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Microbial Diversity in Maras Salterns, a Hypersaline Environment in the Peruvian Andes

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Maras salterns are located 3,380 m above sea level in the Peruvian Andes. These salterns consist of more than 3,000 little ponds which are not interconnected and act as crystallizers where salt precipitates. These ponds are fed by hypersaline spring water rich in sodium and chloride. The microbiota inhabiting these salterns was examined by fluorescence in situ hybridization (FISH), 16S rRNA gene clone library analysis, and cultivation techniques. The total counts per milliliter in the ponds were around 2×10^6 to 3×10^6 cells/ml, while the spring water contained less than 100 cells/ml and did not yield any detectable FISH signal. The microbiota inhabiting the ponds was dominated (80 to 86% of the total counts) by *Archaea*, while *Bacteria* accounted for 10 to 13% of the 4′,6′-diamidino-2-phenylindole (DAPI) counts. A total of 239 16S rRNA gene clones were analyzed (132 *Archaea* clones and 107 *Bacteria* clones). According to the clone libraries, the archaeal assemblage was dominated by microorganisms related to the cosmopolitan square archaeon "*Haloquadra walsbyi*," although a substantial number of the sequences in the libraries (31% of the 16S rRNA gene archaeal clones) were related to *Halobacterium* sp., which is not normally found in clone libraries from solar salterns. All the bacterial clones were closely related to each other and to the γ-proteobacterium "*Pseudomonas halophila*" DSM 3050. FISH analysis with a probe specific for this bacterial assemblage revealed that it accounted for 69 to 76% of the total bacterial counts detected with a *Bacteria*-specific probe. When pond water was used to inoculate solid media containing 25% total salts, both extremely halophilic *Archaea* and *Bacteria* were isolated. Archaeal isolates were not related to the isolates in clone libraries, although several bacterial isolates were very closely related to the "*P. halophila*" cluster found in the libraries. As observed for other hypersaline environments, extremely halophilic bacteria that had ecological relevance seemed to be easier to culture than their archaeal counterparts.

The microbiota of hypersaline environments close to saturation has been studied by both culture and molecular methods. The diversity of this microbiota is very low (9, 30), although there is a considerable microdiversity, since the coexistence of several closely related clones of microorganisms has been detected in such environments (reference 7 and references therein).

Maras salterns are located over the Maras Formation in the Cusco Department (13°18′10″S, 72°09′21″W) in southern Peru at an altitude of 3,380 m in the Andes, and they are 1,000 km from the coast. These salterns have been used for salt production since the time of the Incas. Salt is produced mostly during the dry season from May to November. The salterns consist of more than 3,000 small shallow ponds which are not interconnected, so there is no spatial salinity gradient as there is in multipond marine solar salterns (30). Each pond is filled with hypersaline water from a spring feeding the saltern and empties after salt precipitation, so the ponds act directly as crystallizers.

These salterns, along with many others that have similar characteristics in the Andes, are thought to be of marine origin (18, 21). The origin could be related to the presence in the Maras Formation of underground halite deposits dating to 110 million years ago. Geological studies (18) have indicated that millions of years ago, an ocean covered the central region of Peru. The formation of the Andes would have caused marine waters to remain inland and, by evaporation, form halite deposits. In addition, the presence of fossilized oysters in the central Andean region supports the hypothesis that this region was covered by marine waters (Boletín de la Organización de Estados Americanos [www.oei.es]).

Since there were no previous reports on the microbiota of this very peculiar hypersaline environment, we undertook this study to evaluate the microbial diversity of Maras salterns and to compare this diversity with that of other salterns. We characterized the prokaryotic community inhabiting the ponds by using a combination of microscopy, molecular methods (fluorescence in situ hybridization [FISH] and 16S rRNA gene clone library sequencing), and cultivation techniques. According to this polyphasic approach, this hypersaline environment, as expected, is dominated by haloarchaea and has low diversity, but it has some characteristics not previously found in other hypersaline environments.

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MATERIALS AND METHODS

Sample collection. Samples (500 ml) of the emergent water (sample 1) and water from three different crystallizer ponds (samples 2, 5, and 7) were taken in July 2002. The total salt concentration of each sample was determined in situ with a hand refractometer (Sper Scientific). Two more samples of the emergent

water were taken in October 2003 and February 2004 in order to attempt DNA extraction. Portions of the samples were sent to a commercial water chemistry laboratory for analysis.

DAPI counts and fluorescence in situ hybridization. Sample fixation was carried out as previously described (4), using the protocol optimized for fixation of extremely halophilic microorganisms. Hybridization, 4′,6′-diamidino-2-phenylindole (DAPI) staining, and microscopy were carried out as described previously (35). For every sample, one filter was analyzed, and at least 700 cells were counted. Probe Ph659 (5′-ATTCCACTGCCCTCTGCT-3′) was specifically designed for "*Pseudomonas halophila*"-related sequences (see below) using the probe design tool in the ARB software package (http://www.arb-home.de). The rest of the probes (Arc915 for *Archaea*, EUB338 for *Bacteria*, NON338 for nonspecific hybridization, and EHB586 and EHB1451 for *Salinibacter*) have been described elsewhere (2, 3, 5). Formamide concentrations (0 to 80%) in the hybridization buffer were assayed for probe Ph659 in order to determine the optimum hybridization conditions that resulted in both sufficient specificity and good sensitivity. Cultures of isolate 7Sm5 (see below) were used for probe optimization.

