RESEARCH ARTICLE

Prokaryotic diversity in Tuz Lake, a hypersaline environment in Inland Turkey

Mehmet Burcin Mutlu[1,2], Manuel Martínez-García[1], Fernando Santos[1], Arantxa Peña[1], Kiymet Guven[2] & Josefa Antón[1,3]

[1]División de Microbiología, Universidad de Alicante, Alicante, Spain; [2]Department of Biology, Faculty of Science, Anadolu University, Eskisehir, Turkey; and [3]Multidisciplinary Institute for Environmental Studies Ramón Margalef, Universidad de Alicante, Alicante, Spain

Correspondence: Fernando Santos, División de Microbiología, Universidad de Alicante, Apartado 99, 03080 Alicante, Spain. Tel.: 34 965903870; fax: 34 965909569; e-mail: fernando.santos@ua.es

Received 8 January 2008; revised 14 March 2008; accepted 11 April 2008.
First published online 5 June 2008.

DOI:10.1111/j.1574-6941.2008.00510.x

Editor: Riks Laanbroek

Keywords
Haloquadratum; *Salinibacter*; extremely halophilic microorganisms; Tuz Lake; hypersaline environments; prokaryotic diversity.

Abstract

Tuz Lake is an inland thalassohaline water body located in central Anatolia that contributes to 60% of the total salt production in Turkey per year. The microbiota inhabiting this lake has been studied by FISH, denaturing gradient gel electrophoresis of PCR-amplified fragments of 16S rRNA genes, and 16S rRNA gene clone library analysis. Total cell counts per milliliter (1.38×10^7) were in the range of the values normally found for hypersaline environments. The proportion of *Bacteria* to *Archaea* in the community detectable by FISH was one to three. 16S rRNA gene clone libraries indicated that the archaeal assemblage was dominated by members of the Square Haloarchaea of the Walsby group, although some other groups were also found. *Bacteria* were dominated by members of the *Bacteroidetes*, including *Salinibacter ruber*-related phylotypes. Because members of *Bacteroidetes* are widely present in different hypersaline environments, a phylogenetic analysis of 16S rRNA gene sequences from *Bacteroidetes* retrieved from these environments was carried out in order to ascertain whether they formed a unique cluster. Sequences retrieved from Tuz Lake and a group of sequences from other hypersaline environments clustered together in a branch that could be considered as the 'halophilic branch' within the *Bacteroidetes* phylum.

Introduction

Tuz Lake is an inland hypersaline water body with salinity above 30% NaCl, located in the central plateau of Turkey, 120 km south of Ankara. It is the second largest lake of Turkey, with a length of 90 km, a width of 35 km, and a total surface of 1665 km^2 within a closed basin. The lake started its formation with tensional movements during the Late Cretaceous, followed by compressional episodes that, during the Late Eocene, resulted in the isolation of the basin from an open sea (Dirik & Erol, 2000). Water flows into the Tuz Lake through the Melendiz Stream and drainage channels of the Konya plain. In summer, the lake dries out and a 30-cm layer of salt forms because of the evaporation. In winter, water is not more than 2 m deep. Three hundred thousand tons of salt, which is 60% of the total salt production in Turkey, are obtained per year from the lake (Uygun & Sen, 1978; Çamur & Mutlu, 1995).

In previous studies based on the isolation of heterotrophic microorganisms (Birbir & Sesal, 2003) and identification of cultivable *Archaea*, Tuz Lake was found to contain a 'viable, diverse, potentially and industrially important microbial community' although so far (Birbir *et al.*, 2007; Özcan *et al.*, 2007) only the culturable archaeal assemblage has been partially characterized. Here, we present the study of the prokaryotic community of Tuz Lake using a culture-independent approach that includes FISH, denaturing gradient gel electrophoresis (DGGE) of 16S rRNA gene fragments PCR amplified from DNA extracted from the lake, and 16S rRNA gene library analysis. The aim of this work was to describe the prokaryotic community of a new inland hypersaline environment, uncharacterized previously. Although considerable information has been retrieved from crystallizer ponds from solar salterns (Oren, 2002), inland thalassohaline environments have not been extensively characterized by culture-independent techniques (Oren, 2002; Maturrano *et al.*, 2006).

Many hypersaline environments with salinities approaching saturation (Elevi Bardavid *et al.*, 2008) are dominated by the square archaeon of Walsby [the recently isolated

© 2008 Federation of European Microbiological Societies
Published by Blackwell Publishing Ltd. All rights reserved

Downloaded from https://academic.oup.com/femsec/article/65/3/474/519295 by Anadolu University user on 16 December 2022

Haloquadratum walsbyi (Burns *et al.*, 2007)], with bacteria such as *Salinibacter* and *Salicola* also present as a minor component of the prokaryotic communities (Maturrano *et al.*, 2006; Burns *et al.*, 2007). However, some differences among the microbiota of different hypersaline environments have also been found, such as the presence of many *Halobacterium*-related phylotypes in 16S rRNA gene libraries retrieved from Andean salterns of many *Halobacterium*-related phylotypes (Maturrano *et al.*, 2006) or the dominance of *Halorubrum* spp. in Adriatic salterns (Pašić *et al.*, 2005). The second goal of this study was thus to see whether the Tuz Lake prokaryotic community had the structure found in saltern crystallizer ponds or presented some specific characteristics. In this way, we intended to gain a deeper insight into the differences in the prokaryotic communities inhabiting geographically distant hypersaline environments.

According to our results, the prokaryotic community in Tuz Lake is dominated by *Haloquadratum* and *Salinibacter*, thus resembling crystallizer ponds from artificial solar salterns (Elevi Bardavid *et al.*, 2008). As in other hypersaline environments (Burns *et al.*, 2004; Maturrano *et al.*, 2006), sequences related to other extremely halophilic *Bacteria* and *Archaea* were also found. While *Haloquadratum* phylotypes retrieved from the lake were very closely related to those repeatedly found in salterns around the world (Australia, Peruvian Andes and both sides of the Mediterranean), the *Salinibacter* assemblage was quite site specific.

Materials and methods

Sample collection

Samples were taken from two different locations (W and E, located in the West and the East shores, coordinates N 38°46′25E 33°12′57 and N 38°44′14 E 33°36′01, respectively) of the Tuz Lake in May, July, and October 2005 and named as May W, July W, July E, and October W. Sample July W was chosen for a more complete characterization of the microbial community. The total salt concentration of this sample was 32% as measured using a hand refractometer (Eclipse).

Diamidino-2-phenylindole (DAPI) counts and FISH

Sample fixation was carried out as described previously (Antón *et al.*, 1999) using the protocol optimized for fixation of extremely halophilic microorganisms. Hybridization, DAPI staining, and microscopy were carried out as described previously (Snaidr *et al.*, 1997). At least two filters were analyzed and cells were counted in 30 different microscopic fields. The probes used for *in situ* hybridization were Arc915 for *Archaea*, Eub338 for *Bacteria*, and Non338 for nonspecific hybridization (Amann *et al.*, 1990). The formamide concentration in the hybridization buffer was 35%.

