Haloarchaeal communities in the crystallizers of two adriatic solar salterns

Lejla Pašić, Nataša Poklar Ulrih, Miha Črnigoj, Miklavž Grabnar, and Blagajana Herzog Velikonja

Abstract: Solar salterns operate only for short dry periods of the year in the north shore of the Adriatic Sea because of its relatively humid and cold Mediterranean climate. In a previous paper, we showed that the NaCl precipitation ponds (crystallizers) of Northern Adriatic Sečovlje salterns have different haloarchaeal populations from those typically found in dry and hot climates such as Southern Spain. To check whether there is a common pattern of haloarchaeal diversity in these less extreme conditions, diversity in crystallizers of other Adriatic solar salterns in Ston, Croatia was ascertained by molecular and culture methods. In addition, the cultivation approach was used to further describe haloarchaeal diversity in both salterns. Over the period of two solar salt collection seasons, isolates related to species of the genera *Haloferax*, *Haloarcula*, and *Haloterrigena* were recovered from both salterns. Within the same sampling effort, relatives of the genus *Halorubrum* and a *Natrinema*-like isolate were cultivated from Slovenian Sečovlje salterns while *Halobacterium* related isolates were obtained from the Croatian Ston salterns. Concurrent with our previous findings, a library of Croatian saltern crystallizer PCR-amplified 16S rRNA genes was dominated by sequences related to the genus *Halorubrum*. The microbial community structure was similar in both salterns but diversity indices showed greater values in Slovenian salterns when compared with Croatian salterns.

Key words: 16S rRNA, Haloarchaea, saltern, hypersaline, halophiles.

Résumé : Les salines solaires ne fonctionnent que pendant de brèves périodes de temps sec dans l'année sur la rive nord de la mer Adriatique à cause de son climat méditerranéen relativement humide et froid. Dans un article précédent, nous avons démontré que les bassins de précipitation de NaCl (cristalliseurs) des salines de Sečovlje de l'Adriatique Nord renferment des populations haloarchéenes différentes de celles normalement retrouvées dans des climats chauds et secs tels que le sud de l'Espagne. Pour vérifier s'il existe une configuration commune à la diversité haloarchéene dans ces conditions moins extrêmes, nous avons décrit la diversité dans les cristalliseurs d'autres salines solaires adriatiques – les salines Ston en Croatie – par des méthodes moléculaires et de culture. De plus, l'approche de culture fut employée afin de décrire davantage la diversité haloarchéene des deux salines. Sur une période de deux saisons de collecte de sel solaire, des isolats apparentés à des espèces des genres *Haloferax*, *Haloarcula* et *Haloterrigena* ont été recueillis des deux salines. À l'issue d'un même échantillonnage, des parents du genre *Halorubrum* et un isolat semblable à *Natrinema* ont été cultivés à partir de la saline slovène de Sečovlje alors que des isolats apparentés à *Halobacterium* ont été obtenus des salines Croates de Ston. En conformité avec nos découvertes précédentes, la banque de gènes de ARNr 16S amplifiés par PCR du cristalliseur salin Croate était dominée par des séquences apparentées au genre *Halorubrum*. La structure de la communauté microbienne était semblable dans les deux salines mais les indices de diversité ont démontré des valeurs supérieures dans les salines slovènes comparativement aux salines croates.

Mots clés : ADNr 16S, haloarchéens, salines, hypersalines, halophiles.

[Traduit par la Rédaction]

Introduction

The saturated brines of a saltern crystallizer reach close to 40% total dissolved salts. Despite extreme conditions, such waters contain host organisms able to cope with the saturated NaCl concentration and remain highly productive systems with biomass density reaching over 10^8 cells/mL. Halophilic archaea of the family *Halobacteriaceae* dominate these populations, co-existing with some halophilic bacteria, fungi, and protists. The microbiota of solar salterns represents a precious natural resource in terms of microbial diversity (Anton et al. 2000; Gunde-Cimerman et al. 2000; Benlloch et al. 2002; Estrada et al. 2004; Yeon et al. 2005) and is involved in the quality of salt produced, saltern preservation, and management (Javor 2002). With solar salt manu-

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facturing processes well established worldwide it might be assumed that there are very minor, if any, differences in the microbial populations encountered.

In terms of biomass density, distinct solar saltern microbial populations have been reported from the Bahamas (Davis 1976), and both Mexico and California (Javor 1983). In all cases, observed differences were attributed to a difference in amounts of inorganic nutrients available. Furthermore, results of a 5 year study on solar salterns in Israel and California revealed different patterns when polar lipid and pigment profiles, metabolic potential, and PCR amplicon length heterogeneity were compared (Litchfield et al. 2000, 2002). While the solar saltern system in Israel showed greater consistency, the complexity of the Californian saltern was explained on the basis of prevailing weather conditions; a cooler climate with more rainfall.

In different arid areas of the world with a year-round solar salt production, haloarchaeal community structure was examined using libraries of PCR-amplified 16S rRNA genes. Crystallizers of solar salterns in Spain, Israel, and Australia were described as being endowed with low diversity. Overall microbial community structure was similar with the recently described '*Haloquadratum walsbyi*' dominating the population (Benlloch et al. 2001, 2002; Anton et al. 2002; Ochsenreiter et al. 2002; Burns et al. 2004).

In areas with a mild Mediterranean climate, solar salt production is often limited to the arid part of the year. In the Adriatic region, the arid season extends from May to October. The water retention time in the ponds is thus shortened and the time for microbial community development is limited. To ensure solar salt production, the ponds are unusually shallow, measuring not more than 10 cm in depth. In a previous study of haloarchaeal diversity in one such system, Sečovlje salterns in Slovenia, analysis of 16S rRNA and bacteriorhodopsin gene fragments obtained from environmental DNA samples indicated an unusually diverse haloarchaeal community (Pašić et al. 2005). For the present study, Sečovlje solar salterns were further investigated using a cultivation approach. Furthermore, another Adriatic solar saltern system was chosen for comparison. Located 600 km to the south of the Slovenian salterns, the haloarchaeal diversity in the crystallizers of Croatian Ston solar salterns was investigated by an analysis of a library of PCR-amplified 16S rRNA genes and by complementary cultivation methods. Phylogenetic analysis of the microbial component was used in an attempt to assess whether there is a common pattern of haloarchaeal diversity in these less extreme conditions.

