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Novel haloarchaeal 16S rRNA gene sequences from Alpine Permo-Triassic rock salt

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Abstract Prokaryotic diversity in Alpine salt sediments was investigated by polymerase chain reaction (PCR) amplification of 16S rRNA genes, sequencing of cloned products, and comparisons with culturable strains. DNA was extracted from the residue following filtration of dissolved Permo-Triassic rock salt. Fifty-four haloarchaeal sequences were obtained, which could be grouped into at least five distinct clusters. Similarity values of three clusters to known 16S rRNA genes were less than 90%–95%, suggesting the presence of uncultured novel taxa; two clusters were 98% and 99% similar to isolates from Permo-Triassic or Miocene salt from England and Poland, and to *Halobacterium salinarum*, respectively. Some rock salt samples, including drilling cores, yielded no amplifiable DNA and no cells or only a few culturable cells. This result suggested a variable distribution of haloarchaea within different strata, probably consistent with the known geologic heterogeneity of Alpine salt deposits. We recently reported identical culturable *Halococcus salifodinae* strains in Permo-Triassic salt sediments from England, Germany, and Austria; together with the data presented here, those results suggest one plausible scenario to be an ancient continuous hypersaline ocean (Zechstein sea) populated by haloarchaea, whose descendants are found today in the salt sediments. The novelty of the sequences also suggested avoidance of haloarchaeal contaminants during our isolation of strains, preparation of DNA, and PCR reactions.

Key words Archaea · Haloarchaea · 16S rRNA gene · Phylogeny · Salt mines · Alpine rock salt · Zechstein sea · Prokaryotic longevity

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Introduction

Extremely halophilic bacteria grow optimally in media containing 2.5–5.2 M NaCl (Kushner and Kamekura 1988); they comprise both (eu)bacterial and archaeal genera (Kamekura 1998). These bacteria are found in hypersaline environments such as the Dead Sea, the Great Salt Lake, sabkhas, and natural or artificial salterns (Javor 1989). During several periods in the Earth's history, massive sedimentation of halite and other minerals from hypersaline seas took place; an estimated 1.3 million km^3 of salt was deposited in the late Permian and early Triassic alone (ca. 245–280 million years ago) (Zharkov 1981).

The isolation of viable extremely halophilic microorganisms from subterranean salt deposits of Permo-Triassic age has again come into focus during the past decade and has been reported by Norton et al. (1993), Stan-Lotter et al. (1993), Denner et al. (1994), and Stan-Lotter et al. (1999, 2000). McGenity et al. (2000) recently reviewed the evidence for and against long-term survival of halophilic prokaryotes in ancient salt sediments; potential mechanisms for longevity were discussed in the review by Grant et al. (1998). Most of the strains from rock salt described to the present now appear to belong to the Haloarchaea, with the possible exception of a single *Bacillus* isolate (Vreeland et al. 2000). The study of these unique microorganisms could provide insights into the basis for prokaryotic long-term dormancy. In addition, the recent discoveries of macroscopic halite crystals, water, and traces of KCl in meteorites (Zolensky et al. 1999; Whitby et al. 2000) have extended interest in halobacterial longevity to the search for extraterrestrial life.

Haloarchaea are not known to form spores; thus, it remains to be proven how they can survive for extended times. To approach this question, it is necessary to conduct a survey of the indigenous haloarchaeal community in rock salt. Are only certain types of haloarchaea capable of long-term survival, or could this be a widespread phenomenon? From previous reports (see foregoing and references in Vreeland et al. 1998), it became clear that often only a few or sometimes no microorganisms can be cultured from a

given rock salt sample; in addition, most haloarchaeal isolates grow very slowly on solidified medium, forming visible colonies only after several months of incubation. A faster evaluation of microbial diversity in an environment might be obtained by polymerase chain reaction (PCR) amplification of diagnostic molecules, such as the 16S rRNA genes, and subsequent sequencing of cloned products. This technique obviates culturing of microorganisms and has permitted the detection of novel and unexpected phylogenetic groups, e.g., in ocean samples (Giovannoni et al. 1990; DeLong 1992); in several clusters, some of them deeply branching, of members of the *Halobacteriales* in Antarctic hypersaline lakes (Bowman et al. 2000); and an abundant, but as yet still uncultured, haloarchaeon in a Spanish saltern (Benlloch et al. 1995) that might be the famous square bacterium described by Walsby (Walsby 1980; Anton et al. 1999).