Nucleic acid extraction and purification. For nucleic acid extraction, a combination of the methods of Cifuentes et al. (12) and Nogales et al. (25) was used. Microorganisms were collected by filtration on a 0.22-μm-pore-size GTTP filter (Durapore; Millipore) and washed with 2 ml of cold AE buffer (20 mM sodium acetate [pH 5.5], 1 mM EDTA) in a 15-ml polypropylene tube. Five milliliters of phenol-chloroform-isoamyl alcohol (25:24:1) (PCI) prewarmed at 60°C and 150 μl of 10% sodium dodecyl sulfate were added, and the mixture was incubated at 60°C for 5 min with vortexing every minute. After cooling on ice, the tube was centrifuged at 4,500 rpm for 5 min at 4°C. The aqueous phase was transferred to a new tube, to which 200 μl of 3 M sodium acetate (pH 5.2) and 5 ml of PCI were added, and the contents were vortexed and centrifuged as described above. Removal of the aqueous phase, addition of sodium acetate and PCI, and centrifugation were repeated two or three times until a clear interphase between the aqueous and organic phases was observed. Finally, nucleic acids were precipitated with ethanol and resuspended in 100 μl of sterile deionized water. Crude nucleic acid extracts were purified with a Geneclean II kit (Bio 101) used according to the manufacturer's protocol. To check the quality of the extracted nucleic acids, they were subjected to electrophoresis on 1% agarose (LE; FMC Bioproducts) gels and visualized under UV light after ethidium bromide staining.

PCR amplification of 16S and 18S rRNA genes. 16S rRNA genes were PCR amplified from total DNA using universal primers for *Bacteria* and *Archaea*. The reaction mixtures (total volume, 50 μl) contained 1.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl (pH 9.0), each deoxyribonucleotide triphosphate (dATP, dCTP, dGTP, and dTTP) (Invitrogen) at a concentration of 200 μM, 1 U of TacI DNA polymerase (Invitrogen), each oligonucleotide primer at a concentration of 0.2 mM, and 50 ng of template DNA. The sequences of the forward primers were 5′-TTCCGGTTGATCCTGCCGGA-3′ (13) for the *Archaea* and 5′-AGAGTT TGATCATGGCTCAG-3′ (19) for the *Bacteria*. The sequence of the reverse primer for both domains was 5′-GGTTACCTTGTTACGACTT-3′ (19). The following conditions were used for amplification: one 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, and then an extension step for 7 min at 72°C. Negative controls to which no template DNA was added were included. Five-microliter portions of PCR products were loaded onto 1% agarose gels (LE; FMC Bioproducts) in 1× Tris-acetic acid-EDTA buffer, stained, and visualized as described above. PCR products obtained from every environmental sample were compared by amplified rRNA gene restriction analysis (ARDRA) as described below. Prior to cloning, a low-cycle-number reamplification of a 10-fold-diluted mixed-template PCR product was performed with a fresh reaction mixture as described by Thompsom et al. (39) to eliminate heteroduplexes from multitemplate PCR products.

18S rRNA genes were PCR amplified from total DNA using universal primers for *Eukarya.* The sequences of the forward and reverse primers were 5′-AACC TGGTTGATCCTGCCAGT-3′ and 5′-TGATCCTTCTGCAGGTTCACCTAC-3′, respectively (13). The following conditions were used for amplification: one cycle of 95°C for 5 min, 30 cycles of 95°C for 1 min, 60°C for 1 min, and 72°C for 1 min, and then an extension step for 10 min at 72°C.

Purification and cloning of the PCR products. Total PCR products were electrophoresed on a 2% Metaphor agarose (FMC Bioproducts) gel. Bands that were the appropriate size (1,500 bp) were sliced from the gel and purified with a Geneclean II kit (Bio 101) used according to the manufacturer's protocol. The purified PCR products were then electrophoresed on a 1% LE agarose gel (FMC Bioproducts) to check their quality. Ligation of the PCR products with the pCR2.1 vector, transformation of *Escherichia coli* TOP10F′, and selection of the transformants were carried out with a TOPO TA cloning kit (Invitrogen) used according to the manufacturer's protocol. Two 16S rRNA gene libraries (one library for *Archaea* and one library for *Bacteria*) were generated for each sample (samples 2, 5, and 7). Each library was generated with the pooled products of at least five independent PCRs.

TABLE 1. Ionic composition and pH of the Maras salterns[a]

Parameter	Sample			
	1	2	5	7
Concn (g/liter)				
Na^+	116.40	121.10	118.80	139.30
Mg^{2+}	0.76	1.00	0.78	0.79
Ca^{2+}	1.72	1.13	1.29	1.33
K^+	0.30	0.37	0.30	0.30
Mn^{2+}	<0.005	<0.005	<0.005	<0.005
Cl^-	157.89	186.30	174.20	177.90
SO_4^{2-}	4.57	4.89	4.56	4.59
HCO_3^-	0.03	0.04	0.03	0.01
CO_3^{2-}	<0.005	<0.005	<0.005	<0.005
Salinity (%)[b]	25	30	31	30
pH	7.0	6.5	7.0	7.0

[a] The relative errors for the measurements ranged from 12 to 14% for all the ions analyzed.
[b] Measured in situ with a hand refractometer.

Analysis of libraries and clone selection. Clones were screened for redundancies by ARDRA (40) with the enzymes HinfI and MboI (22). Enzymatic digestion was performed by incubating 10 μl of the amplified insert with 5 U of enzyme and the corresponding enzyme buffer. The digestion products were analyzed in 2% agarose gels (LE; FMC Bioproducts) in 0.5× Tris-boric acid-EDTA buffer, stained, and visualized as described above. Two clones were selected from each restriction profile for sequencing.