Materials and methods

Salterns and biomass collection

Samples from the crystallizer ponds were collected aseptically from multipond solar salterns in Sečovlje (near Portorož), Slovenia (E 13°36′, N 45°28′) and Ston, Croatia (E 17°42′, N 42°49′) in August 2001 and August 2003.

We determined the following physicochemical parameters (Table 1): pH (ISO 10523: 1994E, electrometric method), temperature, and water activity (a_w) of the sample (CX-1 system; Campbell Scientific Ltd., Logan, Utah, USA). Concentrations of nitrate (NO_3^-), ammonium (NH_4^+), and phosphate (PO_4^{3-}) were analyzed using standard colorimetric procedures (Grasshoff et al. 1983).

Table 1. Physicochemical properties of Adriatic solar salterns water samples.

	Sečovlje		Ston	
	2001	2003[a]	2001	2003
pH	8.0	8.0	8.1	8.0
Temperature (°C)	32	30	35	35
Water activity (a_w)	0.798	0.759	0.772	0.727
Concn. (μmol/L) of:				
Nitrate (NO_3^-)	20.01	28.33	8.38	6.69
Ammonium (NH_4^+)	115.35	133.33	48.75	28.88
Phosphate (PO_4^{3-})	2.48	2.04	0.29	0.20
Cell counts (cells/mL)	3.0×10^6	3.1×10^6	1.6×10^6	1.7×10^6

[a]Data from Pašić et al. (2005).

Crystallizer pond water and haloarchaeal isolates were examined by phase-contrast and fluorescence microscopy under a Zetopan Binolux™ microscope (Reichert Inc., Reichert, Germany). For cell counts, cells were stained with acridine orange (Sigma-Aldrich Chemie, GmbH, Steinheim, Germany) and 20–30 microscopic fields were analyzed.

Culture media

A 25% (*m/v*) concentrated salt water (SW) stock solution was used for cultivation experiments. It was prepared by dissolving 200 g NaCl, 25 g $MgCl_2{\cdot}6H_2O$, 29.2 g $MgSO_4$, 5.84 g KCl, 0.66 g NaBr, 0.16 g $NaHCO_3$, and 0.41 g $CaCl_2{\cdot}2H_2O$ in 1 L of MilliQ water. For solid media, Difco Bacto agar (Difco, Detroit, Mich., USA) was added to a final concentration of 1.5% (*m/v*).

The substrates in the two minimal medium were 0.4 mmol/L $(NH_4)_2HPO_4$, 2.5 mL/L SL10 trace element solution (Widdel et al. 1983) and either (*i*) glycerol (0.5%) and succinate (0.05%) or (*ii*) glucose, galactose, and xylose (each 50 μmol/L). For SWYE medium, the SW stock solution was supplemented with 0.5% (*m/v*) yeast extract (Difco). Medium HM contained 1% (*m/v*) yeast extract (Difco), 0.5% (*m/v*) proteose peptone No. 3 (Difco), and 0.1% glucose. Finally, HS medium contained (all *m/v*) 12.5% NaCl, 16% $MgCl_2{\cdot}6H_2O$, 0.5% K_2SO_4, 0.01% $CaCl_2{\cdot}2H_2O$, 0.1% yeast extract (Difco), 0.1% proteose peptone No. 3 (Difco), and 0.2% soluble starch (Arahal et al. 1996). The pH was always adjusted to 7.5.

Aliquots of 100 μL of hypersaline samples were plated on all solid media. Another approach included plating 0.22 μm pore-size filters through which 25–150 mL of hypersaline sample was filtered and propagated in liquid media prior to plating. Plates were incubated in sealed plastic containers at 37 or 42 °C for up to 6 weeks. These were open weekly for colony counts.

Isolate diversity was evaluated using phenotypic characterization. The tests conducted using standard procedures are listed in Table 2. Gram staining was performed by using acetic-acid fixed samples (Dussault 1955). Cell morphology was examined on freshly prepared wet mounts of exponentially growing liquid cultures by phase contrast microscopy. Motility was examined on SWYE media supplemented with Bacto™ agar (Difco; 0.4% final concentration). Growth rates at different salt concentrations were determined by using

Table 2. Phenotypic features of representative strains from the Sečovlje and Ston solar salterns.

	Strain[a]						
Characteristic	Sech7a	Sech10	Sech4	Sech14	Sech8	Sech9	Sech6
EX-B L culture collection No.[b]	194	197	192	198	195	196	193
Proposed phylogenetic affiliation	*Haloferax mediterranei*	*Haloferax* sp.	*Haloferax* sp.	*Natrinema* sp.	*Haloarcula hispanica*	*Haloterrigena thermotolerans*	*Halorubrum* sp.
Cell shape	Flattened disks, cups	Pleomorphic rods	Pleomorphic rods	Pleomorphic rods	Irregular triangles, rectangles	Rods, ovals	Rods
Motility	+	+	+	+	+	+	+
Salt range for growth[c]	1.4–4.5	1.4–4.5	1.4–4.5	2–5.2	2–5.2	3.5–5.2	2–5.2
Pigmentation	Pink	Pink	Pink	Red	Red	Red	Red
Oxidase	+	+	+	+	+	+	+
Catalase	+	+	+	+	+	+	+
Gelatin hydrolysis	+	+	+	+	+	–	–
Starch hydrolysis	+	+	+	–	–	–	–
Casein hydrolysis	–	+	+	+	–	–	–
DNase	–	–	+	+	+	–	+
Acid production from:							
L-Arabinose	–	–	–	–	+	–	–
D-Fructose	+	+	–	–	+	–	–
D-Glucose	+	+	+	–	+	–	+
Glycerol	+	–	+	+	+	–	+
Lactose	–	–	–	–	+	–	–
Maltose	+	–	+	–	+	–	+
Sucrose	+	–	–	–	–	–	–
D-Mannitol	+	–	–	–	–	–	+

[a]The strains were chosen among isolates, obtained in this study and were further characterized.
[b]The microbiological culture collection maintained at the University of Ljubljana.
[c]Concentration (mol/L) of total salts.