We report here for the first time an analysis of the microbial community in several Alpine Permo-Triassic rock salt samples by PCR amplification of 16S rRNA genes.

Materials and methods

Geologic setting

Alpine salt evaporites formed mainly during the Permian and Triassic periods and were overlaid by a succession of marine carbonates, in addition to layers of clay, from the Middle Triassic through the Tertiary period (Zharkov 1981; Sonnenfeld 1984). The folding of the Alps, which started about 100 million years ago (Einsele 1992), raised the salt sediments, and the originally horizontal deposits became salt mountains, which are found mainly in the northeast Alps (Fig. 1). The salt sediments are located at altitudes up to 1,100 m in the so-called Haselgebirge, a tectonic mélange of rocks consisting of calcium sulfates, halites, carbonates, sandstones, and volcanic materials (Sonnenfeld 1984; Spötl and Hasenhüttl 1998).

Complex tectonics often caused deformations and interspersing with large rock accumulations (Fig. 2). The clay and carbonate layers largely prevented the washing-out of the salt during the heavy precipitations of the ice ages. Alpine salt deposits vary in thickness from 250 to 700 m; they contain up to 97% halite, some anhydrite, polyhalite, and traces of iron and other metal ions (Schauberger 1986). The age determination of salt deposits was based on palynological and isotope studies, in connection with stratigraphic information. Klaus (1955, 1974) detected, in dissolved Alpine rock salt, plant spores from extinct species that exhibited well-preserved morphological features and thus could date the sediments to the Upper Permian or the Triassic. Determination of sulfur isotope ratios, which is used for evaporites containing sulfates of marine origin (Holser and Kaplan 1966), confirmed a Permian or Triassic age for the Alpine salt deposits (Pak and Schauberger 1981).

Rock salt and preparation of samples

Samples were obtained from salt mines that are still in operation (Fig. 1), such as Bad Ischl-Perneck (Fig. 1, Fig. 2) and Altaussee, both in Austria, and Berchtesgaden in Germany. The air in salt mines can probably be considered devoid of halophilic microorganisms (McGenity et al. 2000). Two types of samples were used. (1) Pieces of visibly stratified rock salt from a depth of about 650 m were taken in a newly created tunnel in Bad Ischl-Perneck 3 days after blasting operations. The tunnels were dry and there was no evidence for salt efflorescence on the walls, which suggested the absence of salt recrystallization as a result of moisture. These samples were designated BI 2.Hor; they consisted of 93%–97% (by weight) of halite and weighed 450–550 g. (2) Cylindrical bore cores were produced by deep drilling for evaluation of the salt content of sediments (total depth below surface, 700–900 m) from the salt mines in Altaussee and Berchtesgaden. Bore cores were 50 or 80 mm in diameter and of various lengths; weight was at least 180 g.

Both types of samples were thoroughly surface-sterilized by flaming them on all sides with a Bunsen gas burner. Rais-

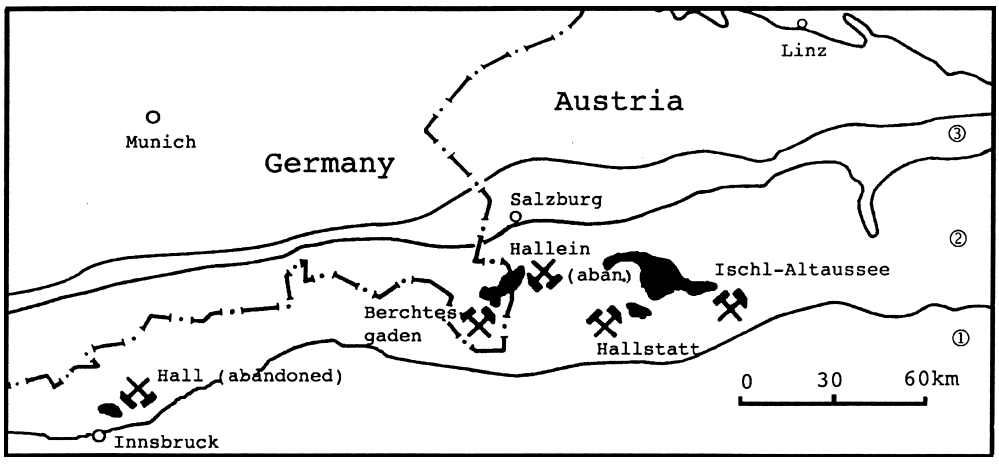

Fig. 1. Locations of several Alpine Permo-Triassic salt deposits and salt mines, some of them abandoned, in Austria and Southern Germany. Salt deposits are depicted in *black*. ①, Central Alps; ②, northern calcareous Alps; ③, Helvetic zone