Sequencing and sequence analysis. The nucleotide sequences of the cloned products were determined from plasmid preparations (Wizard Plus SV Minipreps DNA purification system; Promega). Cloned products and purified PCR products were sequenced using a Big Dye terminator cycle sequencing kit and an ABI PRISM TM 310 DNA sequencer (Applied Biosystems). 16S rRNA gene sequences were initially compared with reference sequences at NCBI (http://www.ncbi.nlm.nih.gov) using BLAST (1). Cloned sequences were checked for possible chimeric structure using the program Chimera_Check at the Ribosome Database Project website (http://rdp.cme.msu.edu/html). The correlation between the primary sequence and the secondary structure was analyzed using the ARB sequence editor (see below). Complete and nearly complete sequences of new clones and isolates obtained in this study were added to an alignment of more than 50,000 primary aligned gene structures available at the website http://db-central.arb-home.de/. New sequences were aligned by using the aligning tool of the ARB program package (20; http://www.arb-home.de). The alignment was based on 16S rRNA primary and secondary structures, which improved the recognition of homologous positions. Sequences not included in the ARB database were added from the GenBank database. These sequences were those of *Halovibrio denitrificans* (accession no. DQ072718), *Halospina denitrificans* (DQ072719), *Pseudomonas* sp. strain GSP65 (AY553126), *Pseudomonas* sp. strain GSP66 (AY553124), and "*Haloquadra walsbyi*" (AY676200). Phylogenetic reconstruction was performed by using the three algorithms as implemented in the ARB package (20) and also by using the Phyml program package (16; http://atgc.lirmm.fr/phyml/). Neighbor-joining, maximum parsimony, and maximum likelihood analyses were performed with ARB by using different data sets and, alternatively, by using masks that removed very frequent gaps, as implemented in the same database. Maximum likelihood analysis with the Phyml program was performed with the data sets shown in Fig. 2 and 3. After the different reconstructions obtained were compared, a consensus tree was drawn, which showed the positions that could not be unambiguously resolved as multifurcations. Bootstrap analysis was performed by using the Phyml program, and the resulting values were based on analysis of 100 replicates for the same database. Bootstrap values below 35% are not shown.

Isolation of microorganisms. Water samples (20 and 50 μl) were plated in duplicate onto six different media: 25% seawater basal salts (32) with 0.1, 0.2, and 0.5% yeast extract (Sb, Sm, and Sa, respectively) and 25% MS salts (0.128 g/liter NaBr, 0.14 g/liter $NaHCO_3$, 0.95 g/liter KCl, 3.32 g/liter $CaCl_2$, 234.0 g/liter NaCl; pH 7.2) (23) with 0.1, 0.2, and 0.5% yeast extract (Mb, Mm, and Ma, respectively). The samples were incubated at 37°C for 4 weeks. Selected colonies

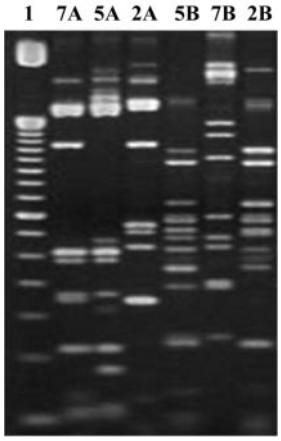

FIG. 1. ARDRA of the prokaryotic community inhabiting Maras salterns: HinfI restriction products of the 16S rRNA gene PCR amplified from DNA isolated from environmental samples using archaeal (lanes 7A, 5A, and 2A) and bacterial (lanes 5B, 7B, and 2B) primers. Lane 1 contained a 1-kb size standard.

were analyzed by 16S rRNA gene PCR amplification, ARDRA, and sequencing as described above for 16S rRNA gene clones.

Nucleotide sequence accession numbers. The 16S rRNA gene sequences determined in this study have been deposited in the GenBank database under accession numbers DQ019934, DQ019935, and AY987818 to AY987851.

RESULTS

Chemical characterization of water samples. The salinities of the samples, as determined in situ with a hand refractometer, were 25% for the emergent water feeding the crystallizer ponds and 30, 31, and 30% for the three ponds (samples 2, 5, and 7, respectively). Chemical analysis of these four water samples indicated (Table 1) that Na^+ and Cl^- were the most abundant ions, followed by SO_4^{2-}, which was present in much smaller amounts.

***Bacteria* and *Archaea* 16S rRNA gene clone libraries.** DNA was extracted from the four samples and PCR amplified with specific 16S and 18S rRNA gene primers for *Bacteria*, *Archaea*, and *Eukarya*. No PCR products were obtained with either set of primers from sample 1, although up to 500 ml of water was used for DNA extraction. For the rest of the samples, products were obtained only with the *Archaea* and *Bacteria* primer sets, and the products were compared by ARDRA in order to evaluate whether the communities in the three crystallizer

