sequences of this cluster were reported previously from subsurface sediments, marine sediments, water and sediments of Qinghai Lake and various other habitats. The abundant clones Hua1-w90 and Hua6-w21 exhibited 91% and 97% sequence identity to the clone BCMS-5 described from prawn farm sediments in China (Shao *et al.*, 2004).

The TMEG was first established by clones from South African gold mines (SAGMA; Takai *et al.*, 2001), but also included sequences retrieved from subsurface and marine sediments. Sequences of TMEG comprised 17% of all clones. This group was clearly predominant in the water of site H4, but was absent from other water samples (Fig. 3). Clone Hua4-w4 had 89% sequence identity to the clone ArcA08 previously retrieved from a hypersaline endoevaporitic microbial mat in Israel and was reported as a member of the 'Halophilic Cluster 2' (Sørensen *et al.*, 2005). In the sediments, TMEG was absent from H1, but present in the other sites, representing 11 phylotypes, with Hua0-s78 being closely related (97% sequence identity) to the clone WCHD3-02, previously found in a hydrocarbon and chlorinated solvent-contaminated aquifer (Dojka *et al.*, 1998).

Unidentified Euryarchaeota that formed novel, and to date, unreported groups, which did not cluster with previously described groups, for example archaeal DGGE sequences previously reported from other wetlands of northern Chile (Demergasso *et al.*, 2004) and that represented 7% of all clones, were found in six out of eight samples (Fig. 3). They formed five different groups (Fig. 4 and Fig. S4). *Group Hua-1* consisted of two phylotypes from sediment samples of site H0 exhibiting a low identity (83–84%) to their closest relative, the clone SBAK-mid-46 retrieved from marine sediments of the Skan Bay in Alaska, classified as 'other *Euryarchaeota*' (Kendall *et al.*, 2007). *Group Hua-2* was represented by a single phylotype

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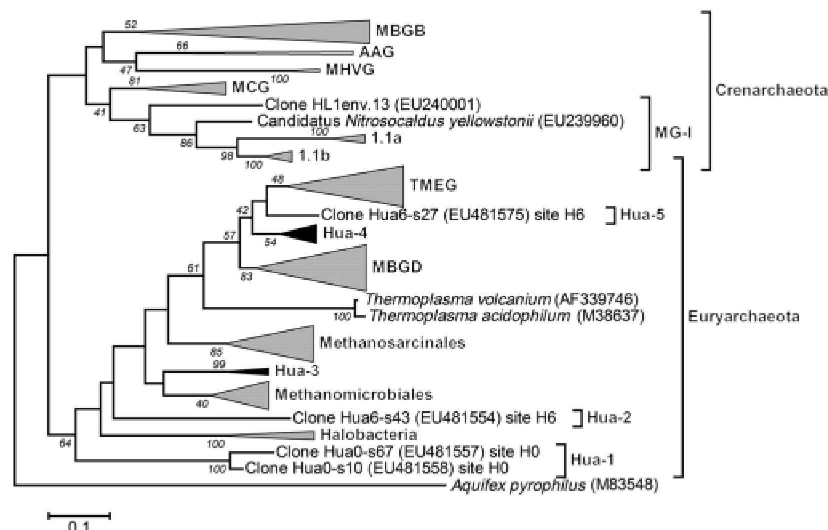

Fig. 4. Phylogenetic tree based on partial 16S rRNA gene sequences (~800 bp) of phylotypes of *Archaea* in water calculated by maximum likelihood analysis. Group designations: MG-I, Marine Group I (DeLong, 1998); MCG, Miscellaneous Crenarchaeotic Group (Teske, 2006); MHVG, Marine Hydrothermal Vent Group (Takai & Horikoshi, 1999); AAG, Ancient Archaeal Group (Takai & Horikoshi, 1999); MBGB, Marine Benthic Group B (Vetriani *et al*., 1999); MBGD, Marine Benthic Group D; TMEG, Terrestrial Miscellaneous Euryarchaeotal Group (Takai *et al*., 2001); Hua-1, Hua-2, Hua-3, Hua-4, Hua-5, Unidentified Euryarchaeota clusters. The scale bar represents a 10% nucleotide sequence difference. *Aquifex pyrophilus* (M83548) was used as an outgroup. Groups in gray include sequences from Salar de Huasco. Groups in black largely consist of sequences from Salar de Huasco. Figure S4 shows the detailed tree.