ing of the temperature of salt pieces during flaming was estimated with a handheld noncontact infrared thermometer (Quicktemp 825-1; Testo, Vienna, Austria). Salt samples were dissolved slowly by adding sterile neutralized water and shaking at 23°C, keeping the solution always close to saturation. These procedures, as well as the DNA extraction and preparations for PCR reactions, were carried out under sterile conditions in a safety hood. Portions of some salt solutions were shaken for 10–12 days at 37°C on a rotary shaker (80 rpm), following addition of 0.05% (w/v) yeast extract (Difco, Augsburg, Germany) and 0.05% (w/v) Hycase (Sigma, St. Louis, MO, USA). Sample aliquots of 12 ml were centrifuged at 6,000 *g*; the pellets, which consisted mainly of undissolved mineral grains, were suspended in about 1 ml 25% (v/v) glycerol in 3 M NaCl and were kept frozen (–70°C) until further use.

DNA extraction from the microbial community in rock salt

The extraction procedure was modified from Benlloch et al. (1996) as follows: 30–250 ml of a solution of dissolved rock salt was passed through a 0.22-µm-pore-size autoclaved membrane filter (Durapore filters; Millipore, Bedford, MA, USA), using a filtration unit (SM 16201/19/20; Sartorius, Vienna, Austria) with low-pressure vacuum. Alternatively, frozen pellets (see foregoing) were diluted 1:10 with 50 mM Tris-HCl/4 M NaCl buffer, pH 7.4, and passed through the Durapore filter. Following storage at –70°C for several days, filters were thawed, cut into small pieces with a sterile scalpel, and vortexed in 5 ml TE buffer [100 mM Tris-HCl, 10 mM ethylenediaminetetraacetic acid (EDTA), pH 8.0], containing 1% sodium dodecyl sulfate (SDS) (w/v), per halved filter.

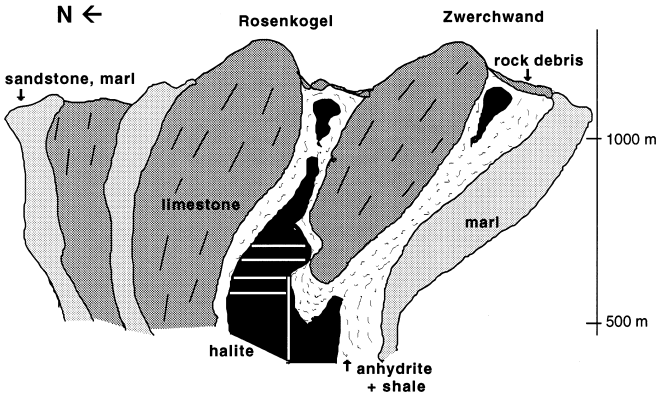

Fig. 2. North–south profile of the salt mountain near Bad Ischl-Perneck, Austria. Several types of rocks are folded together; stratification of some rocks is indicated. Halite is depicted in *solid black*. *Horizontal and vertical straight white lines* indicate tunnels and shafts in the salt mine. (Modified from Mayrhofer 1955)