SWYE medium prepared with salt concentrations (all mol/L) of 0, 0.4, 1, 1.4, 2, 3.5, 4, and 5.2. Catalase and oxidase activities, and the hydrolysis of gelatin, casein, and starch hydrolysis, were performed using standard procedures (Gerhardt et al. 1998; Boone and Castenholz 2001). DNase activity was determined using DNase test agar (Difco) supplemented with 25% SW in accordance with manufacturer's instructions. Production of acid from sugars was determined on solid SWYE medium supplemented with 0.001% (*m/v*) phenol red and the following sterile compounds to a final concentration of 1%: L-arabinose, D-fructose, D-glucose, glycerol, lactose, maltose, sucrose, and D-mannitol.

Genomic DNA extraction and amplification of 16S rDNA of cultured isolates

Genomic DNA was extracted as previously described (Pitcher et al. 1989). Polymerase chain reaction (PCR) amplifications of 16S rRNA were performed using *Taq* DNA polymerase (Fermentas, Vilnius, Lithuania), primers D30 (5′-ATTCCG GTTGATCCTGC) and D56 (5′-GYTACCTTGTTACGACTT) described previously (Arahal et al. 1996), and the following program: 94 °C for 2 min, followed by 30 cycles of 94 °C (45 s), 50 °C (45 s), and 72 °C (90 s), with an additional 5 s added for each cycle with a final 10 min extension step of 72 °C.

The second set of 16S rDNA PCR amplifications was performed using primers Arch21F (5′-TTCCGGTTGATC CYGCCGGA) and Arch958R (5′-YCCGGCGTTGAMTCC AATT) following a previously described protocol (DeLong 1992).

PCR products were then purified using the Qiaquick™ PCR purification kit (Qiagene GmbH, Hilden, Germany) followed by standard ethanol precipitation. The obtained DNA was subsequently used for nucleotide sequence determination.

16S rRNA gene library construction

For DNA extraction, cells from 150 to 300 mL of water were either harvested by centrifugation (10 621*g*, 30 min) or filtrated through 0.45 μm membrane filters (Millipore) until clogging. Extraction of the total microbial community was performed as previously described (Benlloch et al. 1996). Samples were stored in Tris–EDTA buffer (pH 8) at 4 °C.

16S rDNA was amplified from total community DNA following the protocols described above. PCR products of the expected sizes (approximately 950 or 1500 bp) were then cloned using the pGEM-T Easy Cloning kit (Promega, Madison, Wis., USA), according to the manufacturer's directions. To evaluate haloarchaeal clone library diversity, insert-positive clones were randomly chosen. Plasmid DNA for sequencing reactions was purified using Wizard™ Plus SV Minipreps (Promega).

Determination of nucleotide sequences

Amplified 16S rDNA of the saltern isolates and clones positive for inserts were sequenced using primers Arch21F,

Table 2 (*concluded*).

Ston2	Ston3	Ston5	Ston6	Ston11	Ston12	Ston16	Ston28
199	200	201	202	203	204	205	206
Haloarcula sp.	*Haloarcula* sp.	*Haloferax alexandrinus*	*Haloferax alexandrinus*	*Halobacterium salinarum*	*Haloterrigena thermotolerans*	*Haloarcula hispanica*	'*Haloferax larsenii*'
Irregular rods, cocci	Rods	Irregular triangles, rectangles	Irregular triangles, rectangles	Rods	Irregular rods, cocci	Irregular triangles, rectangles	Irregular triangles, rectangles
–	–	+	+	+	–	+	–
2–5.2	2–5.2	1.4–5.2	1.4–5.2	1.4–5.2	1.4–5.2	1.4–4	1.4–5.2
Red	Red	Red	Red	Red	Orange	Orange	Red
–	–	–	+	+	+	–	–
+	–	–	+	–	+	–	+
–	–	+	+	–	–	+	–
–	–	–	–	–	–	+	–
–	–	–	–	–	–	+	–
+	–	+	+	+	–	–	–
+	+	+	+	–	+	+	+
+	+	+	+	–	+	–	+
+	+	+	+	–	–	+	+
+	+	+	+	+	+	+	+
+	+	–	–	+	+	+	+
+	+	+	–	–	–	+	–
+	+	+	–	–	–	–	–
+	+	–	–	+	+	+	+

Arch958R, and the Thermo Sequenase™ Cy5 Dye Terminator Cycle Sequencing kit on ALFexpress™ II DNA Analysis System (Amersham Biosciences, Uppsala, Sweden) in accordance with the manufacturer's protocol. For bidirectional sequencing of 16S rDNA fragments an additional primer, B99 (5′-GTGTTACCGCGGCTGCTG), was used. Low quality sequences were removed and contigs were generated using Vector NTI™ Contig Express (InforMax Inc, Frederick, Md., USA).

Sequence comparisons

Relevant sequences were obtained from GenBank (www.ncbi.nlm.nih.gov) using BLASTN. Alignments of the 16S rDNA region corresponding to nucleotides 74–710 (*Escherichia coli* numbering; Brosius et al. 1981) were created using ClustalX (Thompson et al. 1997). The stability of alignments were assessed through comparison of ClustalW alignments produced under different gap opening/extension penalties using SOAP (Löytynoja and Milinkovitch 2001). This conservative approach assumes that confidence in homology assessment increases with stability to variation in alignment parameters and only unaffected positions across the spectrum of settings are considered to be unambiguously aligned and are kept for phylogenetic analysis. In 16S rDNA analysis gap penalties were incrementally adjusted from seven to 17 in steps of two and extension penalties were adjusted from 0.01 to 0.09 by steps of 0.02.