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

Gene library	No. of clones analyzed	ARDRA		Selected clone(s)	Closest GenBank match		
		Pattern[a]	No. of clones		Taxon	Accession no.	% Identity
2A	40	Iac	14	2A5	"*Haloquadra walsbyi*"	AY676200	98
		IIac	10				
		IIIac	2				
		Vac	8	2A6	*Halobacterium salinarum*	AJ496185	97
		VIac	6	2A1, 2A8	*Halobacterium salinarum*	AJ496185	97
2B	22	IIIbc	8	2B1	"*Pseudomonas halophila*"	AB021383	97
		Vbc	6	2B4	*Pseudomonas* sp. strain GSP65	AY553126	97
		VIIbc	8	2B12	*Pseudomonas* sp. strain GSP65	AY553126	97
5A	46	Iac	16	5A1	"*Haloquadra walsbyi*"	AY676200	98
		IIIac	13	5A7	"*Haloquadra walsbyi*"	AY676200	92
		IVac	10	5A2	*Halobacterium salinarum*	AJ496185	97
		Vac	7	5A9	*Halobacterium salinarum*	AJ496185	96
5B	39	Ibc	8				
		IIbc	5	5B1	*Pseudomonas* sp. strain GSP66	AY553124	97
		IIIbc	6				
		IVbc	6				
		Vbc	5				
		VIbc	9	5B16	*Pseudomonas* sp. strain GSP66	AY553124	99
7A	46	Iac	2				
		IIac	32	7A8, 7A11	"*Haloquadra walsbyi*"	AY676200	98
		IIIac	2				
		IVac	2				
		Vac	4		*Halobacterium salinarum*	AJ496185	97
		VIac	4	7A23	*Halobacterium salinarum*	AJ496185	97
7B	46	Ibc	23	7A16	*Pseudomonas* sp. strain GSP66	AY553124	99
		IIbc	7	7B10, 7B11	*Pseudomonas* sp. strain GSP66	AY553124	98
		IVbc	6	7B12	"*Pseudomonas halophila*"	AB021383	97
		VIbc	5	7B21	*Pseudomonas* sp. strain GSP66	AY553124	99
		VIIbc	5	7B13			

[a] The ARDRA pattern is indicated by a roman numeral followed by ac for archaeal clones and bc for bacterial clones.

TABLE 3. Archaeal and bacterial isolates

Domain	ARDRA		Selected isolate[b]	Closest GenBank match		
	Pattern[a]	No. of isolates		Taxon	Accession no.	% Identity
Archaea	Iai	8	2Sb1	*Haloarcula marismortui*	AY596297	98
	IIai	6	2Ma3	*Haloarcula marismortui*	AY596297	99
	IIIai	15	5Mm6	*Halobacterium salinarum*	AJ496185	96
	IVai	5	7Sb5	*Halorubrum trapanicum*	X82168	98
	Vai	8	5Mm10	*Haloarcula marismortui*	AY596297	98
	VIai	2	5Sa3	*Halogeometricum borinquense*	AF002984	97
	Total	44				
Bacteria	Ibi	4	2Sb7	*Pseudomonas* sp. strain GSP66	AY553124	99
	IIbi	4	7Sm5	*Pseudomonas* sp. strain GSP66	AY553124	99
	IIIbi	2	2Mb5	*Marinococcus halophilus*	X90835	99
	IVbi	2	2Mb1	*Rhodospirillum salinarum*	D14432	94
	Vbi	4	2Mb2	*Salinibacter ruber*	AF323503	99
			5Sm6	*Salinibacter ruber*	AF323503	99
	Total	16				

[a] The ARDRA pattern is indicated by a roman numeral followed by ai for archaeal isolates and bi for bacterial isolates.
[b] The isolates are designated by a number indicating the pond of origin (sample 2, 5, or 7), two letters for the culture medium (Sb, Sm, Sa, Mb, Mm, or Ma), and a number for identification.

ponds were identical. As shown in Fig. 1, the patterns for either *Bacteria* and *Archaea* were different in the three ponds. Therefore, six 16S rRNA gene libraries were constructed, which were designated by numbers corresponding to the environmental samples (samples 2, 5, and 7), followed either by A (for archaeal 16S rRNA genes) or B (for bacterial 16S rRNA genes).

A total of 239 clones (132 *Archaea* clones and 107 *Bacteria* clones) were analyzed by ARDRA, which yielded a total of seven different patterns for *Bacteria* and six different patterns for *Archaea*. As shown in Table 2, all the ARDRA patterns were found in at least two of the three bacterial or archaeal libraries. In other words, no pond-specific pattern was found.

At least one clone per restriction pattern was chosen for complete sequencing. Table 2 shows the best matches with sequences in databases obtained by BLAST analysis of the selected clones. For 69% of the archaeal clones the closest match was with "*H. walsbyi*" (ARDRA patterns Iac, IIac, and IIIac), "the square archaeon" recently isolated by Burns et al. (10) and Bolhuis et al. (8) from Australian and Spanish salterns, respectively. The rest of the archaeal clones were related to *Halobacterium* sp. strain NRC-1. We found no significant differences among the archaeal communities in the three ponds analyzed, since all of them were dominated by "*H. walsbyi*"-related phylotypes (65, 63, and 78% of the archaeal clones for samples 2, 5, and 7, respectively) and contained lower concentrations of the *Halobacterium*-related sequences (35, 37, and 22%, respectively).

For all 107 bacterial clones analyzed the closest matches were with the cultured members of the gammaproteobacterial halophilic branch comprising strain DSM 3050 (15), *Pseudomonas* sp. strains GSP65 and GSP66 (11), and two newly classified species, *Halovibrio denitrificans* and *Halospina denitrificans* (36). Strain DSM 3050 was deposited as "*Pseudomonas halophila*" (15), but its phenotypic description may correspond to the original description of *Halovibrio variabilis* and thus this organism may be renamed (37). DSM 3050 was isolated from a sample from the North Arm of Great Salt Lake in Utah and grew optimally in the presence of about 10% NaCl (15, 37). Both GSP65 and GSP66 were isolated from samples from the great salt plains of Oklahoma, and these strains were recovered together with a collection of 105 isolates belonging to a wide variety of taxa, indicating that their abundance in the cultivable fraction of their community is relatively low (11). Finally, the recently classified species *Halovibrio denitrificans* and *Halospina denitrificans* (36), both of which grow optimally in the presence of 11 to 17% NaCl, have been isolated from sediments of hypersaline lakes in central Asia.