Hua6-s43, which exhibited 83% sequence identity to a clone of the same library (SBAK-shallow-04) classified as 'unaffiliated *Euryarchaeota*' (Kendall *et al*., 2007). *Group Hua-3* was formed by phylotypes Hua1-w46 and Hua1-s26 together with three sequences retrieved previously from wastewater sludge, lake sediments and an anaerobic reactor (Fig. S4). *Group Hua-4* is represented by the largest number of unidentified sequences and was found in H4-w, H6-w, H0-s, H1-s and H6-s. Additional sequences included in this group were recorded from marine sediments, from a sulfur spring and from petroleum-contaminated soil. The two clones from marine sediments (Skan Bay, Alaska) were similar to two different phylotypes of this study. The phylotype sequence Hua6-s37 was 96% similar to the clone SBAK-shallow-06 classified as MBGD (Kendall *et al*., 2007). *Group Hua-5* included a single phylotype (Hua6-s27) that had 90% sequence identity to clone MKCSB-C12, which was the closest relative and was retrieved from mangrove soil (Yan *et al*., 2006).

Evidence of ammonia-oxidizing *Archaea* (AOA) in Salar de Huasco

To evaluate a possible role of *Archaea* in the nitrogen cycle of Salar de Huasco, we tested for the presence of AOA in all samples. PCR products of archaeal *amoA*, the gene coding for ammonia monooxygenase subunit A, were only obtained from the water of site H0. A clone library produced from these products consisted of four phylotypes that were affiliated to sequences retrieved from sediment and water samples (Table 3). Three phylotypes exhibited high identity (97–99%) to two clones of a clone library constructed from water samples from a drinking water distribution system (Van der Wielen *et al*., 2009). The fourth phylotype Hua0-w51 was 99% similar to the clone QLS1-399-A8 retrieved from sediment samples of Qinghai Lake in China (Jiang, 2009).

Discussion

Novelty of the archaeal sequences

A large portion of the sequences were not closely related to any cultured archaeon, and 24% of the sequences had similarities lower than 98% to their closest relatives. Interestingly, the rest of the sequences had clear phylogenetic affiliations, including previously described clusters for uncultured *Archaea*. We described five groups of unidentified *Euryarchaeota*, which exclusively contained representatives from Salar de Huasco (Hua-1, Hua-2 and Hua-5), and of two distinct groups containing representatives from Salar de Huasco (Hua-3 and Hua-4), together with single clone sequences from other sources (Fig. 4 and Fig. S4). Important clusters of other groups (*Halobacteria*, *Methanomicrobiales* and TMEG) including representatives from Salar de Huasco were only distantly related to the sequences available in the databases.

In prokaryotic taxonomy, an identity threshold of 97% is generally accepted in order to separate species (Stackebrandt & Goebel, 1994) and this threshold has even been increased to $>98\%$ (Stackebrandt & Ebers, 2006). Consequently, the five clusters described for uncultured *Euryarchaeota* from Salar de Huasco exhibited similarities lower than 96% to their closest relatives, highlighting the novelty of the sequences retrieved from this location. These results justify considering Salar de Huasco as a unique archaeal habitat and a suitable location for further, more detailed studies. The novelty of sequences and clusters has been reported

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Table 3. Sequence identity of Crenarchaeota *amo*A sequences from Salar de Huasco compared with *Nitrosopumilus maritimus* (NM, accession numbers DQ085098 and AAZ38768) and the first hit in the BLAST search for nucleotide and amino acid sequences

		First hit in BLAST			
Clone	Type	Similarity (%)	Clone name	Habitat	Similarity with NM (%)
Hua0-w20	Nucleotide	97	Clone PLANTB AR RSF-II OTU3 (EU852699)	Drinking water distribution system	89
Hua0-w51	Nucleotide	99	Clone QLS1-399-A8 (EU197155)	Sediment, Qinghai Lake, Tibet, China	74
Hua0-w79	Nucleotide	97	Clone PLANTB AR RSF-II OTU3 (EU852699)	Drinking water distribution system	89
Hua0-w92	Nucleotide	99	Clone PLANTB AR DN OTU5 (EU852705)	Drinking water distribution system	90
Hua0-w20	Protein	99	Clone PLANTA AR DN OTU4 (ACF71541)	Drinking water distribution system	94
Hua0-w51	Protein	100	Clone QLS1-399-A6 (ABZ01900)	Sediment, Qinghai Lake, Tibet, China	82
Hua0-w79	Protein	100	Clone PLANTA AR DN OTU4 (ACF71541)	Drinking water distribution system	94
Hua0-w92	Protein	100	Clone PLANTB AR DN OTU5 (ACF71557)	Drinking water distribution system	94

recently for *Cyanobacteria* in Salar de Huasco (Dorador *et al.*, 2008a, b), for *Bacteria* in Laguna Tebenquiche at the Salar de Atacama (Demergasso *et al.*, 2008) and for *Bacteroidetes* in different saline evaporitic basins of northern Chile (Dorador *et al.*, 2009). Along with the current work, this highlights that wetlands located in northern Chile represent potential reservoirs of undescribed microorganisms, a trend probably due to the unusual physicochemical conditions and geographical isolation of the wetlands, as well as a lack of knowledge regarding the microbial diversity in these environments.