Subsequently, acid-washed glass beads were added; samples were vortexed for 2 min and incubated at 37°C for 1 h after addition of 50 µl lysozyme (Sigma; 1% w/v in TE buffer). Following boiling for 10 min and vortexing for 2 min, 1 ml lysis buffer (4% SDS in 50 mM Tris-HCl, 100 mM EDTA, pH 8.0) and 40 µl proteinase K (Sigma; 10 mg/ml stock solution) were added. Samples were vortexed for 1 min and incubated at 56°C for 1 h. To enhance lysis, a thermal shock was applied consisting of storage at –70°C for 5 min and boiling for 5 min. DNA extraction was carried out by adding 1 vol phenol (saturated with 10 mM Tris-HCl, 1 mM EDTA buffer, pH 8.0), and 1 vol chloroform to the lysate. The solution was centrifuged for a few seconds and the watery phase was taken off carefully; 0.1 vol 2 M sodium acetate, pH 4.8, and 3 vol ethanol (96%) were added, and the samples were divided into aliquots of 2 ml and kept overnight at –20°C. After centrifuging at 12,800 *g* for 45 min at 4°C and removal of the supernatant, pellets were air-dried. The DNA was further purified using the GENE CLEAN II kit (Bio-101, Vista, CA, USA) according to the instructions of the manufacturer.

PCR amplification of 16S rRNA gene fragments

Enzymatic amplification of 16S rRNA gene fragments from the extracted community DNA was performed using Archaea-specific oligonucleotide primers Arch21F (forward) and Arch958R (reverse) (DeLong 1992; Table 1). Two total community DNA extractions served as template for

Table 1. Oligonucleotide primers used for polymerase chain reaction (PCR) amplification and sequencing reactions of archaeal 16S rRNA genes used in this study

Primer	Orientation	Sequence (5′→3′)	Target site	Source or reference
Arch21F	Forward	TTCCGGTTGATCCYGCCGGA	2–21	DeLong (1992)
Arch958R	Reverse	YCCGGCGTTGAMTCCAATT	958–976	DeLong (1992)
U519R	Reverse	GWATTACCGCGGCKGCTG	519–536	Munson et al. (1997)
HF2	Forward	GACGGTGGGGTAACGG	251–265	This work
HF3	Forward	GCGGTAATACCGGCAG	527–539	Stan-Lotter et al. (2000)
HF4	Forward	AACCGGATTAGATACCC	782–795	This work
HR6	Reverse	GGGCCGTTACCCCACC	255–269	This work
Eubac 27F	Forward	AGAGTTTGATCMTGGCTCAG	8–27	DeLong (1992)
Eubac 1492R	Reverse	TACGGYTACCTTGTTACGACTT	1492–1513	DeLong (1992)

Y, M, W, and K indicate C or T, A or C, A or T, and G or T, respectively
Target sites are based on the *Escherichia coli* sequence numbering according to Brosius et al. (1981)

three amplification reactions each, which were performed in a 50-µl reaction volume in a programmable thermal cycler (Biometra, Göttingen, Germany). The reaction mixtures were composed of 50 pmol of each primer, 12.5 nmol deoxynucleotides, 1.5 mM $MgCl_2$, 1 unit Taq DNA polymerase, appropriately diluted DNA polymerase buffer B (both from Promega, Vienna, Austria), and 0.1–1 ng community DNA. Thermal cycling consisted of a primary heating step (94°C, 5 min), followed by 25 cycles (Wang and Wang 1997) of denaturation at 95°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 2 min. Positive controls containing genomic DNA of *Haloferax mediterranei* DSM 1411^T were included along with negative controls, which contained genomic DNA of *Escherichia coli* K12 (DSM 6255), no template DNA, or no polymerase, respectively. PCR reactions with bacteria-specific primers (see Table 1) were carried out as described by DeLong (1992).

Cloning of PCR products

The six amplification reaction mixtures were fractionated by agarose gel electrophoresis; the PCR products were excised and recovered from the gel slices using the NucleoTrap kit (Macherey and Nagel, Düren, Germany) in accordance with the manufacturer's instructions. Purified PCR products were pooled, ligated into vector pGEM-T, and transformed into competent *E. coli* JM 109 (both from Promega) as recommended by the manufacturer. To screen for the presence of inserts, a small amount of each colony was suspended in 30 µl sterile water and 2 µl of this suspension was used as template for a PCR reaction, similar to the method described by Güssow and Clackson (1989); primers and PCR protocol were as described above. Clones that contained the desired insert were grown overnight in 1–2 ml of Luria-Bertani (LB)-Amp medium, and their cell pellets were stored at –70°C until further usage.