Isolation of *Bacteria* and *Archaea*. Colonies were obtained only from samples 2, 5, and 7. The six different media assayed yielded similar numbers of CFU/ml, and the range was 7.5×10^3 to 9.5×10^3 CFU/ml (i.e., around 0.2 to 0.5% of the DAPI counts [see below]). Thus, neither salt composition nor yeast extract concentration had any effect on the number of CFU/ml under the assay conditions used. Sixty colonies were selected on the basis of their morphological characteristics (size, shape, and color) and were characterized by 16S rRNA gene analysis. Forty-four of these colonies were *Archaea* colonies, and 16 were *Bacteria* colonies (Table 3). Selected archaeal isolates were closely related to previously cultured haloarchaea, such as *Haloarcula marismortui*, *Halobacterium salinarum*, *Halorubrum trapanicum*, and *Halogeometricum borinquense*. For one-half of the bacterial strains the best match was with *Pseudomonas* sp. strain GSP66. The rest of the strains were related to *Marinococcus halophilus*, *Rhodovibrio salinarum*, and *Salinibacter ruber*, whose sequences were not found in the clone libraries. Interestingly (data not shown), all the bacterial isolates were extreme halophiles that were able to grow in the presence of 10 to 30% total salts and had salt optima that were higher than those of the reference strains, which are considered moderately halophilic (31).

Comparison of clones and isolates. The phylogenetic affiliations of clones and isolates were studied by inferring independent trees for the *Archaea* and *Bacteria* representatives (Fig. 2 and 3, respectively). To infer each phylogeny, only