Community composition of *Archaea* in Salar de Huasco

Salar de Huasco could be considered as a moderately athalassohaline wetland (Table 1), comparable in its phylogenetic groups with other high-altitude cold aquatic systems (e.g. Tibetan Lakes: Dong *et al.*, 2006; Jiang *et al.*, 2008) or cold saline lakes (e.g. Antarctic lakes: Karr *et al.*, 2006). The abundance and composition of archaeal communities is likely to be driven by the availability of water, and hence, dissolution or concentration effects. The dominance of *Archaea* over *Bacteria* has been reported in habitats with NaCl concentrations close to saturation, where *Halobacteria* are the dominant aerobic heterotrophs (Oren, 2002). However, the levels of *Archaea* detected in this study (summer sampling), including the sediments of the most saline sites, were not associated with halophilic Archaea, but with uncultured marine archaeal groups and methanogens. The presence of extremely halophilic *Archaea* would be predicted only for sites with salt concentrations higher than $150\,g\,L^{-1}$ (Ventosa *et al.*, 1998). Such situations can be found in particular within the dry season when elevated salt concentrations occur due to strong evaporation. Previous studies had described elevated proportions of halophilic *Archaea* in water samples collected from both Salar de Huasco (site H1) and Salar de Ascotán, from northern Chile during winter (dry season) (Dorador, 2007). However, only a single phylotype was reported as a member of the *Halobacteria* in the present study (Figs 3 and 4, and Fig. S4). As the primers and amplification conditions used were the same in both studies, this suggests that these differences reflect temporal climatic variations (e.g. variable salt concentration).

The presence of uncultured archaeal groups in Salar de Huasco is an interesting finding, because these groups were primarily described from marine environments as an important component of the deep-ocean picoplankton (DeLong, 1998). Nevertheless, recently, uncultured marine archaeal groups were also reported from Qinghai Lake, an athalassohaline lake located in the Tibetan plateau, and in other nonmarine saline environments (Sørensen *et al.*, 2005; Yan *et al.*, 2006; Jiang *et al.*, 2008), extending the distribution of these groups.

Crenarchaeota

Recovered sequences related to *Crenarchaeota* were affiliated to marine uncultured archaeal groups with as yet unknown functions in the environment. The only mesophile cultured representative of MG-I, *Nitrosopumilus maritimus* (Group 1.1a), grows by aerobically oxidizing ammonia to nitrite, a metabolic activity previously unknown for *Archaea* (Könneke *et al.*, 2005). Also, members of the MG-I have been reported to demonstrate autotrophic carbon fixation while some can be mixotrophic (Ingalls *et al.*, 2006). In this context, it is interesting that we have found MG-I members in the water of site H0, where the *amoA* gene was also detected (Table 3), although the majority of the 16S rRNA gene sequences were not similar ($< 90\%$) to those of *Nitrosopumilus* sequences, currently the only mesophile representative known to oxidize ammonia. Recently, two new thermophilic members of MG-I (Group 1.1b) that can oxidize ammonia have been described. The moderately thermophilic (46 °C) Candidatus *N. gargensis* (Hatzenpichler *et al.*, 2008) enriched from hot spring microbial mats

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exhibited a higher sequence identity (97%) than *N. maritimus* with Salar de Huasco sequences, while the other thermophilic (74 °C) Candidatus *Nitrosocaldus yellowstonii* (de la Torre *et al.*, 2008) retrieved from hot spring sediments showed lower similarity (< 90%).

Members of the MBGB and MCG have been proposed as the major oxidizers of methane without assimilation of methane-derived carbon in subsurface sediments off Peru (Biddle *et al.*, 2006). The presence of MBGB (sediments of H0, H4 and H6) and MCG (sediments of H0, H1 and H6) confirms the cosmopolitan distribution of this group. Because of their presence under diverse geochemical conditions, it is not possible at present to correlate their presence to particular environmental conditions.