Sequencing and phylogenetic analysis of clones

Plasmids of 54 randomly selected PCR-positive clones were purified with the GFX Micro Plasmid Prep Kit (Amersham Pharmacia Biotech, Piscataway, NJ, USA). Partial insert sequences were obtained with primer U519R (Munson et al. 1997; see Table 1). Sequences were determined by automated dideoxynucleotide methods with the ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit on an ABI Prism 310 Genetic Analyzer (both from Perkin-Elmer Applied Biosystems, Foster City, CA, USA) as recommended by the manufacturer. Searches for the closest relatives of the novel sequences were performed using the Fasta3 (Pearson and Lipman 1988) web interface from GenBank and the BlastN (Altschul et al. 1990) web interface from the European Molecular Biology Laboratory (EMBL). The novel sequences were aligned with Clustal X (Thompson et al. 1997). The same program was used to generate a distance matrix of the region corresponding to nucleotides 90–345 (*E. coli* numbering; Brosius et al. 1981), which was the basis for assigning the clone sequences to sequence clusters (homology less than 95%).

From each of the detected sequence clusters, one to four clones were chosen, and both strands of the inserts (917–919 bp) were sequenced using primers shown in Table 1 or directed against vector sequences. The potential presence of chimerical sequences was examined with the CHIMERA_CHECK program available through the Ribosomal Database Project II (RDP), release 8 (Maidak et al. 2000), and also by looking for taxa that changed positions in neighbor-joining trees based on sequences of 250 nucleotides from both the 5′- and the 3′-ends, similar to the procedure described by Wang and Wang (1997). Based on a subset of aligned archaeal sequences obtained from the RDP, an alignment was created that consisted of 16S rRNA gene sequences from representatives of all known haloarchaeal genera, of strains isolated from salt mines (e.g., brines and rock salt), and of environmental haloarchaeal clones. The sequences of the inserts were fitted in the haloarchaeal alignment with Clustal X; a few minor corrections were made manually. This alignment was subjected to phylogenetic analysis with distance-matrix (Jukes and Cantor 1969), maximum-likelihood, and maximum-parsimony methods, using programs of the PHYLIP package, version 3.5.1 c (Felsenstein 1993).

Other methods

For evaluation of the presence of culturable isolates, samples of 3 ml of dissolved rock salt were streaked on agar plates of 145 mm diameter containing M2 (Tomlinson and Hochstein 1976) or M2A solidified medium (Denner et al. 1994) and incubated at 37°C for 6–10 weeks. Subsequent storage of plates was at 6°C for 12 months or more. Twelve to 15 agar plates were used with each dissolved rock salt sample.

Accession numbers

Full-length insert sequences (917–919 bp) obtained in this study were deposited in the EMBL database under accession numbers AJ278931–AJ278942 (see Table 2 for assignments).

Results

Culturable and uncultured strains

Brief incubation of dissolved rock salt in the presence of dilute nutrients (see Materials and methods) was used in an attempt to dislodge adsorbed microbial cells from small particles of mineral grains that were present in the rock salt solutions and could be observed microscopically (Wieland and Stan-Lotter, unpublished results). This treatment resulted in consistent yields of DNA when the extraction procedure, which comprised filtering of the material and subsequent processing of the filters (see Materials and

Table 2. Sequence similarities of cloned 16S rRNA gene sequences from Bad Ischl rock salt to sequences available in databases

Cluster	Clone designation (accession number)	Most similar 16S rRNA gene sequences				
		Name of strain, organism, or environmental clone	Similarity (%)	Origin	Geological age (Ma)	Reference
I	A148 (AJ278934), A153 (AJ278936),	004.1 (strain), WM2C.13 (strain)	>99	Winsford salt mine brine, UK	235–240	McGenity et al. (2000)
	A157 (AJ278938), A175 (AJ278940)	BbpA.1 (strain), B1bra.5 (strain)	>99	Boulby salt mine brine, UK	235–260	McGenity et al. (2000)
		PW5.4 (strain)	>99	Wieliczka salt mine brine, PL	ca. 20	McGenity et al. (2000)
II	A145 (AJ278933), A174 (AJ278939)	*Halobacterium salinarum*	>98	Salted fish	Recent	Ventosa and Oren (1996)
III	A140 (AJ278932), A177 (AJ278941), A178 (AJ278942)	1MT315 (env)	92.9–93.1	Salt marsh sediment	Recent	Munson et al. (1997)
IV	A151 (AJ278935), A154 (AJ278937)	BbpA.3 (strain), B2S.26 (strain)	91.7–92.6	Boulby salt mine. brine, UK	235–260	McGenity et al. (2000)
		Halorubrum vacuolatum	92.4–92.6	Lake Magadi, Kenya	Recent	Mwatha and Grant (1993)
V	A016 (AJ278931)	T3.1 (strain)	89.2	Khorat Basin salts, TH	60–70	Grant et al. (1998)
		2C129 (env), 2MT16 (env)	89.5–89.8	Salt marsh sediment	Recent	Munson et al. (1997)