Putative chimeric sequences were recognized and omitted from further studies by use of Bellerophon software (Huber et al. 2004) by looking for taxa that changed positions in trees based on sequences of 250 nt from both the 5′ and 3′ end and by looking into sequences with unrealistically long branches or unique branching sites.

To compare our 16S rRNA libraries to published diversity results we used ∫-LIBSHUFF (Schloss et al. 2004). For diversity indices calculations, defining operational taxonomic units (OTUs), and rarefaction analysis computer software DOTUR was used (Schloss and Handelsman 2005).

Phylogenetic reconstruction

Maximum likelihood (ML) searches under the Tamura-Nei substitution model with gamma distributed rate heterogeneity and a proportion of invariable sites (TRN + Γ + *I*) were performed using PHYML (Guindon and Gascuel 2003). Maximum parsimony (MP) searches were performed using PAUP* (Swoford 2001).

Topology supports were assessed using Bayesian inference (Rannala and Yang 1996) and non-parametric bootstrapping. Bayesian posterior probabilities were computed under the same ML model with MrBayes 3.0b4 (Huelsenbeck and Ronquist 2001) with a Metropolis-coupled Markov chain Monte Carlo algorithm (Larget and Simon 1999). Three incrementally heated Markov chains (default heating parameter) and one cold, from which trees were sampled, were run simultaneously. Starting from random trees, the chains were run for 10^6 generations, taking samples every 100 genera-

tions. The initial 6% of trees was discarded as "burn-in" to ensure that the chains have reached stationarity. From the resulting 9400 trees, posterior probabilities were assessed for individual clades based on their observed frequencies.

ML and MP bootstrap support values were assessed by 1000 bootstrap replications. The heuristic branch-swapping algorithm TBR with 10× addition sequences randomized was applied under MP criterion.

Accession numbers

The sequences were deposited in the GenBank sequence database under the accession Nos. AY823950–AY823956 and DQ889324–DQ889339.

Results

Salterns and water samples

The crystallizers of Slovenian Sečovlje salterns (E 13°36′, N 45°28′) and Croatian Ston salterns (E 17°42′, N 42°49′) were sampled during the third week of August of both 2001 and 2003. The water was kept in 0.5 L sterile flasks until further processing in the laboratory. Physicochemical characteristics are presented in Table 1. Water samples were pale yellow in color and samples from Sečovlje salterns were viscous. Light microscopy (phase contrast, 1000× magnification) revealed the rods and polymorphic forms that are typical of halophilic archaea.

Cultivation of crystallizer microorganisms

Microbial growth was examined on several media differing in total salt concentration and carbon sources in an attempt to initiate growth of wide spectrum of halophiles. Samples of the crystallizer pond water were serially diluted to 10^{-2}, plated, and incubated at 37 or 42 °C. Two of the Croatian saltern samples needed to be propagated in liquid media prior to plating, owing to the low density of the sample. To this aim, the samples were either serially diluted to 10^{-6}, inoculated into liquid media, and propagated for 1 week at 37 °C, or up to 150 mL of sample was filtered through 0.22 μm filters and placed on solid media.

After 6 weeks of incubation, viable counts obtained of on all media were comparable (average of 7.5×10^3 CFU/mL) with the exception of the HM medium, on which haloarchaeal growth was poor (average of 2.5×10^3 CFU/mL). All of the plate isolates could be maintained on complex media, supporting the notion that the low-substrate-concentration approach is not necessarily a requirement for hypersaline waters (Burns et al. 2004). All of the media supported fast growth of *Haloferax* species, with colonies appearing during the first week of incubation. While *Haloferax* and *Haloarcula* appeared regardless of media used in this study, the growth of *Haloterrigena* and *Natrinema* species was initially observed on HS medium. The growth was considered absent if no viable counts were obtained in the incubation period.

Based on cell morphology and colony appearance, a total of 60 strains was obtained from both salterns and phenotypically divided into clusters. The phenotypic characteristics of the representatives of each cluster detected in this analysis are presented in Table 2 . Colonies appearing during the first and second week were isolates that fell within the *Haloferax* and *Halorubrum* genera. Colonies that appeared after three weeks were members of *Haloarcula* and *Halobacterium*, while members of *Haloterrigena* and a *Natrinema*-like isolate appeared during the fourth week of incubation. The isolates are maintained in the EX Culture Collection of the Department of Biology, Biotechnical Faculty, University of Ljubljana, Slovenia.

Isolate diversity in Croatian Ston salterns

The diversity of microorganisms was assessed by comparative sequence analysis of the 16S rRNA genes and by phenotypic characterization. 16S rDNA genes of representative isolates from all groups detected in phenotypic characterization were sequenced on both strands and the results are presented in Fig. 1. The 16S rDNA tree was divided into six clades based on the least inclusive monophily composed by Ston isolate sequences and next nearest relatives from GenBank.

Representatives phenotypically affiliated with genus *Haloferax* were most frequently recovered from Ston samples (53.3% of isolates obtained) and formed two distinctive clades. Phenotypic features of the majority of *Haloferax* isolates could not clearly fit any of the known descriptions. These isolates represented 20% of Ston saltern isolates and were phylogenetically related to '*Haloferax larsenii*' an isolate originally cultivated from the Chinese Zhousan Archipelago (isolate Ston28, 99% sequence identity). Representatives of the second *Haloferax* cluster comprising isolates Ston5 and Ston6 formed a separate clade with *Haloferax* sp. HSC4, a species originally cultivated from a saltmarsh. The sequence similarities between the Ston isolates and this species were 98%–99%.