Nucleic acid extraction

Microorganisms were collected by filtration of 50 mL of sample on a 0.22-μm pore size GV filter (Durapore, Millipore). The filter was cut into small pieces with sterile scissors, and placed in RNAse- and DNAse-free 2-mL cryotubes containing 600 μL of extraction buffer (100 mM Tris-HCl, 100 mM EDTA pH 8.0). Six microliters of lysozyme (3 mg mL^{-1}) was added and incubated at 37 °C for 15 min. Then, 9 μL of proteinase K (150 μg mL^{-1}) and 60 μL of 10% sodium dodecyl sulfate (SDS) were added to the tubes and incubated at 37 °C for 30 more minutes. After the addition of 120 μL 5 M NaCl and 90 μL CTAB solution (10% CTAB, 0.7 M NaCl), the tubes were incubated at 65 °C for 10 min, immersed into liquid nitrogen for 2 min, and incubated again for 2 min at 65 °C. The freeze-and-thaw steps were repeated three times. Nine hundred microliters of phenol : chloroform : isoamylalcohol (25 : 24 : 1) (PCI) was added, mixed, and centrifuged at 16 000 ***g*** for 5 min at 4 °C. The aqueous phase was removed and one volume of PCI was added, vortexed, and centrifuged again (two to three times) until a clear interphase between the aqueous and the organic phases was observed. Finally, nucleic acids were precipitated with ethanol and resuspended in 50 μL of sterile deionized water. To check the quality of nucleic acids, they were run in 1% agarose (LE, FMC Products, Rockland, ME) gels and visualized under UV light after ethidium bromide staining. Extracts were stored at − 70 °C until used.

PCR amplification of 16S rRNA genes

16S rRNA genes were PCR amplified from total DNA using universal primers for *Bacteria* and *Archaea*. Reaction mixtures contained 1.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl (pH 9), 200 μM of each deoxyribonucleotide triphosphate (dATP, dCTP, dGTP, and dTTP) (Invitrogen Corporation, Carlsbad, CA), 1 U of Taq DNA Polymerase (Invitrogen), 0.2 mM of each oligonucleotide primer, and 50 ng of template DNA in a total volume of 50 μL. The sequences of the forward primers were 21F 5'-TTCCGGTTGATCCTGCCGGA-3′ (DeLong, 1992) for the *Archaea* and 27f 5′-AGAGTTTGATCATGGCTCAG-3′ (Lane *et al.*, 1985) for the *Bacteria*. The reverse primer for both domains was 1492r 5′-GGTTACCTTGTTACGACTT-3′ (Lane *et al.*, 1985). The following conditions were used for amplification: a cycle of 94 °C for 3 min, 30 cycles of 94 °C for 15 s, 55 °C for 30 s, and 72 °C for 2 min; plus an extension step of 7 min at 72 °C. Negative controls were included with no addition of template DNA. Five microliters of PCR products were loaded onto 1% agarose gels in

Downloaded from https://academic.oup.com/femsec/article/65/3/474/519295 by Anadolu University user on 16 December 2022

© 2008 Federation of European Microbiological Societies
Published by Blackwell Publishing Ltd. All rights reserved

1 × Tris–acetic acid–EDTA (TAE) buffer, stained, and visualized as above.

16S rRNA genes were PCR amplified from all the samples for DGGE analysis with the following primer sets: 341F-GC (5′-GCclamp-CCTACGGGAGGCAGCAG) and 907R (5′-CCGTCAATTCCTTTRAGTTT-3′) for *Bacteria*; 344F-GC (5′-GCclamp-ACGGGGCGCAGCAGGCGCGA) and 907R for *Archaea*. (Muyzer *et al.*, 1993). The PCR program for the *Bacteria* primer set was: 94 °C for 5 min, 65 °C 1 min, 72 °C 3 min, and nine touchdown cycles of: 94 °C for 1 min, 65 °C (with the decreasing 1 °C each cycle) 1 min, 72 °C 3 min, followed by 20 cycles of: 94 °C for 1 min, 55 °C 1 min, and 72 °C 3 min. During the final cycle, the length of the extension step was increased to 10 min. The PCR program for the *Archaea* primer set was: 94 °C for 5 min (initial denaturation), and 30 cycles of 94 °C for 30 s, 56 °C for 45 s, and 72 °C for 2 min. The length of the extension step was increased to 7 min in the last cycle.

Cloning of PCR products

Ligation of the PCR products with the pCRII-TOPO vector, transformation of *Escherichia coli* TOP10, and selection of the transformants were carried out using the TOPO TA cloning kit (Invitrogen Corporation) according to the manufacturer's protocol. Two (one for *Archaea*, one for *Bacteria*) 16S rRNA gene libraries were generated from sample July W. Each library was generated with the pooled products of at least three independent PCR reactions.

Analysis of libraries and clone selection

Clones were screened for redundancies by amplified rDNA restriction analysis (ARDRA) (Vaneechoutte *et al.*, 1992) with the enzymes HinfI and MboI (New England Biolabs). Enzymatic digestions were performed by incubating 10 μL of the amplified inserts with 5 U of enzyme and the corresponding enzyme buffer. The digestion products were analyzed in 2% agarose gels in 0.5 × Tris–boric acid–EDTA (TBE) buffer, stained, and visualized as above. Clones representing the different restriction patterns were selected for sequencing.

DGGE

DGGE was performed with the DCode System (Bio-Rad, Hercules, CA). Electrophoresis conditions were 70 V for 16 h, with a linear gradient of denaturing agents from 40% to 70% (100% denaturing agents were 7 M urea and 40% deionized formamide) in a 6% 37.5 : 1 acrylamide : bis-acrylamide 0.75 mm thick gel, with 1 × TAE as running buffer. Gels were photographed with a Typhoon 9410 (Amersham Biosciences) imaging system after ethidium bromide staining. Selected bands were excised, resuspended in 20 μL Milli Q water, and incubated at 4 °C overnight. An aliquot of the supernatant was used for PCR reamplification with the original primer set, and 50 ng of reamplified PCR products were used for the sequencing reaction.