Similarity values were calculated using Fasta (Pearson and Lipman 1988)
Ma, 10^6 years; UK, United Kingdom; PL, Poland; TH, Thailand; env, environmental sequence

methods), was carried out. Four aliquots of the samples designated BI 2.Hor (see Materials and methods) were extracted independently. Streaking of portions of the samples (BI 2.Hor), after freezing and thawing, on complex solidified medium did not yield growth within more than 1 year of incubation, except for several strains that were similar to *Halococcus salifodinae* on the basis of colonial pigmentation, cellular morphology, and whole-cell protein patterns (Stan-Lotter et al. 1999).

As noted earlier, these halococci are comparatively fast-growing haloarchaea that produce visible colonies after 4–6 weeks (Stan-Lotter et al. 1999). For the purposes of this report, the novel (nonhalococcal) sequences we describe here are to be considered as stemming from strains as yet uncultured. From the bore core samples from Altaussee and Berchtesgaden, no colonies or very few colonies on agar plates were obtained, amounting to a total of not more than 10 cfu/kg of rock salt, as was observed previously (Stan-Lotter et al. 2000). No amplifiable DNA was obtained from these latter samples with eight independent extraction experiments. We deliberately used rather large samples (see Materials and methods) and surface-sterilized them by thorough flaming; heat transfer during this treatment, as measured with a noncontact instrument, was not more than approximately 8°C across a thickness of 5 cm and thus would have contributed only insignificantly to a destruction of biomass within the rock salt samples.

Novel sequences

By using standard primers specific for archaeal 16S rRNA genes with DNA from BI 2.Hor, we obtained a single PCR product of the expected size (approximately 920 bp), as judged from ethidium bromide staining. In no case did we observe the formation of PCR product in any of the negative controls (see Materials and methods). The same template did not yield a PCR product with bacteria-specific standard PCR primers. Purified PCR products of six independent amplifications from two DNA extractions were pooled and a clone library was constructed; 54 clones were randomly chosen for further analysis. Partial 16S rRNA sequences were determined using primer U519R for a single sequencing reaction, and the region corresponding to nucleotides 90–345 of the *E. coli* 16S rRNA gene sequence was analyzed. The partial sequences formed five clusters, which had sequence similarity values of less than 95% (data not shown). Cluster I contained 30 clones, which corresponds to 56% of the analyzed clones. The respective values for clusters II, III, and IV were 4 (7%), 15 (28%), and 4 (7%); cluster V was formed by a single sequence (1.9%).

The inserts of 12 clones representing each cluster were fully sequenced. The length of the inserts was between 917 and 919 bp. As judged by the results of the CHIMERA_CHECK program and comparison of distance matrix trees based on sequences of 250 nucleotides from the 5′- and 3′-ends, none of these sequences was a potential PCR artifact. When compared to each other, the different clusters had sequence similarities of 86.2%–89.5%; only clusters I and II were more closely related (approximately 96% sequence similarity). Sequences forming one cluster had similarities of 99.1%–99.9%. By comparison with sequences available in the EMBL and GenBank databases, all five clusters could be affiliated with the *Halobacteriaceae* (Table 2). Cluster I was more than 99% similar to strains isolated from brines in British (strains BbpA.1, B1bra.5, 004.1, and WM2C.13) and Polish (strain PW5.4) salt mines, which were isolated by W.D. Grant and coworkers (Grant et

al. 1998; McGenity et al. 2000); similarities to *Hb. salinarum* were somewhat lower (96%). Cluster II shared more than 98% identity with this latter strain. In phylogenetic trees that were constructed using distance-matrix/neighbor-joining (Fig. 3), maximum-likelihood, and maximum-parsimony methods (not shown), cluster II was placed close to *Hb. salinarum,* and cluster I formed, together with strains from salt mine brines, a distinctive branch within the *Halobacterium* branch.

