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Diversity of Archaea in hypersaline environments characterized by molecular-phylogenetic and cultivation studies

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Abstract The diversity of Archaea from three different hypersaline environments was analyzed and compared by polymerase chain reaction (PCR)-based molecular phylogenetic techniques and cultivation approaches. The samples originated from a crystallization pond of a solar saltern in Spain (FC); an alkaline lake in Nevada, USA, (EMF); and a small pond from a slag heap of a potassium mine in Germany (DIE). Except for two 16S rDNA sequences that were related to crenarchaeota from soil and did not apparently belong to the indigenous halophilic community, all sequences recovered from environmental DNA or cultivated strains grouped within the *Halobacteriaceae*. Mostly 16S rDNA sequences related to the genera *Halorubrum* and *Haloarcula* were detected in sample FC, and organisms belonging to these genera were also recovered by cultivation. In contrast, sequences related to five different groups of halophilic archaea were amplified from sample DIE (including novel lineages with only uncultivated phylotypes), but the organisms that were cultivated from this sample fell into different groups (i.e., *Natronococcus*, *Halorubrum*, or unaffiliated) and did not overlap with those predicted using the culture-independent approach. With respect to the highly alkaline sample, EMF, four groups were predicted from the environmental 16S rDNA sequences, two of which (*Natronomonas* and *Haloarcula*) were also recovered through cultivation together with *Natronococcus* isolates. In summary, we found that halophilic archaea dominate the archaeal populations in these three hypersaline environments and show that culturability of the organisms predicted by molecular surveys might strongly depend on the habitat chosen. While a number of novel halophilic archaea have been isolated, we have not been able to cultivate representatives of the new lineages that were detected in this and several other environmental studies.

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Introduction

Halophilic archaea thrive in natural lakes and sediments of high salinity or alkaline soda lakes, but also in solar salterns and other hypersaline environments of anthropogenic origin. The salt concentrations in these environments range from 15% to saturation, with pH values from slightly acidic to alkaline (pH 6–11). Members of the family *Halobacteriaceae* are the dominant inhabitants, besides halophilic bacteria and halotolerant eukaryotes such as the alga *Dunaliella*. Halophilic archaea accumulate potassium ions intracellularly to balance the high salt content of the environment. This ability differentiates them from halophilic bacteria that usually produce compatible solutes (betaine, ectoine) to counteract the high external salt concentrations (da Costa et al. 1998).

Most halophilic archaea are aerobic chemoorganotrophs and depend on the organic material supplied by the primary producers such as *Dunaliella* or halophilic cyanobacteria. Anaerobic growth by fermentation or by respiration with the electron acceptor nitrate has been shown for a few species (e.g., *Haloarcula vallismortis*). *Halobacterium* is also able to grow photoheterotrophically under low oxygen tension using the retinal protein bacteriorhodopsin as a light-driven proton pump (Oesterhelt and Stoeckenius 1973). Until recently, bacteriorhodopsin was treated as trait solely found in halophilic archaea. However, a gene encoding a related rhodopsin has recently been detected on a DNA fragment derived from an alpha-proteobacterium in marine plankton (Beja et al. 2000).

To date 14 different genera of halophilic archaea containing about 35 validly described species are known and have been characterized (Oren 1998). In addition, a few studies have recently assessed the diversity and distribution of halophilic archaea by molecular-phylogenetic techniques that are independent of laboratory cultivation of the organ-

isms. By amplifying 16S rDNA sequences directly from environmental samples, novel phylotypes of the halophilic archaea have been detected in crystallizer ponds of a marine saltern (Benlloch et al. 1995; Antón et al. 1999), in Lake Magadi, an alkaline soda lake (Grant et al. 1999), in the sediments of hypersaline Antarctic lakes and coastal salt marsh (Bowman et al. 2000; Munson et al. 1997), and in the aerobic epilimnion and the sulfide-rich hypolimnion of the hypersaline stratified Solar Lake (Cytryn et al. 2000). Although a novel and abundant group of bacteria has been detected in a crystallizer pond from a solar saltern (Antón et al. 2000), all environmental studies indicate that halophilic archaea dominate the populations of aerobic hypersaline environments, and that a considerably greater diversity of halophilic archaea occurs than previously assumed. As in other molecular phylogenetic studies, most phylotypes found did not correspond to any previously described organism, indicating that even in a system with relatively low diversity cultivation might be biased towards minority species, and that most of the community is composed of hitherto uncultured organisms.

The aim of the research presented here was to assess and compare the diversity of archaea in three different hypersaline environments with molecular and culture-based approaches. One habitat sampled was an alkaline soda lake in Nevada, while two other samples came from habitats of anthropogenic origin, namely a crystallization pond of a solar saltern near Fuencaliente (La Palma, Spain), and a salty pond that arose on the bottom of a slag heap of a former potassium mine (Diekholzen, Germany).