Sequencing and sequence analysis

The nucleotide sequences of the cloned products were determined from plasmid preparations (Wizard Plus SV Minipreps DNA Purification System, Promega, Madison, WI). Cloned products and purified PCR products were sequenced using the Big Dye Terminator Cycle Sequencing kit and an ABI PRISM 3100 Genetic Analyser (Applied Biosystems, Foster City, CA). 16S rRNA gene sequences were initially compared with reference sequences at NCBI (http://www.ncbi.nlm.nih.gov) using BLAST (Altschul *et al.*, 1990). The correlation between primary and secondary structures was analyzed using the ARB sequence editor (Ludwig *et al.*, 2004). Nearly complete (for the 16S rRNA gene clones) and partial (for the DGGE bands) sequences obtained in this study were added to an alignment of over 50 000 primary aligned gene structures available at http://db-central.arb-home.de/. Sequences not included in the ARB database were drawn from the GenBank database. Phylogenetic analysis was performed using the three algorithms implemented in the ARB package: maximum-likelihood using FASTDNAML and TREE-PUZZLE programs (Strimmer & Von Haeseler, 1996; Schmidt *et al.*, 2002), neighbor joining, and maximum parsimony. Partial sequences from DGGE analysis were added to the tree using the ARB parsimony tool. All the sequences obtained in this study were analyzed simultaneously by MALLARD 1.02 software (Ashelford *et al.*, 2006) in order to detect chimeras and other artifacts. Furthemore, each individual sequence was screened by PINTAIL 1.1 software according to the authors (Ashelford *et al.*, 2005) to confirm the presence of chimeric sequences.

Isolation of microorganisms

In an attempt to isolate new *Salinibacter* spp., the following medium was used: to a liter of a solution of salts, named as 25% SW and containing ($g\,L^{-1}$): NaBr 0.65, $NaHCO_3$ 0.17, KCl 5, $CaCl_2$ 0.72, $MgSO_4 \cdot 7H_2O$ 49.5, $MgCl_2 \cdot 6H_2O$ 34.6, NaCl 195, 1 g yeast extract (YE), and 20 g of agar were added. Several dilutions (from 10^{-1} to 10^{-5}) of the original water sample were used to inoculate the plates by a 'top agar' technique. Briefly, 0.1 mL of inoculum and 3 mL of 0.7% agar in 25% SW were mixed and the mixture was immediately poured onto plates containing SW 25% and 0.1% YE. After incubation of the plates for 60 days at 37 °C, the 269 colonies grown in the plate inoculated with dilution 10^{-4} were restreaked in the same medium. DNA from each colony was extracted by boiling and used as a template for two independent PCRs with primers specific for *Bacteria*

© 2008 Federation of European Microbiological Societies
Published by Blackwell Publishing Ltd. All rights reserved

Downloaded from https://academic.oup.com/femsec/article/65/3/474/519295 by Anadolu University user on 16 December 2022

and *Archaea*, respectively, as described above. Colonies yielding PCR products only with either *Bacteria*- or *Archaea*-specific primers were identified, respectively, as *Bacteria* or *Archaea*. The 16S rRNA genes from the eight bacterial colonies were sequenced, while 29 colonies were randomly selected among the 261 archaeal colonies for ARDRA and sequencing of selected clones from each ARDRA pattern as described above.

Nucleotide sequence accession numbers

The 16S rRNA gene sequences determined in this study have been deposited in the GenBank database under accession numbers EF459702–EF459730.

Results

DGGE

Bacterial and archaeal communities from the water samples were compared by DGGE analysis of PCR amplified 16S rRNA gene fragments, as shown in Fig. 1. Selected bands were cut from the gel, reamplified, and sequenced (Table 1). Bands that could be successfully sequenced are labeled with arrows. All the bacterial bands (labeled with a number followed by 'B') analyzed corresponded to members of the *Bacteroidetes*, most of them (bands 2B, 4B, 7B, and 8B) to phylotypes among the *Salinibacter* cluster. Band 1B was related to environmental sequence NCh63AT12 retrieved from Lake Tebenquiche in the Salar de Atacama in Northern Chile (C. Demergasso *et al.*, unpublished data), with no match to cultured bacteria. Band 4B was related to a sequence retrieved from the athalassohaline Lake Chaka, in Northwestern China (Jiang *et al.*, 2006), with *Salinibacter ruber* Pola 18 as the closest cultured relative. Band 8B had the highest similarity to the uncultured *Salinibacter* phylotype EHB-2 (Antón *et al.*, 2000). Three out of the eight archaeal (number plus 'A') bands analyzed (i.e. 4A, 7A and 8A) were related to *H. walsbyi* DSM 16790, 'the square archaeon' recently isolated from Spanish and Australian salterns (Burns *et al.*, 2007). Band 11A was distantly related to a group (ss057) of environmental sequences retrieved from soil samples taken along a salinity gradient (Walsh *et al.*, 2005), with *Halorubrum* sp. as the closest cultured relative. Band 12A was related to the environmental sequence A54 obtained from a Permo-Triassic rock salt (Radax *et al.*, 2001) and also to ss057sequences; *Halorubrum sacharovorum* was the closest cultured relative. Band 14A was related to the haloarchaeon CSW2.24.4, isolated from an Australian crystallizer pond, that branched just outside the Square Haloarchaea of the Walsby (SHOW) group. In fact, the cultured archaeon closest to band DGGE14A was *H. walsbyi*, with 92% similarity. Sequences related to CSW 2.24.4 were also retrieved from a Spanish coastal multi-pond saltern (Legault *et al.*, 2006). Bands 16A and 17A were very closely related to *Halorubrum orientale* and to the haloarchaeon CSW5.28.5, isolated from the above-mentioned Australian crystallizer pond (Burns *et al.*, 2004). Sequences very closely related to CSW5.28.5 have also been retrieved from crystallizers of an Adriatic solar saltern (Pašic *et al.*, 2005). Because the number of bands in the DGGE for sample July W appeared to be higher than in the rest of the samples, it was chosen for a more detailed analysis.

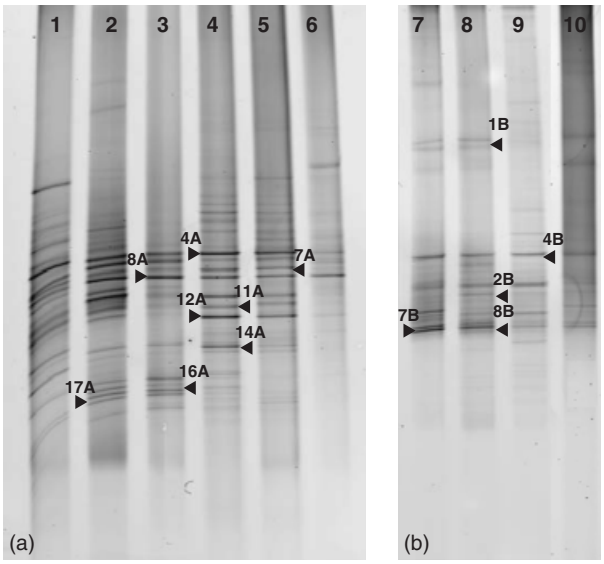

Fig. 1. DGGE analysis of archaeal (a) and bacterial (b) diversity in Tuz Lake samples. a: PCR-amplified archaeal 16S rRNA gene fragments from different Tuz Lake samples [May W (4), July W (1), July E (5), October W (6)]. Lanes 2 and 3 correspond to a coastal solar saltern in Turkey. b: PCR-amplified bacterial 16S rRNA gene fragments from samples: May W (7), July E (8), July W (10), and October W (9).