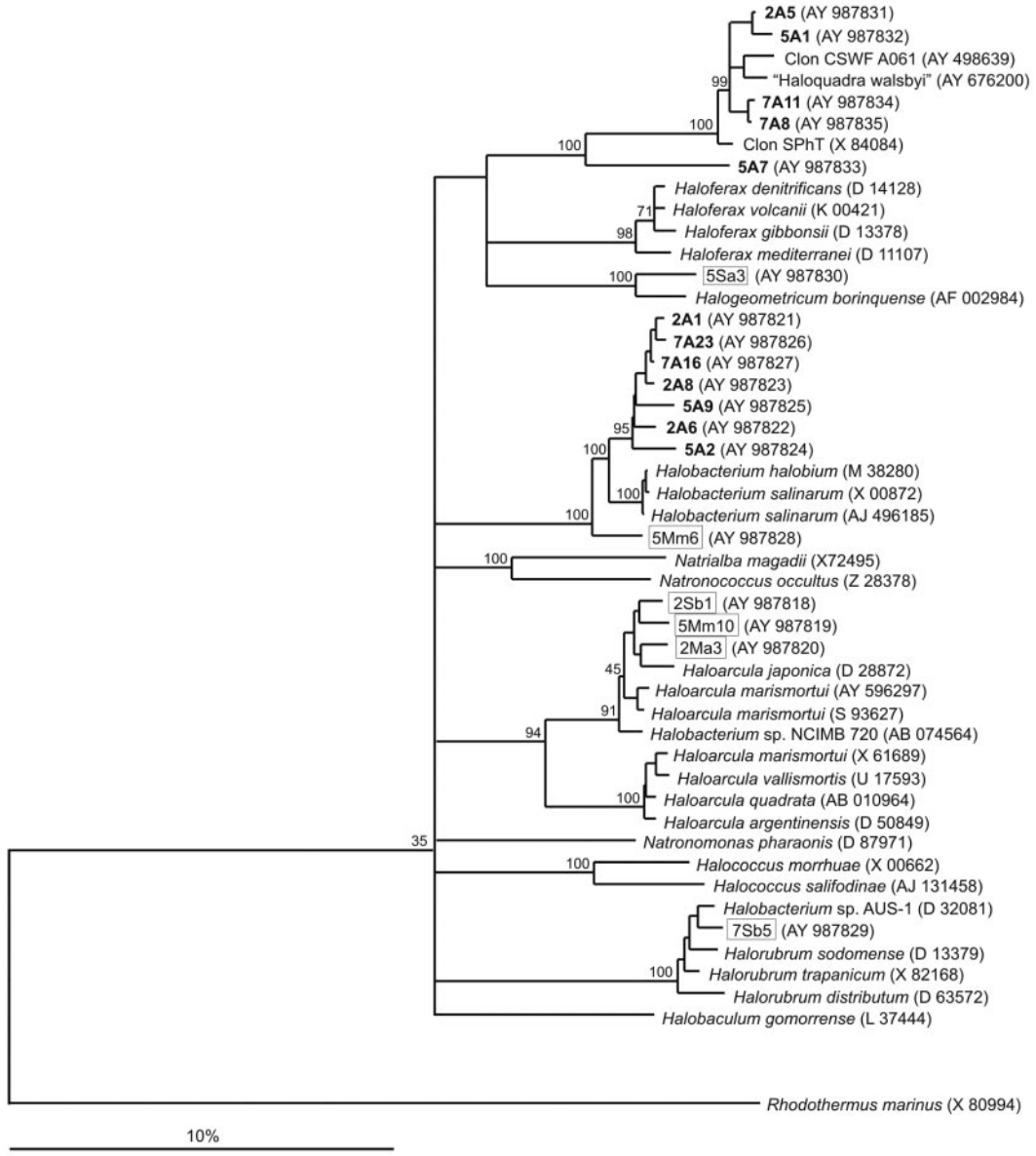

FIG. 2. Phylogenetic inferences based on 16S rRNA gene sequences from strains (enclosed in boxes) and clones (indicated by boldface type) belonging to the halophilic *Archaea*. The sequence of *Rhodothermus marinus* was used as the outgroup. The tree is based on the results of neighbor-joining inferences for only complete or nearly complete sequences. The tree topology was corrected based on the results of additional maximum parsimony and maximum likelihood analyses. Bootstrap values were obtained after maximum likelihood analysis by using the Phyml program (16). The values at the nodes are the highest bootstrap values observed for pairwise branching orders. For branches without bootstrap values the values were less than 35%. Bar = 10% estimated sequence divergence. The numbers in parentheses are accession numbers.

almost complete sequences were used, and the trees were the result of evaluation of multiple trees with different data sets and different algorithms. Multifurcations were included for the branching patterns that could not be resolved.

For both *Archaea* and *Bacteria*, we found more diversity among the isolates than among the clones, an observation that has been made previously for hypersaline environments (6). However, we were able to culture the representatives of the group that was most frequently retrieved in the clone libraries only for *Bacteria*.

Archaeal 16S rRNA gene clones related to "*H. walsbyi*" (2A5, 5A1, 7A11, and 7A8) grouped together with levels of similarity of more than 98.26%. Clones associated with *Halobacterium* sp. also formed a very homogeneous phylogenetic group, with levels of similarity of more than 97.91%. Isolate 5Mm5, although not a member of the clone clade, had levels of similarity of 96.94 to 97.43% with the clones related to *Halobacterium* sp.

All the sequences (either from clones or isolates) related to *Pseudomonas* sp. strains GSP65 and GSP66 or "*P. halophila*" DSM 3050 exhibited more than 95.8% similarity and formed two different clusters; 7B10, 7B11, 5B16, 7B13, 2Sb7, 7Sm5,