Materials and methods

Sample collection and chemical analysis

Samples were collected in flasks of different sizes and kept aseptically until analyzed. Liquid with salt crystals from the crystallizer pond of a solar saltern next to Fuencaliente, La Palma, Spain, was collected in summer 1998 (sample FC). Brine from the hypersaline alkaline lake "Eight Mile Flat", 150 km east of Reno, Nevada, USA, was collected in summer 1998 (sample EMF). Salt water from a small pond on the bottom of a slag heap of the former potassium mine "Kali and Salz AG", Diekholzen, Germany was collected in winter 1998 (sample DIE). The salinity of each sample was determined with a refractometer (A. Krüss Optronic, Hamburg, Germany). From each sample 1 ml was used for optical emission spectrometry (ICP-OES, inductive coupled plasma-optical emission spectrometry) to determine the inorganic ion composition (Spectroflame Modula; Spectro, Kleve, Germany).

Microscopic inspection and cell counts

Samples (5 µl) were analyzed with 400-fold magnification in a Zeiss Axiovert 100 microscope (Zeiss, Germany). For cell counts, cells were stained with DAPI (4′,6-diamidino-2-phenylindole; Sigma-Aldrich, Munich, Germany) and 20–30 microscopic fields were analyzed.

Preparation of media and enrichment cultures

Media were either composed based on the chemical analysis of the samples (see tables), or they were modified standard media. For the enrichment of organisms from samples FC and DIE, media were designed following the main salt concentrations as determined by optical emission spectrometry. The medium for sample DIE had the following composition: NaCl 2.8 M, KCl 0.11 M, $MgCl_2$ 0.7 M, $(NH_4)_2SO_4$ 3.5 mM, $CaCl_2$ 0.05 mM; Medium for sample FC contained: NaCl 2.3 M, KCl 0.3 M, $MgCl_2$ 1 M, $(NH_4)_2SO_4$ 3.5 mM, $CaCl_2$ 0.05 mM. In addition, Hutners modified salts (1 ml/l), were added as well as K_2HPO_4 0.5 mM, ferrous-citrate 0.01% and vitamin solution no. 6 (Staley 1968) 1 ml/l. A modified *Natronobacterium* medium (medium 371, DSM, Catalogue of strains 1993, NA-medium in tables) was supplemented with yeast extract (0.1%, w/v), casamino acids (0.075%, w/v), 0.17% sodium citrate, KCl 26.8 mM, $MgSO_4$ 8.3 mM, NaCl 3.4 M, Na_2CO_3 173.6 mM, $MnCl_2$ 18 µM, $FeSO_4$ 1.8 mM, and was adjusted with NaOH to pH 11. A modified *Haloarcula* medium (Medium 372, DSM, Catalogue of strains 1993, HA-medium in tables), supplemented with peptone (0.15% w/v), contained NaCl 4.3 M, $MgSO_4$ 166.1 mM, KCl 26.8 mM, Tris-HCl 50 mM and was adjusted with HCl to pH 6. For solid media agar was added to 1.5% (w/v). Portions of 0.1–1 ml of samples were either directly plated on solid media or in soft agar overlay (0.8%), or the sample was first enriched by adding 0.001% tryptone/yeast (5 : 3) or 0.1% of pyruvate:2-ketoglutarate:2-ketoisovalerate (1 : 1 : 1), or 0.15% ferrous-citrate:$FeSO_4$/citrate (20 : 15). Incubation of the enrichments both on solid and in liquid media was carried out aerobically at 42°C for 7–14 days.

DNA extraction

Nucleic acids were extracted by filtering the sample onto a nitrocellulose filter (0.45 µm) and resuspending the organic material in lysis-buffer (Tris-HCl pH 8, 20 mM, EDTA 40 mM, NaCl 100 mM, Na-citrate 1 mM, lysozyme 2 mg/ml). After incubation at 37°C for 90 min, proteinase-K (0.2 mg/ml) was added, and the sample was incubated at 55°C for 30–60 min. We subsequently applied the freeze-and-thaw technique using liquid nitrogen. The lysate was extracted with an equal volume of buffered phenol (Roth, Karlsruhe, Germany) followed by an extraction with phenol : chloroform : isoamyl alcohol (25 : 24 : 1). Nucleic acids were precipitated with 0.1 vol 3 M sodium acetate and 0.7 vol isopropanol (98%) at room temperature and washed with ethanol 70% (w/v). Nucleic acids were resuspended in 100–500 µl TE-buffer (Tris-HCl 20 mM pH 7.5; EDTA 1 mM). The resuspended nucleic acids were subsequently dialyzed against TE-buffer using microconcentrators (Centricon10; Amicon, Beverly, MA, USA) to reduce the salt content.