Bacteria and *Archaea* 16S rRNA gene clone libraries from sample July W

A total of 200 clones (100 for *Archaea* and 100 for *Bacteria*) were analyzed by ARDRA, which yielded a total of nine different patterns for *Bacteria* and nine for *Archaea*. At least one clone per restriction pattern was chosen for complete sequencing. Using the chimera sequence detection softwares described in Ashelford *et al.* (2005, 2006), two ARDRA groups from the archaeal library (corresponding to 13 clones) and none for the bacterial were identified as possible chimeric sequences and were thus eliminated from the analysis. Table 2 shows the best match with the sequences in databases obtained by BLAST analysis of the selected clones. Most (82%) of the bacterial clones were related to different uncultured members of the *Bacteroidetes*. For all these sequences, except 4-01B, *S. ruber* was the closest cultured relative. Sequence 4-01B did not match with any cultured

© 2008 Federation of European Microbiological Societies
Published by Blackwell Publishing Ltd. All rights reserved

Downloaded from https://academic.oup.com/femsec/article/65/3/474/519295 by Anadolu University user on 16 December 2022

Table 1. DGGE bands and their closest matches in GenBank

DGGE band	Closest GenBank match
Bacteria	
1B	95% Environmental 16S rRNA gene clone NCh63AT12 (AY862804)
2B	98% *Salinibacter ruber* POLA 18 (AF323502)
4B	98% Environmental 16S rRNA gene clone LCKW-B8 (DQ129882)/94% *Salinibacter ruber* POLA 18 (AF323502)
7B	97% *Salinibacter ruber* POLA 18 (AF323502)
8B	98% Uncultured *Salinibacter* EHB-2 (AJ242998)/94% *Salinibacter ruber* POLA 18 (AF323502)
Archaea	
4A	99% *Haloquadratum walsbyi* DSMZ16790 (AM180088)
7A	98% *Haloquadratum walsbyi* DSMZ16790 (AM180088)
8A	100% *Haloquadratum walsbyi* DSMZ16790 (AM180088)
11A	88% Environmental 16S rRNA gene clone ss057t (AJ969857)/88% *Halorubrum* sp. CGSA-14 (DQ822454)
12A	90% Environmental 16S rRNA gene sequence A154 (AJ278937)/90% *Halorubrum saccharovorum* (U17364)
14A	97% Haloarchaeon CSW2.24.4 (AY498650)/92% *Haloquadra walsbyi* DSMZ16790 (AM180088)
16A	99% Haloarchaeon CSW5.28.5 (AY498647)/99% *Halorubrum orientale* CECT7145 (AM235786)
17A	99% Haloarchaeon CSW5.28.5 (AY498647)/99% *Halorubrum orientale* CECT7145 (AM235786)

Table 2. *Bacteria* and *Archaea* 16S rRNA gene clones analyzed by ARDRA and identification of the almost complete sequence of selected clones

ARDRA pattern	No Clones per type	Selected clone (length)	Closest GenBank match
Bacteria			
IB	6	4-62B (1404)	96.8% *Halanaerobacter lacunarum* (U32593)
IIB	15	3-45B (1440)	93.7% Uncultured *Bacteroidetes* AT12OctA10 (AY862788)/92.7% *Salinibacter ruber* POLA 13 (AF323503)
IIIB	18	4-48B (1513)	95.8% Uncultured halophilic eubacterium EHB-2 (AJ242998)/95% *Salinibacter ruber* POLA 18 (AF323502)
IVB	11	4-01B (1499)	95.1% Uncultured halophilic bacterium AT12OctB3 (AY862793)
VB	9	3-75B (1477)	97% *Halanaerobacter lacunarum* (U32593)
VIB	12	3-55B (1519)	94.1% Uncultured halophilic eubacterium EHB-2 (AJ242998)/93.7% *Salinibacter ruber* POLA 18 (AF323502)
VIIB	8	4-35B (1518)	99.3% Uncultured halophilic eubacterium EHB-2 (AJ242998)/97.3% *Salinibacter ruber* POLA 18 (AF323502)
VIIIB	3	3-12B (1493)	99.1% *Acinetobacter* sp. TUT1001 (AB098569)
IXB	18	3-17B(1520)	99.2% Uncultured halophilic eubacterium EHB-2 (AJ242998)/97.7% *Salinibacter ruber* POLA 18 (AF323502)
Archaea			
IA	12	1-13A (1441)	99.4% *Haloquadratum walsbyi* DSM 16790 (AM180088)
IIA	8	1-39A (1440)	94% Uncultured haloarchaeon WN-FWA-186 (DQ432537)/92% *Halosimplex carlbadense* (AJ586107)
IIIA	15	3-3A (1411)	99.5% *Haloquadratum walsbyi* DSM 16790 (AM180088)
IVA	10	3-7A (1425)	92.6% Uncultured haloarchaeon MSP1 (AB012049)/89% *Halorhabdus utahensis* (AF071880)
VA	16	3-9A (1441)	99.3% *Haloquadratum walsbyi* DSM 16790 (AM180088)
VIA	14	3-46A (1441)	99.4% *Haloquadratum walsbyi* DSM 16790 (AM180088)
VIIA	8	3-4A (1442)	95.6% Halophilic archaeon CSW1.15.5 (AY498641)

bacterium, although it was affiliated to the *Bacteroidetes* group. In addition, 15 clones were related to *Haloanaerobacter lacunarum*, which was isolated from hypersaline Lake Chokrak on the Kerk Peninsula (Zhilina *et al*., 1992). Finally, three bacterial clones were very closely related to *Acinetobacter* sp. TUT1001, a proteobacterium isolated from a household biowaste composting reactor (Hiraishi *et al*., 2003), as well as to 16S rRNA gene sequences obtained from oil- or phenol-degrading bacteria.