Sequence similarities of the remaining three clusters to known 16S rRNA gene sequences were less than 94%. The sequences that represented cluster III were related most closely to environmental sequences recovered from salt marsh sediment samples (clone 1MT315) (Munson et al. 1997; see Fig. 3), with which they shared approximately 93% sequence similarity; the closest cultured relatives were *Haloferax* and *Halogeometricum.* Cluster IV was located in one branch with *Halorubrum* and strains from a brine in Boulby salt mine (strains BbpA.3 and B2S.26; McGenity et al. 2000); sequence similarities with these strains were in the range 91.7%–92.6%. Similarities of clone A016, the sole constituent of cluster V, to sequences from databases were less than 90%. Its closest relatives were environmental clones from coastal salt marsh sediments (clones 2C129 and 2MT16; Munson et al. 1997), and strain T3.1, which was isolated from a salt mine in Thailand (Grant et al. 1998).

So far, we have not detected any *Halococcus* sequence in our rRNA clone library. However, this lack was not caused by a failure of the primers since the same PCR procedure, which was used to amplify the archaeal 16S rRNA gene sequences from rock salt DNA, yielded products of the expected size with genomic DNA or whole cells of *Hc. salifodinae* DSM 8989^T as template (data not shown).

Discussion

The prokaryotic content of dissolved rock salt can be determined with ease, using the classical methods of enrichment