PCR amplification of rDNA genes and clone library construction

Archaeal 16S rDNA genes were amplified in an OmnE cycler (Hybaid, Heidelberg, Germany) for 32 cycles (5 min denaturing step at 94°C in the first cycle; 0.5 min denaturing at 94°C, 1 min annealing at 54°C and 1 min elongation at 72°C, with a final extension step at 72°C for 10 min) using Archaea-specific primers (20f 5′ TTCCGGTTGATCCY GCCRG 3′; 958r 5′ YCCGGCGTTGAMTCCAATT 3′; DeLong 1992). Each reaction (40 µl) contained 20 pmol of the primers, dNTPs 0.2 mM, $MgCl_2$ 2 mM, 4 µl of PCR buffer and 1 U of Ampli-Taq (Perkin Elmer, Rodgau-Jügesheim, Germany) or Goldstar Polymerase (Eurogentec, Cologne, Germany).

The amplification products were purified from a 1% agarose gel using the Geneclean II kit (BIO 101, Vista, CA, USA). The purified PCR products were ligated into TA-vector pCR 2.1 (Invitrogen, Groningen, The Netherlands) or pGEM-T vector (Promega, Mannheim, Germany) and subsequently used to transform *E. coli* TOP10F. The resulting clones were screened for 16S rDNA inserts by colony-PCR using 20 pmol M13 forward and M13 reverse primers. PCR-fragments of correct size (about 1,100 bp) were further analyzed by restriction digests with *Bsp*143I and *Nde*II (MBI Fermentas, St Leon-Rot, Germany) on a 3% agarose gel to evaluate the diversity of the clone library.

Identification of cultivated organisms

Cultivated organisms were identified using colony polymerase chain reaction (PCR), with the Archaea-specific primers described above. Single colonies were picked from the agar plate with a sterile pipette tip and dipped into a PCR-mix. The PCR reactions and cloning of PCR products were performed as described above.

Sequencing of 16S rDNA environmental clones

The nucleotide sequences of 12 environmental clones and 11 cultivated isolates of sample EMF, of 21 environmental clones and 14 isolates of sample FC and of 14 environmental clones and 6 isolates of sample DIE were completely determined. PCR products were purified by ethanol precipitation. Precipitated nucleic acids were resuspended in 20 µl water. Four microliters of the purified PCR product were used in the following cycle sequence protocol: 2 pmol IRD800 labeled M13, 1 µl SequitherTherm ExcellII Polymerase (Biozym, Hess Oldendorf, Germany), 8 µl buffer, 3.7 µl water. Sequences that showed less than 1% divergence from each other were considered as one group and only one representative was used for the phylogenetic reconstructions described below.

Phylogenetic analyses

The ARB software package (Ludwig et al. 1998) was used for phylogenetic analyses with an alignment of approximately 10,000 sequences, including the next relatives to sequences, from this survey (according to BLASTN searches in the EMBL database). The tool ARB-edit from the ARB software package was applied for the alignment. The alignment of 41 sequences used in this study was corrected manually. For the detection of chimeric sequences, several tests were applied: (1) The Check-Chimera program of the Ribosomal Database Project was used (Maidak et al. 1999), (2) every sequence was checked manually in the alignment, and (3) phylogenetic trees were screened for sequences with unrealistic long branches or unique branching sites. In these analyses two apparent chimeric sequences were recognized as putative PCR chimeras and were omitted from further studies. The reconstruction of phylogenetic trees was based on partial sequences of 739–916 bp. Tree topologies were evaluated using the maximum parsimony, the maximum likelihood (fastDNAml) and the distance matrix (Felsenstein correction) method with different alignment filters (gap-filter, positional variability filter, maximum frequency filter, ARB software package).

Accession numbers

The sequences were deposited in the EMBL Nucleotide Sequence Database under the accession numbers AJ307999 through AJ308019.

Results

Sample characteristics

Three samples from different locations and origin were chosen for our study. Their salinity was above 32% as determined by a refractometer. Sample EMF from the alkaline soda lake had a pH of 11 and exhibited the typical red color indicative of halophilic archaea. Microscopic inspection revealed cell counts above 10^8/ml with various prokaryotic coccoid and rod-shaped morphotypes. The sample FC from the crystallization pond of a solar saltern had similar cell counts and was of red and turbid appearance. Different prokaryotic morphotypes as well as larger, polarly flagellated cells reminiscent of the halophilic alga *Dunaliella* were observed by microscopic inspection. The third sample stemmed from a salt pond that arose on the bottom of a slag heap of a former potassium mine in Dieckholzen, Germany (sample DIE). It was colorless and indicated an acidic pH of 5.6. The cell counts of around 10^6/ml were considerably lower than in the other two samples.