Many (69%) archaeal clones were very closely related (more than 99% similarity) to *H. walsbyi* DSM 16790. The rest of the sequences had their closest matches to uncultured haloarchaea. ARDRA pattern IIA, represented by clone 1-39 A, was related to a sequence (WN-FW-186) retrieved from alkaline hypersaline lakes of the Wadi An Natrun in Egypt (N.M. Mesbah *et al*., unpublished data). The closest cultured organism was *Halosimplex carlbadense*, a halophilic archaeon isolated from unsterilized salt crystals taken from the

© 2008 Federation of European Microbiological Societies
Published by Blackwell Publishing Ltd. All rights reserved

Downloaded from https://academic.oup.com/femsec/article/65/3/474/519295 by Anadolu University user on 16 December 2022

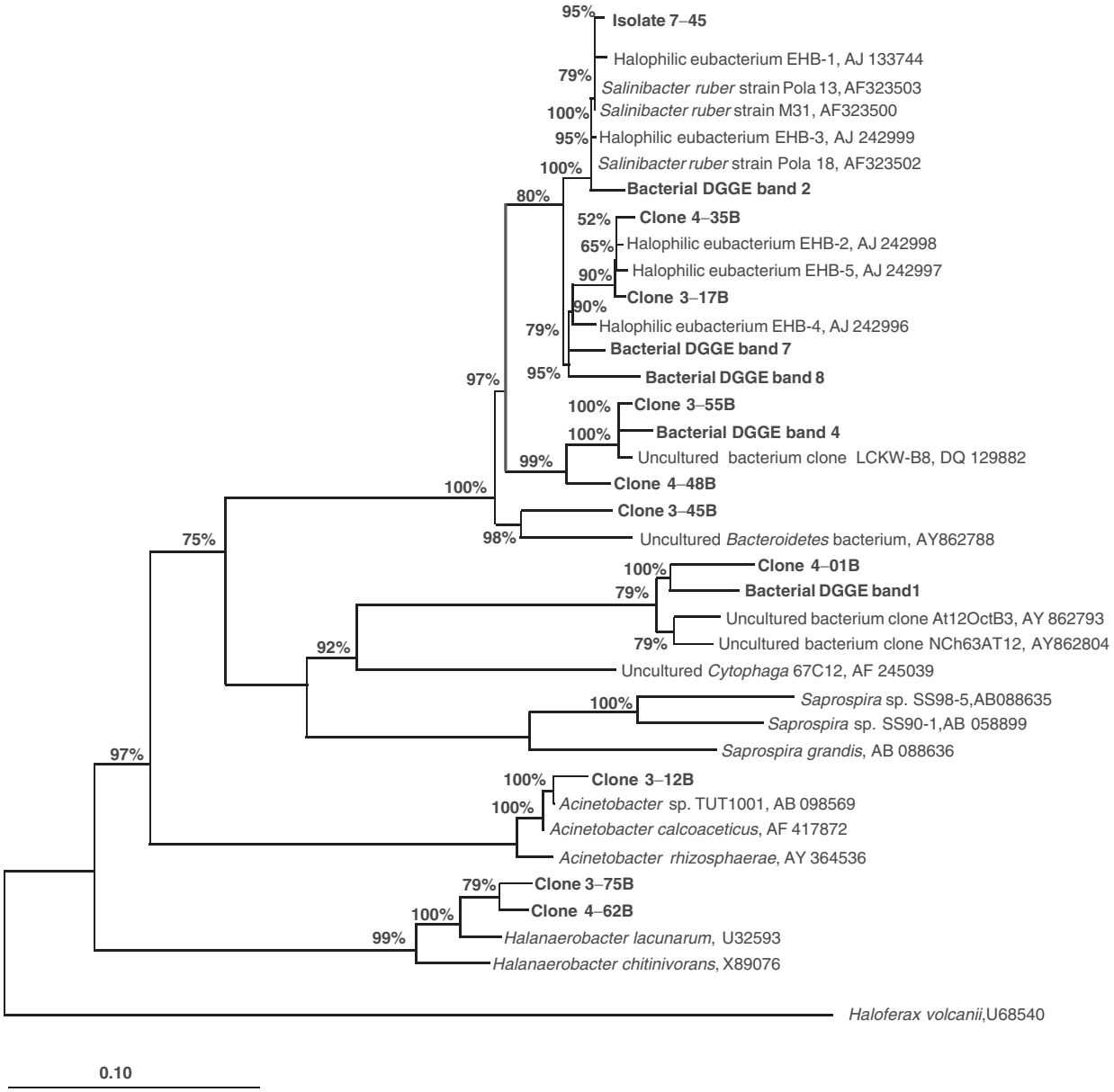

Fig. 2. Phylogenetic inferences based on 16S rRNA gene sequences from clones, isolates, and DGGE bands (indicated by bold face type) belonging to the halophilic *Bacteria*. The tree is based on maximum likelihood analysis for nearly complete sequences of the clones and isolates. Denaturing gradient gel electrophoresis band sequences were added by parsimony onto the tree. Quartet puzzling support values for each internal branch are shown at the branch nodes. The sequence of *Haloferax volcanii* was used as the outgroup. The horizontal scale bar represents the number of substitutions per site.

250-million-year-old Salado formation in southeastern New Mexico (Vreeland *et al.*, 2002). Sequences related to *H. carlbadense* have also been recovered in 16S rRNA gene libraries obtained from a crystallizer pond in a solar saltern from Alicante, Spain (Legault *et al.*, 2006). Ten archaeal clones (ARDRA pattern IVA) were related to the uncultured haloarchaeon MSP1, an archaeal phylotype detected in an East African alkaline saltern (Grant *et al.*, 1999), and to *Halorhabdus utahensis*, an extremely halophilic archaeon isolated from the Great Salt Lake in Utah (Wainø *et al.*, 2000). Finally, eight clones (ARDRA pattern VIIA) were related to the halophilic archaeon CSW1.15.5, isolated from an Australian crystallizer pond (Burns *et al.*, 2004), which belongs to the novel ADL group that had been detected previously only in the Antarctic Deep Lake (Bowman *et al.*, 2000).

© 2008 Federation of European Microbiological Societies
Published by Blackwell Publishing Ltd. All rights reserved

Downloaded from https://academic.oup.com/femsec/article/65/3/474/519295 by Anadolu University user on 16 December 2022