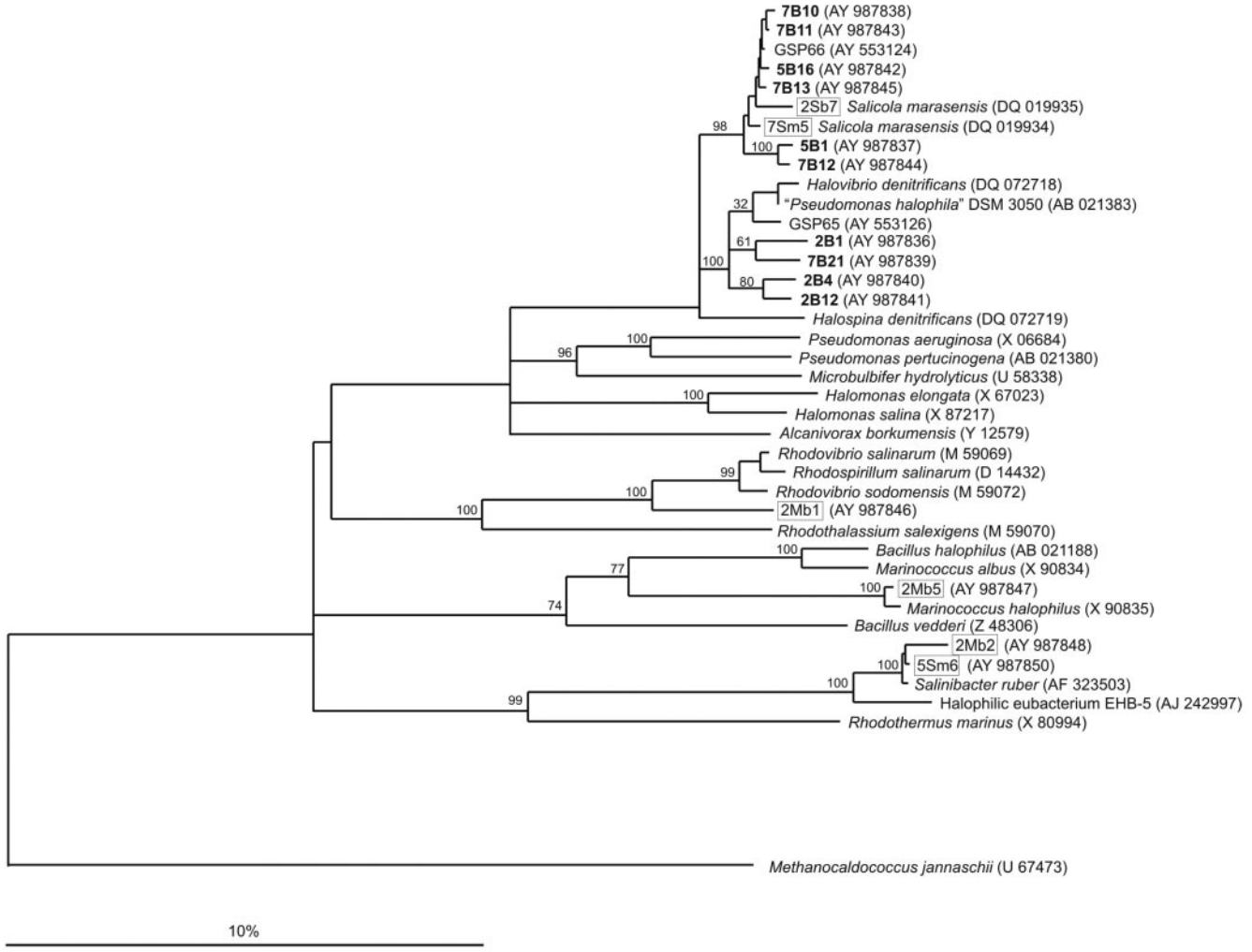

FIG. 3. Phylogenetic inferences based on 16S rRNA gene sequences from strains (enclosed in boxes) and clones (indicated by boldface type) belonging to the halophilic *Gamma-* and *Alphaproteobacteria*, *Firmicutes*, and *Bacteroidetes*/*Chlorobi*. The sequence of *Methanocaldococcus jannaschii* was used as the outgroup. The tree is based on the results of neighbor-joining inferences for only complete or nearly complete sequences. The tree topology was corrected based on the results of additional maximum parsimony and maximum likelihood analyses. Bootstrap values were obtained after maximum likelihood analysis by using the Phyml program (16). The values at the nodes are the highest bootstrap values observed for pairwise branching orders. For branches without bootstrap values, the values were less than 35%. Bar = 10% estimated sequence divergence. The numbers in parentheses are accession numbers.

5B1, and 7B12 were more than 97.99% similar to each other, and 2B1, 7B21, 2B4, and 2B12 were more than 96.31% similar to each other. Isolates belonging to this clade have recently been described as *Salicola marasensis* gen. nov., sp. nov. (24).

FISH analysis. The community was dominated by *Archaea* (68 to 77% of the total counts) (Table 4), although high percentages (10 to 13%) of *Bacteria* were also present. *Bacteria* detected with probe Ph659 in samples 2, 5, and 7 accounted for

TABLE 4. Quantification of *Bacteria*, *Archaea*, *Salinibacter ruber*, and "*Pseudomonas halophila*"-related bacteria in different samples of the Maras salterns

Sample	Salinity (%)	Total DAPI counts/ml (mean ± SD)	% EUB338	% Arc915	% EHB586/EHB1451	% Ph659	% of detected counts[b]
1	25	$9.2 \times 10 \pm 0.8$	ND[a]	ND	ND	ND	ND
2	30	$3.1 \times 10^6 \pm 0.5 \times 10^6$	12.9	77.4	ND	9.2	90.3
5	31	$2.5 \times 10^6 \pm 0.6 \times 10^6$	12.0	68.10	ND	8.3	80
7	30	$2.1 \times 10^6 \pm 0.6 \times 10^6$	10.0	76.19	ND	7.6	86.19

[a] ND, not detectable (less than 1%).
[b] Percentage for *Bacteria* plus *Archaea.*

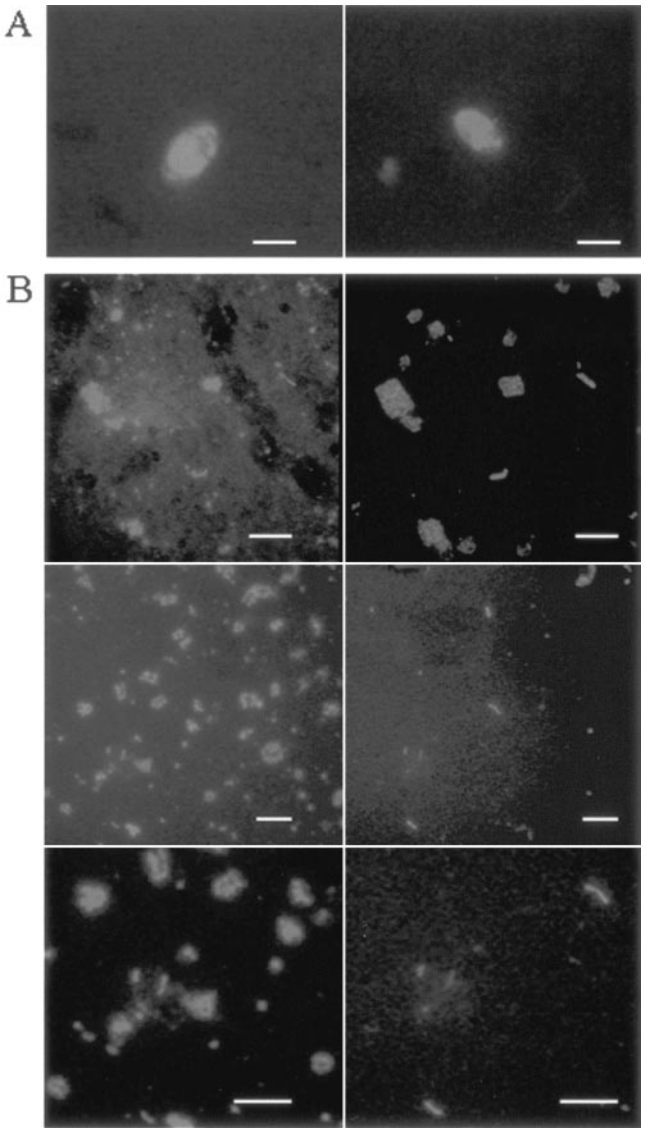

FIG. 4. Cells in the water feeding the salterns (A) and FISH of samples from crystallizer ponds (B). The left micrographs in panel B show DAPI staining, and the right micrographs show hybridization signals for the same fields obtained with probes Arc915 (top), EUB338 (middle), and Ph659 (bottom). Scale bars = 5 μm.