The inorganic composition of the three samples was determined by optical emission spectrometry (Table 1). In this analysis, the total ion concentration of EMF was 4.6 N, of FC it was 5.1 and of DIE it was 3.9 N.

The content of the divalent ions (Mg^{2+} and Ca^{2+}) were low in EMF, as usually found in alkaline lakes, while the relative Mg^{2+} concentrations in the other two samples were rather high as compared with marine water or media

Table 1. Inorganic composition of the samples from an alkaline lake in Nevada, USA (EMF), a small pond from a slag heap of a potassium mine in Germany (DIE), and a crystallization pond of a solar saltern in Spain (FC) as determined by optical emission spectrometry

Cation/anion	EMF[a]		DIE		FC		Seawater[b]	
	(g/l)	(M)	(g/l)	(M)	(g/l)	(M)	(g/l)	(M)
Na	17	0.74	52.3	2.3	64.9	2.8	90.5	3.9
K	<3.4	–	12.4	0.3	4.3	0.11	3.25	0.08
Mg	<1.2	–	17	0.7	24	1	10.88	0.45
Ca	<0.9	–	<0.09	–	<0.9	–	3.43	0.08
Cl	163	4.7	137.3	3.9	180	5.1	162.7	4.6
SO_4	0.03	0.3×10^{-3}	0.36	3.75×10^{-3}	0.04	0.42×10^{-3}	17.6	183×10^{-3}
Mn	4×10^{-3}	0.07×10^{-3}	1.4×10^{-3}	0.025×10^{-3}	8×10^{-3}	0.14×10^{-3}	0.086×10^{-3}	0.002×10^{-3}
Co	7×10^{-3}	0.11×10^{-3}	0.6×10^{-3}	0.009×10^{-3}	7×10^{-3}	0.11×10^{-3}	0.77×10^{-3}	0.012×10^{-3}
Fe	13×10^{-3}	0.23×10^{-3}	0.7×10^{-3}	0.012×10^{-3}	13×10^{-3}	0.23×10^{-3}	0.17×10^{-3}	0.003×10^{-3}

[a]For the sample EMF we were not able to detect all necessary counter ions
[b]For comparison, seawater projected from 3.5% to 30% salinity (from Weast 1977)

typically used for the enrichment of halophilic archaea. As expected, the potassium ion concentration was high in the slag heap sample DIE from the salt mine. In all three samples the sulfate ion concentration was extremely low (<3.8 mM). Extraordinarily high amounts of trace elements, especially iron, copper, and manganese, were found in the samples EMF and FC.

16S rDNA analyses

Total DNA was isolated from the three different samples, and 16S rDNA clone libraries were generated with PCR primers targeting phylotypes of the archaeal domain (DeLong 1992). The 16S rDNA inserts of these clone libraries were amplified and analyzed with two different restriction enzymes (not shown). Based on these RFLP patterns, the most different clones were subjected to DNA sequence determination. Phylogenetic analysis revealed that with two exceptions from sample DIE (DIE 15 and DIE 8) all 16S rDNA sequences grouped within the *Halobacteriaceae*. The phylogenetic tree shown in Fig. 1 represents phylotypes obtained in this study and their closest relatives as well as representatives of all distinct halobacterial phyla. Sequences from cultivated halobacterial strains isolated in this study (see below) are represented in boxes, while uncultivated phylotypes from the 16S rDNA libraries are unboxed. Only one representative each of a group of closely related sequences that exhibited <1% divergence was chosen for the phylogenetic calculations and is represented in the tree. Depending on the phylogenetic method used, variable branching orders were found for the different halobacterial genera within the phylum of the *Halobacteriaceae*, but the clustering within each genus (*Haloarcula*, *Halorubrum* etc.) remained stable, i.e., reproducible, and was found to be identical to that defined by Kamekura (1999).

The sequences from the soda lake sample EMF (pH 11) were affiliated with the genera *Natronomonas*, *Halorubrum*, and *Haloarcula*. One group of sequences (represented by EMF3 in Fig. 1) did not have any significant similarity to cultivated or uncultivated halophilic archaea but tended to group together with *Halococcus morrhuae* in most phylogenetic calculations (87% sequence similarity to this species). The dominant groups of the second sample, FC, from the crystallizer pond, represented relatives of *Halorubrum* and *Haloarcula*. One sequence, however (FCII3), was weakly affiliated with uncultivated phylotypes from a lake in Antarctica and from sample DIE (85% and 86% sequence similarity to ORGANIC1 and DIE3, respectively). Together, these sequences were termed cluster 1 in Fig. 1. We did not find any sequences related to the SPhT phylotype that was detected as an abundant organism in another solar saltern in Alicante, Spain (Benlloch et al. 1995; Antón et al. 1999).