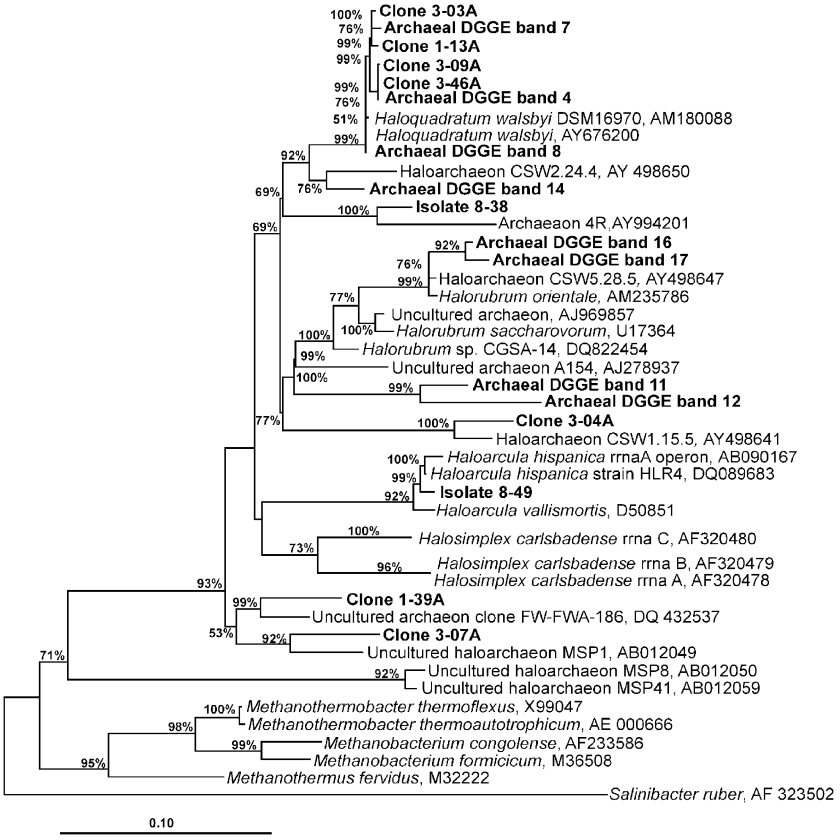

Fig. 3. Phylogenetic inferences based on 16S rRNA gene sequences from clones, isolates, and DGGE bands (indicated by bold face type) belonging to the halophilic *Archaea*. The tree is based on maximum likelihood analysis for nearly complete sequences of the clones and isolates. Denaturing gradient gel electrophoresis band sequences were added by parsimony onto the tree. Quartet puzzling support values for each internal branch are shown at the branch nodes. The sequence of *Salinibacter ruber* was used as the outgroup. The horizontal scale bar represents the number of substitutions per site.

FISH analysis

FISH analysis of sample July W indicated that the prokaryotic community was dominated by *Archaea* (58% of the total counts, three-fourths of the community detectable by FISH), although *Bacteria* were also present in high proportions (21.7% of the total counts). DAPI counts of the Tuz Lake samples were 1.38×10^7 cells mL^{-1}. Square cells and curved rods, the characteristic morphologies of *H. walsbyi* and *S. ruber*, respectively (Antón *et al.*, 1999, 2000; Maturrano *et al.*, 2006), were observed in the samples.

Isolation of microorganisms

In an attempt to isolate the new phylotypes of *Salinibacter* found in the clone library, a water sample was used to inoculate the medium that had been used previously for the isolation of *S. ruber* (Antón *et al.*, 2000). Out of the 269 colonies obtained, eight were *Bacteria* and 261 were *Archaea*. All the *Bacteria* isolates had the same ARDRA pattern that, as revealed by sequencing of two representatives (7-45 and 7-46 in Fig. 2), corresponded to *S. ruber* EHB-1. Twenty-nine archaeal colonies were randomly chosen and analyzed by ARDRA, which yielded two different patterns. The most frequent pattern (25 out of the 29 analyzed colonies) was represented by sequence 8-49 (see Fig. 3) and was very closely related (99% identity) to *Haloarcula hispanica* strain HLR4, isolated from a salt mountain in Taiwan (M. Lai & Y. You, unpublished data). The other ARDRA pattern was represented by sequence 8-38 (Fig. 3), which was found to be related (97%) to archaeon 4R (M. Aponte *et al.*, unpublished data), isolated from salted anchovies in Salerno, Italy. The closest cultured relative was '*Halorubrum californiensis*' (P.T. Pesenti *et al.*, unpublished data), with 92% nucleotide identity in the 16S rRNA gene.

Comparison of clones, isolates, and DGGE patterns

The phylogenetic affiliations of clones and isolates were studied by inferring trees for *Archaea* and *Bacteria* (Figs 2 and 3, respectively), using only almost complete 16S rRNA gene sequences. DGGE band sequences were added to the trees by parsimony.

For *Bacteria*, clone library analysis gave a larger picture of diversity than DGGE because all the DGGE bands had their counterparts in the library, while many clones were not related to DGGE bands. The culture approach we used did not allow for the isolation of the more abundant members of

© 2008 Federation of European Microbiological Societies
Published by Blackwell Publishing Ltd. All rights reserved

Downloaded from https://academic.oup.com/femsec/article/65/3/474/519295 by Anadolu University user on 16 December 2022

Fig. 4. Comparison by maximum likelihood analysis of the complete 16S rRNA gene sequences from clones and isolates belonging to the phylum *Bacteroidetes* retrieved from the following hypersaline and non hypersaline environments: (i) Hypersaline (labeled as H in the tree): Salar de Atacama (Chile), Lake Wadi An Natrun (Egypt), Lake Tuz (Turkey, in bold), Lake Mono (CA, USA), Maras salterns in the Peruvian Andes, coastal solar salterns from Spain, endoevaporitic microbial mats from Eilat (Israel), and endoevaporitic salt crusts from California (USA) and New Mexico (USA); (ii) Nonhypersaline: freshwater lakes, deep-sea, hydrothermal vent sediments, seawater, soils, Antarctic lakes and sediments, contaminated aquifers, abdominal setal tuft from a decapode, human oral cavity, industrial biofilters, and bioreactors. Bootstrap values for each internal branch are shown at the branch nodes. The sequence of the bacterium *Halomonas halodenitrificans* was used as the outgroup. The horizontal scale bar represents the number of substitutions per site.

the community detected by cloning since only some phylotypes of *S. ruber* related to cultured bacteria could be retrieved. Although, according to the molecular data, *Salinibacter* spp. appear to be an important component of the bacterial assemblage, the isolates we obtained were not very closely related to the clones and DGGE sequences. Also, a 'Tuz Lake *Salinibacter* spp. group' was found to be clearly different from the previously *Salinibacter* described phylotypes EHB-1 and EHB-2. Members of this group could not be retrieved by cultivation from the Tuz lake water samples. This Tuz Lake cluster is separated from the EHB-2 group with a puzzle quartet value of 97%, higher than the value that supports the separation between clusters EHB-1 and EHB-2. This group presents the following signature sequence in positions 1015–1030 (*E. coli* numbering): 5′-TCCGCAAGGACCCC-3′.

Both molecular approaches revealed that haloarchaea belonging to the SHOW (Square Haloarchaea of Walsby) were a very important part of the microbial community. DGGE bands 4, 7, and 8 were identical or almost identical (above 99% in the complete length of the DGGE band, positions 436–801 in the clones) to clones 1-13A, 3-3A, 3-09, and 3-46A. The other DGGE bands, affiliating within the *Halorubrum* sp. cluster, did not have matches with the analyzed archaeal clones. However, there were some sequences (1-39A and 3-07A, corresponding to 20% of the clones) that represented new archaeal groups and did not have counterparts in the DGGE analysis. The archaeal

© 2008 Federation of European Microbiological Societies
Published by Blackwell Publishing Ltd. All rights reserved

Downloaded from https://academic.oup.com/femsec/article/65/3/474/519295 by Anadolu University user on 16 December 2022

diversity recovered by plating on 25% total salts 0.1% yeast extract plates was, as expected, quite low, although we were able to culture a completely new archaeon, corresponding to sequence 8-49 (Fig. 3), whose phenotype is presently being characterized.