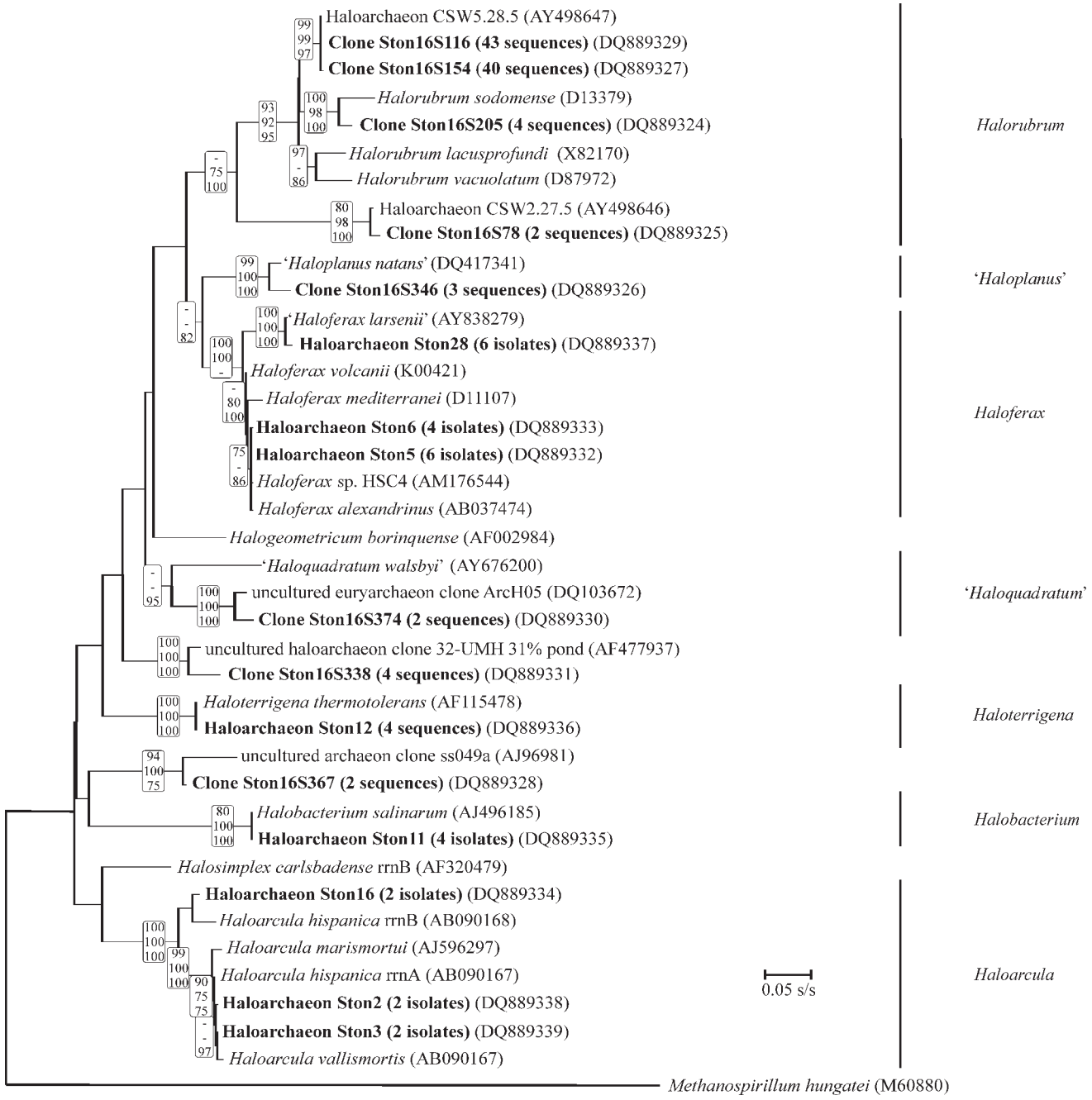
Twenty percent of isolates recovered from the Ston salterns were phenotypically affiliated with the genus *Haloarcula*. The 16S rRNA gene sequence of the chosen representative was 98% identical to the 16S rRNA gene sequence of *H. hispanica* and clustered with this species in all phylogenetic calculations (Fig. 1, isolate Ston16). Representatives of the second *Haloarcula* cluster, isolates Ston2 and Ston3, clustered within the *H. marismortui* branch, to which they shared sequence identities of 98% (Ston2) and 99% (Ston3).

A group of isolates was phenotypically affiliated with the genus *Haloterrigena*. Their 16S rDNA sequences, represented by isolate Ston12, clustered with *H. thermotolerans*. Finally, 16S rDNA sequence of isolate Ston11, a representative of *Halobacterium* group, was identical to *H. salinarum* DSM3754^T. Both groups represented 13.3% of isolates obtained.

Isolate diversity in Sečovlje salterns

Among 30 isolates recovered from the Slovenian salterns, seven separate clades were detected (Fig. 2). As in the Croatian salterns, 16S rDNA sequences of representative isolates branched with *Haloarcula hispanica* (16.6%), *Haloterrigena thermotolerans* (13.3%), *Haloferax* sp. HSC4 (10%) and '*Haloferax larsenii*' (20% of isolates recovered). However, the majority of isolates (33.3%) formed a phenotypically uniform group showing the features of *Haloferax mediterranei*. Phylogenetic placement of the representative isolate Sech7a next to *H. mediterranei* (99% sequence similarity) was also supported in all analyses. One isolate was associated with members of genus *Natrinema*, a group which comprises both alkaliphilic and neutrophilic haloarchaea. Strain Sech14, which is most similar to neutrophilic *Natrinema* sp.

Fig. 1. Phylogenetic reconstruction of 16S rRNA sequences of isolates (bold signatures) and clone sequences (prefix Clone Ston, bold signatures) recovered from Croatian Ston solar salterns. The most likely topology shown here was obtained under the Tamura-Nei substitution model with gamma distributed rate heterogeneity and a proportion of invariable sites (TRN + Γ + *I*). Scale represents the expected number of substitutions per site. Numbers represent the bootstrap values for the node of special interest. From top to bottom: maximum likelihood, maximum parsimony, and posterior probability (expressed as percentages).