The highest diversity was found in the third sample, DIE. The sequences grouped into five different halobacterial clusters: beside relatives of *Halobacterium salinarum* (genus *Halobacterium*) and *Halorubrum trapanicum* (placed within the genus *Natronobacterium*), phylotypes more distantly related to *Haloferax mediterranei* (DIE28, 89% similarity) were found. One sequence (DIE3) formed a separate cluster (cluster 1 in Fig. 1) together with a phylotype found in an Antarctic, hypersaline lake (ORGANIC1). Two more sequences (represented by DIE1) grouped in a separate cluster (cluster 2, Fig. 1) that was exclusively represented by uncultivated phylotypes detected in salt marshes (Munson et al. 1997) and hypersaline Antarctic lakes (Bowman et al. 2000). Two additional sequences, that did not group within the *Halobacteriaceae* (represented by DIE 15 in Fig. 1), were found to be closely related (96% similarity) to a specific cluster of non-thermophilic, uncultivated *Crenarchaeota*, that have been detected repeatedly in different soils in Michigan (Bintrim et al. 1997; Buckley et al. 1998) and also in various soils in Germany (Sandaa et al. 1999; T. Ochsenreiter and C. Schleper, unpublished). Therefore, it seems to be very likely that the respective organisms are not extreme halophiles but instead are soil inhabitants that have been washed into the sample from the surrounding area.