Discussion

The composition of the Tuz Lake microbiota resembles in many aspects that of crystallizers from coastal solar salterns both in the abundance and the diversity of prokaryotes. The total counts in Tuz Lake are within the range reported for this kind of environments (Elevi Bardavid *et al.*, 2008). The proportion of *Bacteria* in Tuz Lake water is one of the highest reported for hypersaline environments (although there are only very few studies providing these kind of data: Antón *et al.*, 2000; Rosselló-Mora *et al.*, 2003; Maturrano *et al.*, 2006).

DGGE and 16S rRNA gene libraries indicate that *Haloquadratum* spp. and members of the *Bacteroidetes* (most of them related to *Salinibacter*) are presumably the main components of the microbiota in Tuz Lake, as reported in some crystallizers (Elevi Bardavid *et al.*, 2008). The presence of archaeal phylotypes related to sequences recently retrieved from an Australian crystallizer (Burns *et al.*, 2004) or Adriatic salterns is also noteworthy (Pašic *et al.*, 2005). The tolerance of *H. walsbyi* to high solar irradiance and extremely high magnesium concentrations (Bolhuis *et al.*, 2006) has been used to explain the worldwide success of this archaeon. Indeed, representatives of this phylum have been found in hypersaline environments of different characteristics around the world including Andean salterns as well as Australian and Mediterranean coastal salterns, and now an inland hypersaline environment in Western Asia.

Members of the *Bacteroidetes* are a very important part of the bacterial community in Tuz Lake. Although not present in all the salterns studied around the world (Maturrano *et al.*, 2006), members of this phylum have been found in coastal salterns (Antón *et al.*, 2000; Benlloch *et al.*, 2002), in the athalassohaline Lake Chaka (32.5% salinity) in Northwestern China (Jiang *et al.*, 2006), as well as in athalassohaline lakes from Los Andes with a wide range of salt concentrations and compositions (Demergasso *et al.*, 2004) and in an evaporite crust on the bottom of salt pools at the Badwater site in Death Valley, CA (Elevi Bardavid *et al.*, 2007). As these authors pointed out, 'hypersaline waters (both thalassohaline and athalassohaline) apparently constitute an important environment' for *Bacteroidetes*. In order to ascertain whether there is a group of *Bacteroidetes* phylotypes associated with hypersaline environments, we constructed phylogenetic trees including all the sequences retrieved from these kind of systems but did not find a unique cluster exclusively formed by 'halophilic' sequences (Fig. 4). However, a group of sequences retrieved only from extreme environments was found; this group included all the *Salinibacter* sequences as well as sequences from deep sea and from the hypersaline alkaline Mono Lake and could be considered as a monophyletic halophilic branch within the *Bacteroidetes* phylum.

Acknowledgements

This work was supported by grant CGL2006-12714-C02-01 (Spanish Ministry of Science and Education) and Anadolu University Research Foundation No. 041016. M.B. was a student of the EEC Erasmus program.

References

Altschul SF, Gish W, Miller W, Myers EW & Lipman DJ (1990) Basic local alignment search tool. *J Mol Biol* **215**: 3389–3402.

Amann RI, Binder BJ, Olson RJ, Chisholm SW, Devereux R & Stahl DA (1990) Combination of 16S rRNA-targeted oligonucleotide probes with flow cytometry for analyzing mixed microbial populations. *Appl Environ Microbiol* **56**: 1919–1925.

Antón J, Llobet-Brossa E, Rodríguez-Valera F & Amann R (1999) Fluorescence *in situ* hybridization analysis of the prokaryotic community inhabiting crystallizer ponds. *Environ Microbiol* **1**: 517–523.

Antón J, Rosselló-Mora R, Rodríguez-Valera F & Amann R (2000) Extremely halophilic *Bacteria* in crystallizer ponds from solar salterns. *Appl Environ Microbiol* **66**: 3052–3057.

Ashelford KE, Chuzhanova NA, Fry JC, Jones AJ & Weightman AJ (2005) At least 1 in 20 16S rRNA sequence records current held in public repositories is estimated to contain substantial anomalies. *Appl Environ Microbiol* **71**: 7724–7736.

Ashelford KE, Chuzhanova NA, Fry JC, Jones AJ & Weightman AJ (2006) New screening software shows that most recent large 16S rRNA gene clone libraries contain chimeras. *Appl Environ Microbiol* **72**: 5734–5741.

Benlloch S, López-López A, Øvreås L *et al.* (2002) Prokaryotic genetic diversity throughout the salinity gradient of a coastal solar saltern. *Environ Microbiol* **4**: 349–360.

Birbir M & Sesal C (2003) Extremely halophilic bacterial communities in Şereflikoçhisar Salt Lake in Turkey. *Turk J Biol* **27**: 7–22.

Birbir M, Calli B, Mertoglu B, Elevi Bardavid R, Oren A, Ogmen MN & Ogan A (2007) Extremely halophilic Archaea from Tuz Lake, Turkey, and the adjacent Kaldirim and Kayacik salterns. *World J Microbiol Biotechnol* **23**: 309–316.

Bolhuis H, Palm P, Wende A, Falb M, Rampp M, Rodríguez-Valera F, Pfeiffer F & Oesterhelt D (2006) The genome of the square archaeon *Haloquadratum walsbyi*: life at the limits of water activity. *BMC Genom* **7**: 169.

Bowman JP, McCammon SA, Rea SM & McMeekin TA (2000) The microbial composition of three limnological disparate hypersaline Antarctic lakes. *FEMS Microbiol Lett* **183**: 81–88.

© 2008 Federation of European Microbiological Societies
Published by Blackwell Publishing Ltd. All rights reserved

Downloaded from https://academic.oup.com/femsec/article/65/3/474/519295 by Anadolu University user on 16 December 2022

Burns DG, Camakaris HM, Janssen PH & Dyall-Smith ML (2004) Combined use of cultivation-dependent and cultivation independent methods indicates that members of most haloarchaeal groups in an Australian crystallizer pond are cultivable. *Appl Environ Microbiol* **70**: 5258–5265.

Burns DG, Janssen PH, Itoh T, Kamekura M, Li Z, Jensen G, Rodríguez-Valera F, Bolhuis H & Dyall-Smith ML (2007) *Haloquadratum walsbyi* gen. nov., sp. nov., the square archaeon of Walsby, isolated from saltern crystallizers in Australia and Spain. *Int J Syst Evol Microbiol* **57**: 387–392.