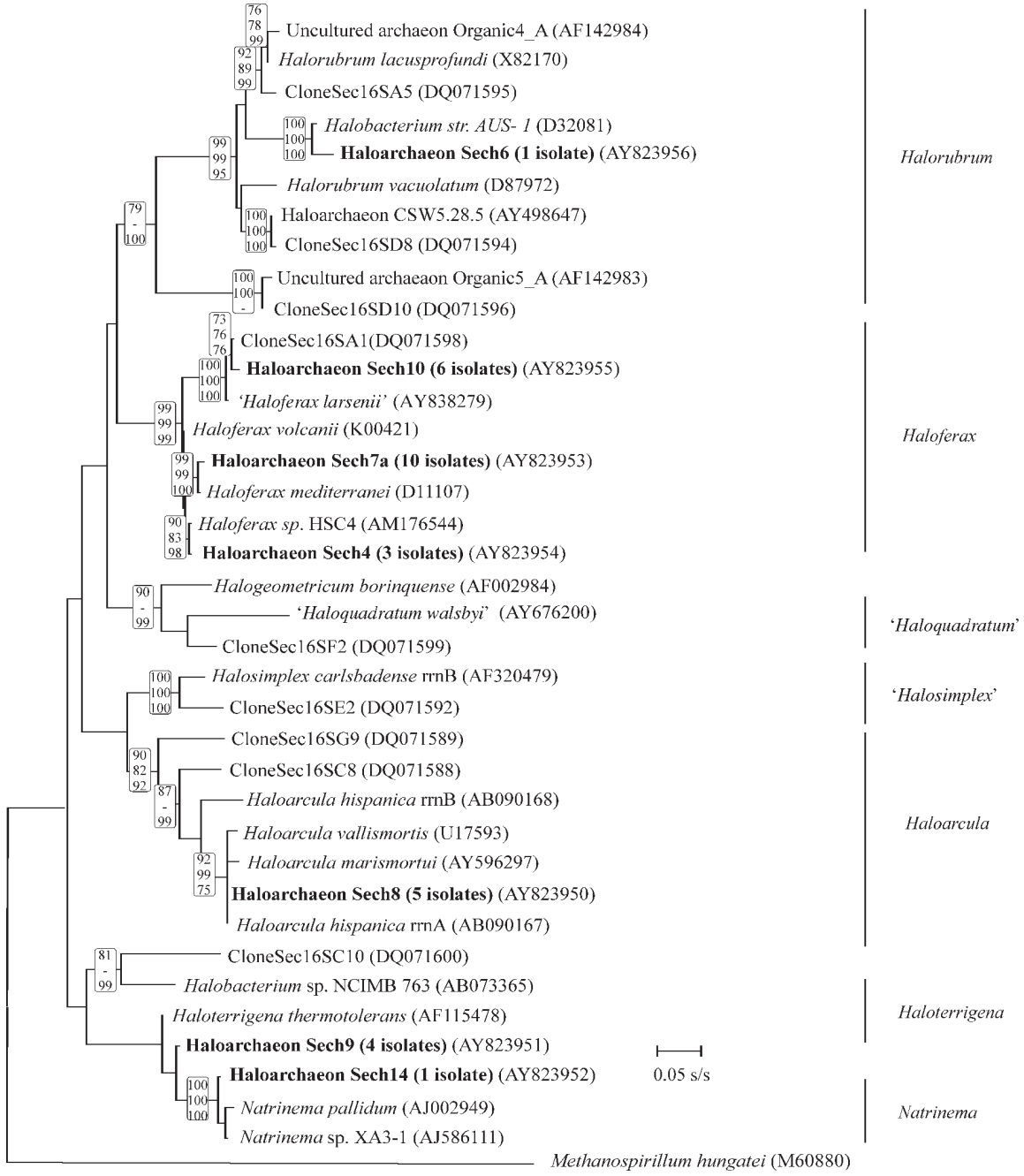


XA3-1 and *N. pallidum* (98% sequence identity to both) branched just outside the *Natrinema* group. Isolate Sech6, a representative of genus *Halorubrum*, branched with *Halorubrum* AUS-1, originally cultivated from Australian Cheetham salterns.

Sequence analysis of 16S rRNA gene fragments recovered from Ston salterns

A sample of 105 clones from three 16S rRNA libraries was randomly selected and sequenced. Five sequences were recognized as chimeric and were discarded. A PHYLIP

Fig. 2. Phylogenetic reconstruction for 16S rRNA sequences of isolates recovered from Slovenian Sečovlje solar salterns (bold signatures). Sequences from a previous study on Sečovlje solar salterns all have prefix Clone Sec. The most likely topology shown here was obtained under the Tamura-Nei substitution model with gamma distributed rate heterogeneity and a proportion of invariable sites (TRN + Γ + *I*). Scale represents the expected number of substitutions per site. Numbers represent the bootstrap values for the node of special interest. From top to bottom: maximum likelihood, maximum parsimony, and posterior probability (expressed as percentages).



generated distance matrix was used to assign sequences to OTUs using furthest neighbor algorithm at a distance of 3% as implemented in DOTUR (Schloss and Handelsman 2005). Within this sample, seven OTUs were detected, all represented by at least two clones. Representative clones from all phylotypes detected in the phylogenetic analyses were sequenced on both strands. The results are presented in Fig. 1. All of the recovered sequences shared similarities with halo-archaeal sequences previously detected in hypersaline environments.

The aligned 16S rRNA sequences of 83 clones were highly homologous with pairwise sequence similarities ranging from 98% to 99%. Represented by clones Ston16S116 and Ston16S154, these sequences formed a distinct branch with high affinity to isolate CSW5.28.5. This isolate represents almost half (49%) of the environmental 16S rRNA sequences

recovered from Australian Cheetham salterns (Burns et al. 2004). Furthermore, related sequences represent the second most recovered group of environmental 16S rRNA sequences from Slovenian salterns (Pašić et al. 2005).

The second cluster within the *Halorubrum* branch was formed by four clone sequences, represented by clone Ston16S205. These sequences were found to be related to *Halorubrum sodomense* (≥96% sequence similarity). In addition, two sequences were found to be related to *Halorubrum*-placed members of the Antarctic Deep Lake (ADL) sequence group. Described by Bowman et al. (2000), the ADL group also comprises environmental sequences and isolates from solar salterns. Two sequences recovered from Ston solar salterns (clone Ston16S78), branched with Australian isolate CSW2.27.5, where members of the ADL group represented 16% of total sequences obtained (Burns et al. 2004).