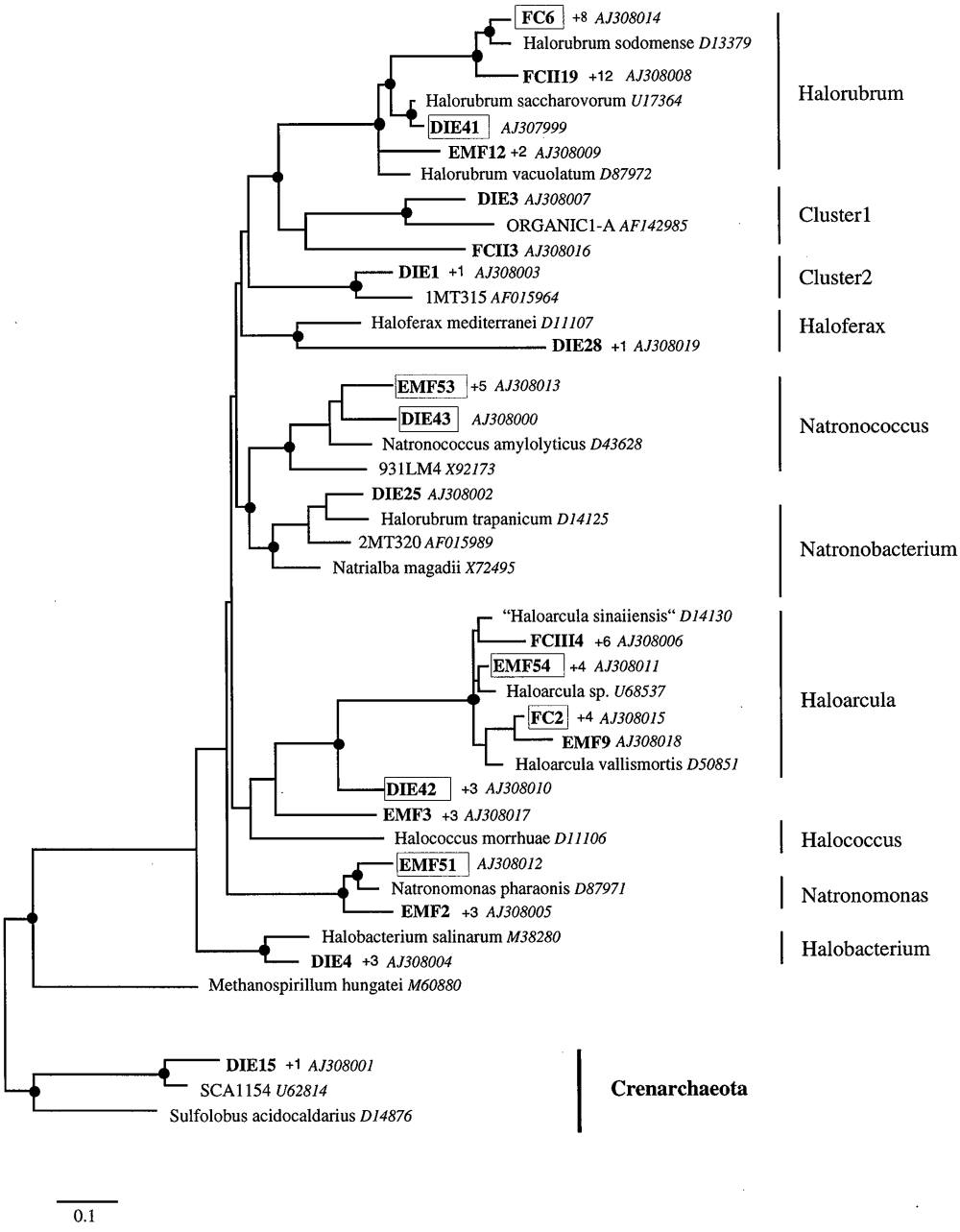

Fig. 1. Phylogenetic tree based on archaeal partial 16S rDNA sequences from environmental clones (*bold*) and cultivated isolates (*bold*, *boxed*) recovered from three different habitats: (1) salty pond of a slag heap from a potassium mine (*DIE*), (2) crystallizer pond from a solar saltern (*FC*), and (3) hypersaline alkaline lake (*EMF*). Sequences with <1% divergence were considered one group and are represented by only one sequence in the tree. Published reference sequences were chosen to represent the diversity within the *Halobacteriaceae* and to show next relatives to sequences found in our study. Different alignment filters were used to evaluate the phylogenetic reconstructions. The tree topology shown here is based on a maximum parsimony analysis. *Closed circles* indicate branching points supported by three different phylogenetic methods (maximum parsimony, maximum likelihood, and distance matrix)

Enrichment cultures and isolation of halophilic archaea

Different enrichment strategies in liquid and on solid media were used to assess the diversity of cultivatable halophilic archaea. For two of the three samples (FC and DIE), media were designed that followed the major ion composition of the natural habitats as determined by optical emission spectrometry (see Materials and methods). While this strategy was successful for the FC sample from the saltern pond, no isolates were obtained from the slag heap sample DIE (see Table 2). Another approach involved the use of classical growth media for halophilic archaea and haloalkaliphiles amended with different carbon sources in varying concentrations (from 0.001% to 0.15%), and at different pH values. Samples were either directly plated on regular agar plates or poured into agar soft layers (for microaerophilic organisms), or they were enriched in liquid media prior to plating. Only from sample FC did we obtain colonies by directly spreading the original sample, while the other two samples had to be propagated in liquid media first. Table 2 lists the enrichment and culture conditions of the isolates obtained and their characteristics. Whenever the enrichments were positive, usually several dozen or hundreds of colonies mostly of similar color and morphology appeared. Based on colony appearance and on microscopic inspection of the cells, the most diverse isolates, between five and ten different ones per enrichment were chosen for further purification and characterization. Purified isolates were directly subjected to PCR analysis with Archaea-specific primers and the PCR products were cloned and compared by

Table 2. Enrichment conditions and characterization of cultivated halophilic microorganisms

Source	pH	Media[1]	Carbon source	Colony color	Morphology	Representative isolate	Affiliation
DIE	6	Ha, overlay	Peptone 0.15%	Red/orange	Small cocci	DIE42	*Haloarcula*
DIE	11	Na	Peptone 0.15%	Red	Small cocci in packages of ≥2	DIE43	*Natronococcus*
DIE	11	Na	Peptone 0.15%	White	Pleomorphic small rods	DIE41	*Halorubrum*
EMF	11	Na	Yeast 0.1%, caa 0.075%	nd	nd	EMF51	*Natronomonas*
EMF	11	Na	Yeast 0.1% caa 0.075%	Red	Cocci in packages	EMF53	*Natronococcus*
EMF	11	Na	Yeast 0.1% caa 0.075%	Red	Motile rods	EMF54	*Haloarcula*
FC	8	Fc	Ferrous-citrate 0.15%	Light red	nd	FC6	*Halorubrum*
FC	8	Fc	Tryptone/yeast 0.001%	Red	nd	FC6.1	*Halorubrum*
FC	8	Fc	Organic acids 0.1%	Light red	nd	FC6.2	*Halorubrum*
FC	8	Fc	Tryptone/yeast 0.001%	Orange	nd	FC2	*Haloarcula*
FC	8	Fc	Organic acids 0.1%	Light red	nd	FC2.1	*Haloarcula*

Isolates were cultivated on agar plates (or in soft agar) at 42°C for 7 to 14 days. Organisms from DIE and EMF were enriched in liquid culture prior to plating on solid media

[1] Ha, modified *Haloarcula* medium; Na, modified *Natronobacterium* medium; Fc, medium designed from the chemical analysis of sample FC; caa, casamino acids; nd, not determined