Euryarchaeota

Sequences of MBGD were particularly abundant in water and sediment samples from site H6 and also water samples from H1. This group (equivalent to Marine Group III, as defined by DeLong, 1998) has been reported as being metabolically active in subsurface sediments (e.g. Sørensen & Teske, 2006), and recently, a metagenomic analysis of bathypelagic plankton revealed that members of Marine Group III *Euryarchaeota* might also utilize the oxidation of ammonia to gain energy (Martin-Cuadrado *et al.*, 2008). The TMEG group was also reported as being metabolically active in subsurface sediments (e.g. Sørensen & Teske, 2006), but a specific physiological function of this group has not yet been found.

Role of *Archaea* in high-altitude saline wetlands: methanogenesis and evidence of archaeal ammonia oxidizers

Methanogenic *Archaea* dominated the 16S rRNA gene clone libraries of sediment samples from sites H0, H1 and H4, and probably represents an indicator of elevated methanogenic activity. In sediments, the sequences clustered with four genera of *Methanosarcinales*: *Methanosarcina*, *Methanosaeta* (aceticlastic methanogens), *Methanomethylovorans* and *Methanolobus* (methylotrophic organisms), highlighting the diverse substrates used by methanogens in these environments. Considering the high sulfate concentrations reported in Salar de Huasco (Risacher *et al.*, 1999), sulfate reduction could be expected, especially at the saline sites H4 and H6, where the presence of 16S rRNA gene sequences related to sulfate-reducing bacteria has been reported previously (Dorador, 2007). Site H4 was anoxic at the time of sampling (Table 1) and exhibited the highest sulfate concentration in water ($4\,g\,L^{-1}$, almost double that of seawater, for example Holmer & Storkholm, 2001); under these conditions, almost all sequences from water samples matched into the TMEG (Fig. 3). Contrastingly, in sediment samples, *Methanomicrobiales* were the most abundant group (77% of the sequences). These contrasting differences in the water chemistry can help in future studies to understand the functional role of uncultured *Archaea* and the interaction with methanogens and sulfate-reducing bacteria. Also, it is interesting to note the low identity of the methanogenic clone sequences with the described species, highlighting the potential discovery of new species of methanogenic *Archaea*.

The presence of methanogens (e.g. *Methanosarcinales*) and benthic *Archaea* (MBGD) in water samples from site H1, which exhibited dissolved oxygen concentrations of $10\,mg\,L^{-1}$, is intriguing. The water levels in different sites of Salar de Huasco are shallow (few cm) and the typically strong afternoon winds in the Altiplano can result in a well-mixed water column, and therefore the presence of anaerobic and benthic microorganisms in water samples. At site H6, methanogens were absent from clone libraries, and the most abundant group in water and sediment samples was MBGD. The functional role of this group is not yet known. Recently, it has been demonstrated that microorganisms that perform anaerobic oxidation of methane (AOM) can use alternative electron acceptors to sulfate, including Mn and Fe. It has been shown experimentally that the MBGD represents the most abundant group in anaerobic sediments treated with different electron acceptors, indicating a potential role in methane cycling (Beal *et al.*, 2009). Interestingly, site H6 receives water from a stream different from the other sites of Salar de Huasco (Risacher *et al.*, 1999) and has contrasting water chemistry (Fig. S3), indicating that the chemical conditions of the water (Table 1) might favor the development of members of the MBGD instead of the methanogens recorded from other sites.

Nitrogen limitation has been reported in several wetlands in the Altiplano and has also been reported for the sites H1 and H4 in Salar de Huasco (Dorador *et al.*, 2008a). The study of the N-cycle in this system has included analysis of enrichment cultures of ammonia-oxidizing bacteria (AOB) that are dominated by *Nitrosomonas* (Dorador *et al.*, 2008a). Here, we reported AOA sequences from site H0 from Salar de Huasco, indicating the potential role of this archaeal group in the ammonia oxidation. AOA has also been reported as being more abundant than AOB in other saline mountain lakes (Qinghai Lake: Jiang *et al.*, 2009). Specific studies related to the functional role of uncultured *Archaea* (including AOA) are promising in Salar de Huasco because of the exceptional environmental conditions.

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Supporting Information

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

Fig. S1. Rarefaction curves of archaeal 16S rRNA gene clone libraries from water (a) and sediment samples (b) show saturation in all samples.
Fig. S2. UPGMA clustering of DGGE band patterns of archaeal 16S rRNA gene from water (w) and sediment (sed) samples of the four sites in Salar de Huasco.
Fig. S3. Group average clustering of chemical analysis of water samples in Salar de Huasco taken from Table 1 show marked physicochemical differences between sites.
Fig. S4. Phylogenetic tree based on partial 16S rRNA gene sequences (~800 bp) of phylotypes of *Archaea* in water calculated by maximum likelihood analysis.

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