71, 69, and 76%, respectively, of the bacterial community. Thus, as indicated by the clone library analysis, the bacterial assemblage was dominated by microorganisms related to "*P. halophila*" DSM 3050. The concentration of microorganisms in the emergent water (sample 1) (Table 4) was very low (less than 100 cells/ml), and these microorganisms did not yield a FISH signal when they were hybridized with probes for *Bacteria* and *Archaea*. Figure 4A shows the morphology and dimensions of the cells observed in sample 1; these eukaryote-like cells were not observed in the rest of the samples analyzed. The DAPI counts for the crystallizer ponds (samples 2, 5, and 7) ranged from 2×10^6 to 3×10^6 cells/ml. As shown in Fig. 4B, archaeal square cells were conspicuous in the samples.

DISCUSSION

Hypersaline environments can be classified as thalassohaline and athalassohaline. Thalassohaline waters are "concentrated seawaters with NaCl as the major salt," while athalassohaline waters are "saline waters that are rich in anions other than chloride and/or cations other than sodium" (31). Therefore, on the basis of their salt compositions, Maras salterns are thalassohaline hypersaline environments. To the best of our knowledge, this is the first report on the microbiota inhabiting inland salterns of marine origin at such a high altitude, although athalassohaline lakes from the Andes have recently been studied by Demergasso et al. (14). These authors sampled lakes with a wide range of salt concentrations and compositions and analyzed their prokaryotic microbiota by denaturing gradient gel electrophoresis.

The total cell counts in the crystallizer ponds were around 1 order of magnitude lower than the total cell counts obtained for different solar salterns. Salterns in Eilat, Israel (27), Santa Pola, Spain (4, 5), and coastal Australia (9) contained 1.3×10^7, 1×10^7 to 5×10^7, and 1.2×10^7 cells/ml, respectively, while hypersaline environments in Delta del Ebro (Spain) harbored up to 10^8 cells/ml (17) and solar salterns in La Palma (Canary Islands, Spain) contained more than 10^8 cells/ml (26). These high numbers of cells in many hypersaline environments are thought to be a result of the lack of predation and high nutrient levels (30). The low levels of cells in Maras salterns compared to the levels in coastal salterns could be related to lower temperatures (temperatures are very rarely above 20°C in Maras) and/or to the organic matter content in the feeding water (as expected, much lower in spring water than in concentrated coastal marine waters).

The cell counts for the water feeding the salterns were remarkably low, as were the DNA contents. These findings, together with the fact that the only cells observed with a microscope had a eukaryote-like morphology, pose interesting questions concerning the origin of the prokaryotic microbiota inhabiting the salterns; the questions are even more interesting if we consider the fact that this microbiota includes species which are very widespread in marine salterns, as discussed below.

As previously observed in coastal salterns (4, 5, 34), the prokaryotic community of Maras salterns was dominated by *Archaea*, and the assemblage related to "*H. walsbyi*" was the assemblage that was most frequently retrieved in the clone libraries. "*H. walsbyi*" and closely related sequences have been isolated from widely separated hypersaline environments, such as western Europe, Australia, western Asia, and now South America (31, 9). However, Maras salterns also harbor a group of *Archaea* related to *Halobacterium* sp. which, although easily culturable (28, 30), are not normally detected in clone libraries from coastal salterns, as shown by studies of salterns in Israel (33), mainland Spain (5, 7), the Canary Islands (26), and Australia (9). However, sequences related to *Halobacterium* sp. were retrieved by Ochsenreiter et al. (26) when they analyzed a slag heap of a potassium mine in Germany.

As revealed by FISH analyses, *Bacteria* related to the γ-proteobacterium "*P. halophila*" DSM 3050 also constitute an important (around 10%) part of the prokaryotic community inhabiting Maras salterns. Marine salterns in Mallorca (34) and

Alicante (4, 5) also contain relatively high numbers of *Bacteria*, although in these cases *S. ruber* is the most abundant bacterium. In fact, although hypersaline waters seem to be an important environment for planktonic *Bacteroidetes* (14), we did not find sequences related to this phylum in the clone libraries, nor did we detect *S. ruber* with FISH specific probes (Table 4). In contrast, sequences related to "*P. halophila*" are not normally retrieved in 16S rRNA clone libraries from coastal salterns (7), although Souza et al. (38) found such sequences when they analyzed hypersaline environments with high selenium concentrations. Bacteria related to "*P. halophila*" have been cultured from solar salterns (7), and they seem to grow in coculture with the recently isolated square archaeon (8). Interestingly, both *S. ruber* and the extremely halophilic bacterium related to "*P. halophila*" (now validly described as *Salicola marasensis* [24]), the most abundant bacteria in their hypersaline environments, have been cultured very easily from environmental samples. This contrasts with the difficulty of culturing the most abundant archaeon in the same environments.

Conclusion. Thus, Maras salterns are similar to other hypersaline environments, such as crystallizer ponds in coastal solar salterns, in several respects: their microbial diversity is very low (overall, two groups of *Archaea* and one group of *Bacteria*), they are dominated by *Archaea*, although *Bacteria* account for around 10% of the community, and they harbor a very large population of square haloarchaea. On the other hand, there are also some features unique to this system, including the abundance of *Halobacterium*-related sequences in the 16S rRNA gene clone libraries and the presence in the community of a population of extremely halophilic *Bacteria* that so far have not been considered relevant in other hypersaline environments. Thus, even for such low-diversity and extreme systems, studies of new environments can provide unexpected findings. As anticipated by Oren and Rodríguez-Valera (29), crystallizer ponds at different geographical locations may indeed harbor microbial communities with different structures.

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