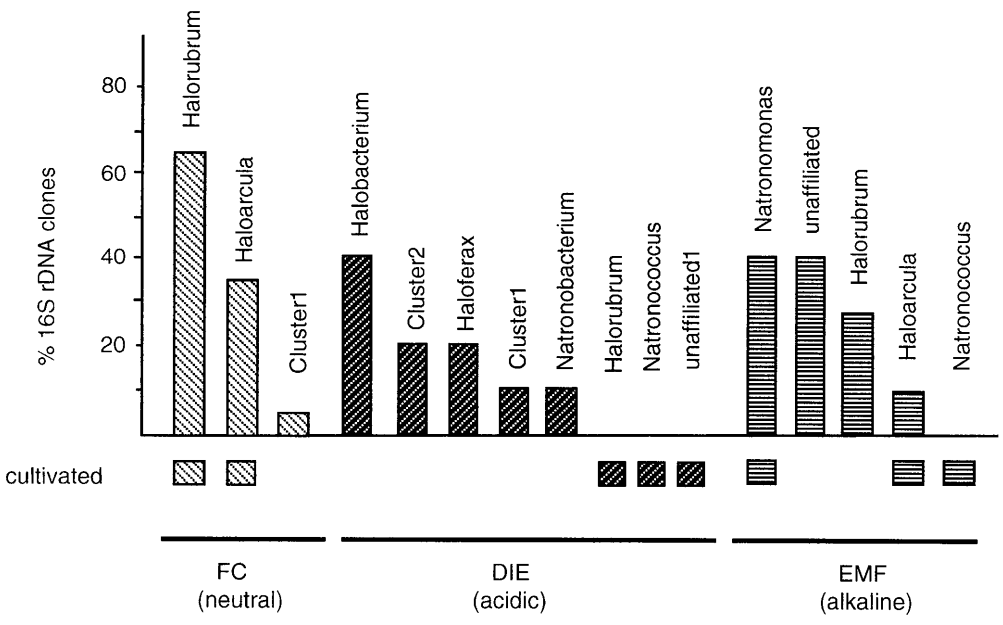

Fig. 2. Comparison of 16S rDNA survey and cultivation approach. The affiliation of sequences with known genera of Archaea or with novel clusters is based on the phylogenetic reconstructions calculated in Fig. 1

restriction analyses. The 16S rDNA sequences of selected fragments were subsequently determined. Phylogenetic placement of the cultivated isolates is shown in Fig. 1 (boxed signatures) and in Fig. 2. We identified exclusively archaea but no bacteria in our enrichments. Strains belonging to the genus *Halorubrum* were obtained from samples FC and DIE, *Natronococci* were obtained from EMF and DIE and relatives of *Natronomonas* were isolated from the EMF sample. Relatives of *Haloarcula* were isolated from EMF and FC and some more distantly related isolates to this genus were also obtained from DIE (DIE 42 with 92%–93% sequence similarity to species of *Haloarcula*, see Table 2 and Fig. 1).

Discussion

The aim of this study was to describe the diversity of archaea in different hypersaline habitats in a culture-independent molecular phylogenetic approach and to compare these results to the diversity of halobacterial cultures obtained from the same samples by classical enrichments.

Phylogenetic analyses suggest that archaea adapted to hypersaline environments form a monophyletic group within the kingdom of the *Euryarchaeota*, the *Halobacteriaceae*, which comprises 14 different genera. Culture-independent, molecular phylogenetic studies of hypersaline

habitats have indicated more phylotypes and novel lineages within the *Halobacteriaceae* (Benlloch et al. 1995; Munson et al. 1997; Grant et al. 1999; Cytryn et al. 2000; Bowman et al. 2000). However, additional sequences were recently isolated from anaerobic, hypersaline environments that cluster within the *Euryarchaeota* but not within the *Halobacteriaceae*, suggesting that adaptation to high-salt environments might be more widespread in the archaeal domain (Eder et al. 1999; Cytryn et al. 2000). Some of these novel sequences were isolated from methane- and sulfide-rich habitats, suggesting that the respective organisms could represent halophilic methanogens (Cytryn et al. 2000).

Almost all sequences obtained in our study by 16S rDNA amplification from the environment grouped within the *Halobacteriaceae*, either within the genera of cultivated halophilic archaea or within clusters detected earlier in environmental surveys. Only two sequences were identified with a different affiliation. These sequences grouped within the *Crenarchaeota* together with a number of environmental clones obtained from soil samples in Northern America (Bintrim et al. 1997; Buckley et al. 1998) and Germany (Sandaa et al. 1999; T. Ochsenreiter and C. Schleper, unpublished). Since our sample was taken from a small salt pond that had formed on the bottom of a slag heap in direct contact with soil, we suppose that the crenarchaeal sequences most probably do not stem from halophilic organisms but rather from archaea typically found in soil. The finding of these sequences also demonstrates that our PCR primers and conditions were suitable to amplify very diverse archaeal sequences.