Four sequences of OTU represented by clone Ston16S338 shared sequence similarities of <96% to known GenBank sequences, while their pairwise sequence similarities were ≥99%. These sequences were most similar to the environmental 16S rRNA sequence recovered from the crystallizers of Spanish solar saltern (clone 32-UMH 31% pond, accession number AF477937). This group was followed in abundance by sequences represented by clone Ston16S346 (3% of total sequences recovered, pairwise sequence similarities ≥98%), which formed a monophyletic clade within *Halobacteriaceae*. These sequences shared 97% sequence identity with the 16S rRNA of '*Haloplanus natans*' strain RE-103, an isolate recently cultivated from the Dead Sea (David R. Elevi-Bar, A. Oren, and L. Mana, Department of Plant and Environmental Sciences, The Hebrew Institute of Jerusalem, The Institute of Life Sciences, unpublished data).

The remaining two OTUs as determined at 3% sequence distance, both represented 2% of 16S rRNA environmental sequences recovered. These sequences grouped with uncultured haloarchaeal clones ss042 originating from saline soil (clone Ston16S367, pairwise sequence similarities ≥99%) and clone ArcH05 originating from a hypersaline endoevaporitic mat (clone Ston16S374, pairwise sequence similarities ≥99%).

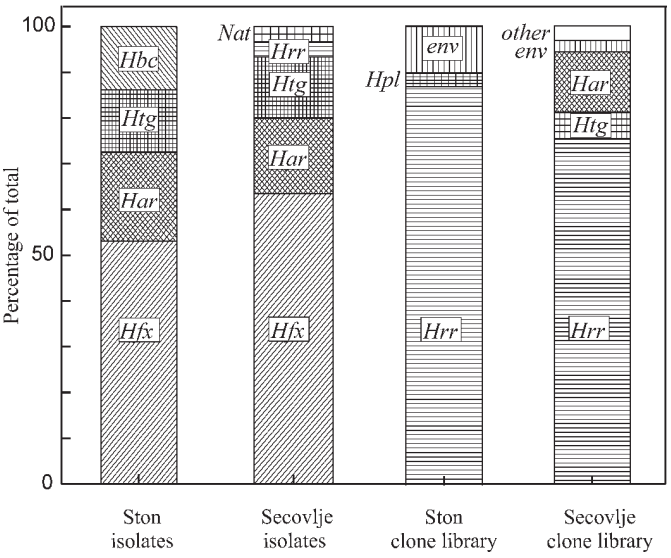
Sequence comparisons, diversity indices, and rarefaction analysis

Comparisons of microbial community structure encountered in Ston and Sečovlje salterns are presented in Fig. 3.

Representatives of the dominant *Halorubrum* group recovered from the Ston salterns 16S rRNA gene library (Fig. 1, clones Ston16S116 and Ston16S154) shared ≥98% sequence similarity with the second most abundant cluster recovered from salterns in Sečovlje (Fig. 2, clone Sec16SD8). The sequence similarities between the remaining clone sequences from both salterns 16S rRNA gene libraries were <98%. Therefore, to statistically evaluate the possibility that the Ston 16S rDNA gene library is a subset of previously published Sečovlje 16S rDNA gene library, the libraries were compared using the integral form of statistic in ∫-LIBSHUFF with 10 000 randomizations. The obtained $P < 0.001$ suggested that there is a high probability that the 16S rDNA libraries constructed in this and a previous study contain different taxonomical lineages.

Furthermore, indices indicating diversity (Shannon–Weaver index), dominance (Simpson index), and species richness

Fig. 3. Diversity of isolates and 16S rRNA library sequences. The bar chart compares the diversities of isolates and clone libraries obtained for Slovenian Sečovlje and Croatian Ston salterns in this and a previous study (Pašić et al. 2005). The labels denote the various sequence groups recovered as follows: *Hfx*, *Haloferax* group; *Hrr*, *Halorubrum* group; *Htg*, *Haloterrigena* group; *Har*, *Haloarcula* group; *Hbc*, *Halobacterium* group; *Nat*, *Natrinema*-like group; *Hpl*, "Haloplanus" group; *env*, environmental clones obtained in other hypersaline environments; *other*, other haloarchaea.



(Chao 1 and ACE indices) showed higher diversity in the Sečovlje salterns compared with the Ston salterns (Table 3).

Finally, rarefaction analysis was applied to evaluate whether the screening of 100 16S rDNA clones obtained from the Ston solar salterns was sufficient to estimate diversity within this clone library (Fig. 4). The rarefaction curves did not reach a clear plateau, indicating that additional sequencing would have revealed further diversity.

Discussion

Given the limitation of data available on different solar saltern systems worldwide, the aim of this study was to lay the groundwork for further ecological research by establishing haloarchaeal diversity in the crystallizers of Adriatic solar salterns. In comparison to well studied salterns in arid parts of the world, the salterns in the Adriatic region operate only during the dry summer season, have shallow ponds measuring not more than 10 cm in depth, and lack typical red coloration. The absence of coloration is a consequence of low densities of haloarchaeal populations. The total cell counts in the crystallizer ponds (10^6 cells/mL) is at least an order of magnitude lower than the 10^7–10^8 cells/mL reported from arid solar salt systems in Eilat, Israel (Litchfield et al. 2000), Santa Pola, Spain (Benlloch et al. 2002), and coastal Australia (Burns et al. 2004), and $>10^8$ cells/mL in solar salterns in Fuencaliente, La Palma, Spain (Ochsenreiter et al. 2002). We speculate that the low levels of cells in the Adriatic solar salterns, compared with the levels in arid salterns, could be

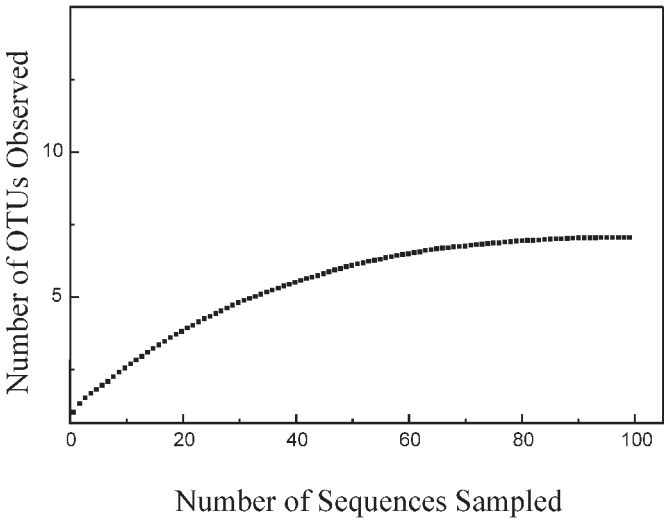
Table 3. Diversity indices for Ston and Sečovlje solar salterns.