Çamur MZ & Mutlu H (1995) Tuz Gölündeki mineral çökeliminin termodinamik değerlendirimi. *Geolog Bull Turk* **38**: 67–73.

DeLong E (1992) *Archaea* in costal marine environments. *Proc Natl Acad Sci USA* **89**: 5685–5689.

Demergasso C, Casamayor EO, Chong G, Galleguillos P, Escudero L & Pedrós-Alió C (2004) Distribution of prokaryotic genetic diversity in athalassohaline lakes of the Atacama Desert, northern Chile. *FEMS Microbiol Ecol* **48**: 57–69.

Dirik K & Erol O (2000) Tectonomorphologic evolution of Tuzgölü and surrounding area, central Anatolia-Turkey. Haymana-Tuzgölü-Ulukışla Basenleri Uygulamalı Çalışma (workshop), 9-11 Ekim 2000, Aksaray-Türkiye. *Turk Assoc Petroleum Geolog Special Publ* **5**: 27–45.

Elevi Bardavid R, Ionescu D, Oren A, Rainey FA, Hollen BJ, Bagaley DR, Small AM & McKay C (2007) Selective enrichment, isolation and molecular detection of *Salinibacter* and related extremely halophilic *Bacteria* from hypersaline environments. *Hydrobiologia* **576**: 3–13.

Elevi Bardavid R, Khristo P & Oren A (2008) Interrelations between *Dunaliella* and halophilic prokaryotes in saltern crystallyzer ponds. *Extremophiles* **12**: 5–14.

Grant S, Grant WD, Jones BE, Kato C & Li L (1999) Novel archaeal phylotypes from an East African alkaline saltern. *Extremophiles* **3**: 139–145.

Hiraishi A, Narihiro T & Yamanaka Y (2003) Microbial community dynamics during start-up operation of flowerpot-using fed batch reactors for composting of household biowaste. *Environ Microbiol* **5**: 765–776.

Jiang H, Dong H, Zhang G, Yu B, Chapman LR & Fields MW (2006) Microbial diversity in water and sediment of Lake Chaka, an athalassohaline lake in Northwestern China. *Appl Environ Microbiol* **72**: 3832–3845.

Lane DJ, Pace B, Olsen GJ, Stahl D, Sogin M & Pace NR (1985) Rapid determination of 16S ribosomal RNA sequences for phylogenetic analyses. *Proc Natl Acad Sci USA* **82**: 6955–6959.

Legault BA, López-López A, Alba-Casado JC, Doolittle WF, Bolhius H, Rodríguez-Valera F & Papke RT (2006) Environmental genomics of "*Haloquadratum walsbyi*" in a saltern crystallizer indicates a large pool of accesory genes in a otherwise coherent species. *BMC Genom* **4**: 171.

Ludwig W *et al.* (2004) ARB: a software environment for sequence data. *Nucleic Acids Res* **32**: 1363–1371.

Maturrano L, Santos F, Rosselló-Mora R & Antón J (2006) Microbial diversity in Maras salterns, a hypersaline environment in the Peruvian Andes. *Appl Environ Microbiol* **72**: 3887–3895.

Muyzer G, De Waal EC & Uitterrlinden AG (1993) Profiling in complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Appl Environ Microbiol* **59**: 695–700.

Oren A (2002) *Halophilic Microorganisms and their Environments*. Kluwer Academic Publishers, Dordrecht, the Netherlands.

Özcan B, Ozcengiz G, Coleri A & Cokmus C (2007) Diversity of halophilic archaea from six hypersaline environments in Turkey. *J Microbiol Biotechnol* **17**: 985–992.

Pašić L, Bartual SG, Ulrih NP, Grabnar M & Velikonja BH (2005) Diversity of halophilic archaea in the crystallizer of an Adriatic solar saltern. *FEMS Microbiol Ecol* **54**: 491–498.

Radax C, Gruber C & Stan-Lotter H (2001) Novel haloarchaeal 16S rRNA gene sequences from Alpine Permo-Triassic rock salt. *Extremophiles* **5**: 221–228.

Rosselló-Mora R, Lee N, Antón J & Wagner M (2003) Substrate uptake in extremely halophilic microbial communities revealed by microautoradiography and fluorescence *in situ* hybridization. *Extremophiles* **7**: 409–413.

Schmidt HA, Strimmer K, Vingron M & Von Haeseler A (2002) TREE-PUZZLE: maximum likelihood phylogenetic analysis using quartets and parallel computing. *Bioinformatics* **18**: 502–504.

Snaidr J, Amann R, Huber I, Ludwig W & Schleifer KH (1997) Phylogenetic analysis and *in situ* identification of bacteria in activated sludge. *Appl Environ Microbiol* **63**: 2884–2896.

Strimmer K & Von Haeseler A (1996) Quarter puzzling: a quarter maximum likelihood method for reconstructing tree topologies. *Mol Biol Evol* **13**: 964–969.

Uygun A & Şen E (1978) Tuz Gölü havzası ve doğal kaynakları. *Geolog Bull Turk* **21**: 113–120.

Vaneechoutte M, Rossau R, Vos PD, Gillis M, Janssens D, Paepe N, De Rouck A, Fiers T, Claeys G & Kersters K (1992) Rapid identification of bacteria of the *Comamonadaceae* with amplified ribosomal DNA-restriction analysis (ARDRA). *FEMS Microbiol Lett* **93**: 227–234.

Vreeland RH, Straight S, Krammes J, Dougherty K, Rosenzweig WD & Kamekura M (2002) *Halosimplex carlsbadense* gen. nov., sp. nov., a unique halophilic archaeon, with three 16S rRNA genes, that grows only in defined medium with glycerol and acetate or pyruvate. *Extremophiles* **6**: 445–452.

Wainø M, Tindall BJ & Ingvorsen K (2000) *Halorhabdus utahensis* gen nov., sp. nov., an aerobic, extremely halophilic member of the Archaea from Great Salt Lake, Utah. *Int J Syst Evol Microbiol* **50**: 183–190.

Walsh DA, Papke RT & Doolittle WF (2005) Archaeal diversity along a soil salinity gradient prone to disturbance. *Environ Microbiol* **7**: 1655–1666.

Zhilina TN, Miroshnikova LV, Osipov GA & Zavarzin GA (1992) *Halobacteroides lacunaris* sp. nov., new saccharolytic, anaerobic, extremely halophilic organism from the lagoon-like hypersaline lake Chokrak. *Mikrobiologiya* **60**: 714–724.

Downloaded from https://academic.oup.com/femsec/article/65/3/474/519295 by Anadolu University user on 16 December 2022

© 2008 Federation of European Microbiological Societies
Published by Blackwell Publishing Ltd. All rights reserved