The microbiota of crystallizer ponds from solar salterns have been well studied by culture-dependent and also by molecular-phylogenetic studies. In both approaches, relatively few phylotypes have been described, supporting the view that this hypersaline environment harbors a very low prokaryotic diversity. However, the organisms detected as dominant representatives by 16S rDNA analysis and through fluorescent in situ hybridization (Benlloch et al. 1995; Antón et al. 1999, 2000) were different from those isolated through classical cultivation (mostly species of *Halorubrum* and *Haloarcula*; Benlloch et al. 1995, 2001).

In contrast, in our analysis of the salt pond sample FC we found that with one exception (FCII3, which groups in cluster 1) organisms of the same genera, i.e., *Halorubrum* and *Haloarcula*, were detected with both the culture-independent study and the cultivation approach (Fig. 2). We assume that these organisms, which were cultivated in medium based on the salt composition of the collected sample, might represent an actively growing and perhaps dominant population in the natural habitat. In order to draw conclusions on the abundance of specific phylotypes, direct cell counting using in situ hybridization (FISH) with specific probes will be needed. Recently, Antón et al. (1999) have shown by FISH analysis that the dominant archaeon in crystallizer ponds of a solar saltern in Alicante, Spain, was the square-shaped gas-vesicle-containing prokaryote detected earlier by Walsby (1980). Although we have occasionally observed square-shaped prokaryotes during microscopic inspection, they did not seem to represent a dominant fraction in our sample. Furthermore, the SPhT phylotype described by Antón et al. was not amplified in our study, although the primers used for PCR amplification perfectly match the SPhT sequence. We have also not found any sequences that are specifically related to the SPhT phylotype and therefore have to conclude that the prokaryotic community in our sample is of different composition than that characterized by Antón et al. (1999).

In contrast to our findings from the solar saltern sample, quite different results were obtained with sample DIE, which was collected on the bottom of a slag heap from a potassium mine. This sample harbored the greatest diversity of 16S rDNA sequences with a relatively even distribution of phylotypes into five different genera or clusters (*Haloferax*, *Halorubrum trapanicum*/*Natronobacterium*, *Halobacterium*, cluster 1, and cluster 2). However, none of the cultivated isolates was related to the sequences found in the culture-independent approach (Fig. 2). They were affiliated with the genera of *Halorubrum* and *Natronococcus* or were unaffiliated (DIE42). This finding indicates that our molecular study was not comprehensive enough to cover the naturally occurring diversity. Also, bias in the PCR approach might have been introduced by incomplete cell lysis, e.g., of *Natronococcus* species that are difficult to break open. The larger diversity of sample DIE might be explained by a relative instability of this habitat compared to the crystallizer pond (FC) and the alkaline lake (EMF). Temperature, pH, or nutrient supply could change more frequently in this small biotope and give rise to the proliferation of differently adapted microorganisms. Interestingly, no colonies were recovered by direct plating of the original sample, whereas several isolates were obtained after plating from primary enrichments that had been set up in liquid media.

We obtained a mixed result for the alkaline lake sample EMF. Representatives of two of the four genera predicted by the molecular phylogenetic approach were recovered through cultivation together with a third genus, *Natronococcus*, that was not seen in the phylogenetic survey.

Of the six genera containing alkaliphilic organisms (*Natronobacterium*, *Natronococcus*, *Natronorubrum*, *Natronomonas*, *Halorubrum*, and *Natrialba*) only two were represented in our 16S rDNA survey (*Natronomonas* and *Halorubrum*). Other sequences grouped with *Haloarcula* or were unaffiliated (EMF3 in Fig. 1), predicting that alkaliphilic organisms should be more diverse. In fact, besides members of the genus *Natronomonas* and *Natronococcus*, relatives of *Haloarcula* were also cultivated from media at pH 11 (Table 2). It might not be too surprising that many of the 16S rDNA sequences were more closely related to sequences of non-alkaliphilic microorganisms. Also within the Eubacteria, the diversity of alkaliphilic organisms is high and alkaliphilic representatives are found in very diverse lineages (Jones et al. 1998). These observations suggest that the adaptation to alkaliphilic environments has evolved several times independently. Recently two novel lineages, one belonging to the *Halobacteriaceae*, but the second one being only distantly related to known Euryarchaeota, were detected in an alkaline saltern of Lake Magadi (Grant et al. 1999).

In summary, our study indicates that the culturability of a specific group of microorganisms detected by molecular

approaches is not easily predictable and might depend on the habitat chosen. We were able to obtain many isolates of genera that were seen in the phylogenetic study, even those including novel phenotypes such as the alkaliphilic species belonging to the genus *Haloarcula*. On the other hand, we have not been able to cultivate representatives of the novel lineages that have appeared in this and other molecular studies, (i.e., cluster 1 and 2 in Fig. 1) and that are exclusively represented by environmental sequences.

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