Diversity index	Sečovlje[a] 16S rRNA library	Ston 16S rRNA library	Sečovlje isolates	Ston isolates
Diversity (*H′*)	2.24	0.49	1.47	1.19
Dominance (*SI′*)	0.14	0.75	0.25	0.33
Species richness (Chao)	27	3	7	4
Species richness (ACE)	20.32	3	7.32	4

Note: Diversity indices were determined at a distance of 3%, as implemented in DOTUR (Schloss and Handelsman 2005).

[a]Data from Pašić et al. (2005).

Fig. 4. Rarefaction curve determined at a distance of 3% as implemented in DOTUR (Schloss and Handelsman 2005) using 16S rRNA gene sequences from Croatian Ston salterns.



related to the short water retention time, preventing maturation of the haloarchaeal community. Likewise, low cell counts have been obtained from high altitude Maras salterns in the Peruvian Andes (Maturrano et al. 2006), which also operate only during the dry season.

The haloarchaeal community of saltern crystallizers as depicted by analysis of PCR-amplified environmental 16S rRNA is by far dominated by relatives of genus *Halorubrum* (66% and 87% of total sequences recovered from the Slovenian Sečovlje and Croatian Ston salterns, respectively). Closely related sequences were previously reported as the dominant group in the sequence library of the Australian Cheetham salterns crystallizer, yet there they represented 49% of sequences recovered (Burns et al. 2004). In contrast, the haloarchaeal communities in the solar saltern crystallizer represent near monocultures of sequences closely related to '*Haloquadratum walsbyi*,' the recently cultivated square archaeon (Bolhuis et al. 2004). Indeed, these assemblages are the most frequently recovered from clone libraries of geographically distant salterns in Asia, Europe, and South America (Benlloch et al. 1996; Litchfield et al. 2000; Benlloch et al. 2001; Oren 2002; Maturrano et al. 2006). Apart from Adriatic salterns, salterns in San Diego, California have been reported as devoid from '*Haloquadratum walsbyi*,' yet the dominant community representatives belong to genus *Halobacterium* (Bidle et al. 2005).

Although the dominant groups in both of the salterns studied belong to the genus *Halorubrum*, comparative sequence analysis at a distance of 0.03 as implemented in LIBSHUFF indicates that there is a high probability that the libraries constructed in this and a previous study contain different taxonomical lineages. Furthermore, the diversity indices of Croatian Ston salterns, determined at a distance of 0.03 as implemented in DOTUR are lower than previously determined for Slovenian Sečovlje salterns or Spanish Santa Pola saltern (Pašić et al. 2005). This drop in diversity might be a consequence of a relatively more oligotrophic water source for the Ston salterns as compared with the Sečovlje salterns.

The levels of microbial diversity discerned by the sequence library and by cultivation differed significantly. Such a difference in organisms retrieved by 16S rRNA analysis and those obtained through classical cultivation has been previously reported (Benlloch et al. 2001; Ochsenreiter et al. 2002). However, classical cultivation methods remain an important part of the description of haloarchaeal communities. The set of active growing taxa that carries out most ecosystem functions and suffers intense losses through predation and viral lysis is readily retrieved by molecular techniques, yet is difficult to obtain in pure culture. On the other hand, many of the rare taxa (not growing or slow growing) are seldom retreived by molecular techniques, yet many can be cultivated. Therefore, although easily culturable, members of some haloarchaeal genera (primarily *Halobacterium* and *Haloferax*) are not normally detected in clone libraries (Pedros-Alio 2006).

While isolates of the genus *Halorubrum* have been most frequently recovered from Spanish and Australian solar salterns (Benlloch et al. 2001, Burns et al. 2004), isolates resembling relatives of the genus *Haloferax* are most frequently recovered from Adriatic salterns. Given the high culturability of *Haloferax* sp. compared with other haloarchaea, we cannot exclude the possibility of overestimation of its proportion of the diversity. The 16S rRNA sequences of the majority of Sečovlje isolates were most similar to *Haloferax mediterranei*, an isolate originally cultivated from the Spanish solar salterns. In Ston solar salterns, two major groups of *Haloferax* isolates are observed. One group of isolates was related to '*Haloferax larsenii*,' an isolate originally cultivated from the Chinese Zhoushan Archipelago. Isolate sequences sharing >98% identity with representatives of the second *Haloferax* group were recently reported from another Adriatic hypersaline habitat, a salt marsh in Greece (isolate *Haloferax* sp. HSC4, AM176544). Furthermore, members of the genus *Haloterrigena* make up the second largest group within the isolates obtained. Although differing in pheno-

typic characteristics, all of the isolates phylogenetically clustered with *Haloterrigena* thermotolerans, a species originally cultivated from solar salterns in Puerto Rico (Montalvo-Rodriguez et al. 2000). The remaining isolates belonged to the genera *Haloarcula* and *Halobacterium*, commonly isolated from hypersaline environments. Although the cultivation techniques we used were similar to those used previously (Burns et al. 2004), we had only limited success in cultivating members of genus *Halorubrum*.

Our work confirms that NaCl saturated systems can vary widely in the microbiota they possess and that climate can play a role in the communities found even in these very specialized and extreme environments. One of the systems studied that is more oligotrophic shows less diversity, strengthening the potential value that the study of diverse hypersaline environments could possess.

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