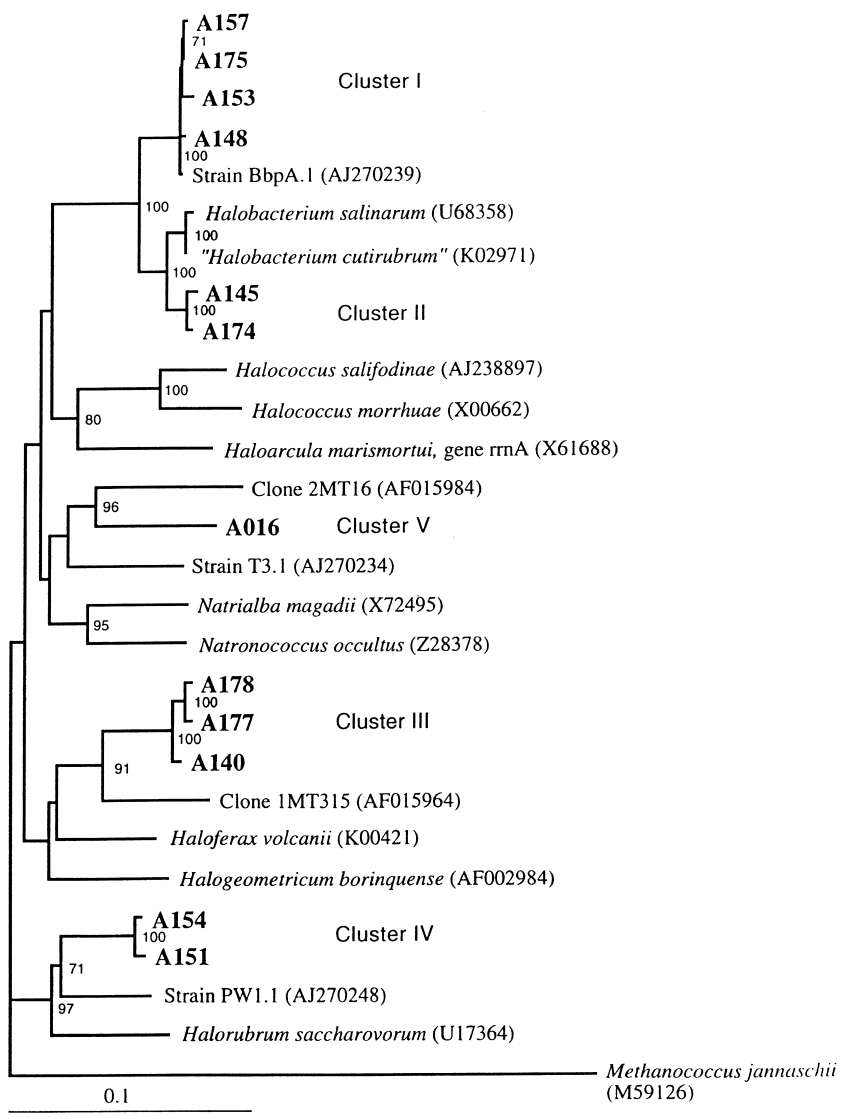

Fig. 3. Phylogenetic tree showing the relationships among 16S rRNA gene sequences from rock salt obtained in this study, which corresponded to sequence positions 2–976 of the *Escherichia coli* 16S rRNA gene sequence (Brosius et al. 1981) (*bold letters*; see Table 2 for accession numbers), and among various species of the *Halobacteriaceae* as well as strains and environmental sequences affiliated with this family (accession numbers in *parentheses*). The tree was constructed using the neighbor-joining method of Saitou and Nei (1987). Bootstrap values are indicated at *nodes* supported by more than 70 of 100 replicate trees. *Scale bar* represents 10% nucleotide sequence difference

procedures and plate counts. However, these approaches could also suffer from the phenomenon of the "great plate count anomaly" (Staley and Konopka 1985), as known from numerous environmental sites, because only a fraction of the existing subsurface microbial community can be cultured in the types of nutrient media currently used (Amann et al. 1995). To obtain more information on the true distribution and diversity of halobacteria in salt sediments, we used extraction of DNA, following filtration of dissolved rock salt, and amplification of genes with archaeal and bacterial primers by PCR. The data suggested that several novel sequences of 16S rRNA genes, which are similar to those of known haloarchaea, are present in Permo-Triassic salt sediments and that some of them, interestingly, correspond to cultured haloarchaeal isolates from ancient salt deposits in other parts of Europe.

The results corroborated our earlier finding that similar strains of *Hc. salifodinae* can be isolated from geographically distant subterranean salt deposits (Stan-Lotter et al. 1999). The salt sediments from which the samples were taken are now located far apart in different countries and at different altitudes. One hypothesis that could explain the finding of similar microorganisms, or their genes, would be the existence of a large hypersaline ocean (Zechstein sea) that was a habitat for Haloarchaea. On the evaporation of this sea, microorganisms may have become trapped in the salt crystals, possibly within fluid inclusions (Norton and Grant 1988), and certain genera that are found today might be descendants of the original populations in the sediments.

No *Halococcus* sequences were present in the 54 clones that were analyzed, although several *Hc. salifodinae* strains were isolated repeatedly from Alpine and Zechstein rock salt (Stan-Lotter et al. 1999). Similarly, Munson et al. (1997) reported detection of several haloarchaeal sequences in salt marshes, but also lack of *Halococcus* sequences. The missing *Halococcus* sequences could perhaps be ascribed to morphological changes of Archaea enclosed in rock salt or soil, such as reduction of cell size and thus loss of cells on filtering, or the presence of a thick envelope, as has been reported with haloalkaliphilic Archaea from soil (Kostrikina et al. 1991), that may preclude lysis during the DNA extraction procedure.

Fredrickson et al. (1997) did succeed in amplifying haloarchaeal 16S rRNA genes from geologically recent salt deposits, but not from the waste isolation pilot plant (WIPP) site in the Salado salt formation of New Mexico, which is of similar age as the European Zechstein sediments (Zharkov 1981). However, as discussed by the authors, heterogeneous distribution of halophiles in the rock salt and/or methodological problems might have caused this negative result.

Samples from different salt mines representing different strata of sediments gave different results; sometimes neither amplifiable DNA nor colony-forming units were obtained. This finding could result from the geologic heterogeneity of the Alpine salt sediments (see Fig. 2), differences in pressure and temperature, and variations in the influx of freshwater and resedimentation over the millennia.

Reports about the isolation of viable microorganisms from salt deposits millions of years old, or other very ancient materials, are rightfully viewed with skepticism by the scientific community because contamination of cultures, which is a constant possibility, would invalidate results. We demonstrated with the data presented here that we did not encounter any haloarchaeal contamination. Although in the same laboratory isolation and identification of Haloarchaea and halophilic bacteria are carried out continuously, requiring culturing and handling of almost all halophilic type strains in addition to unknown strains (Nguyen et al. 1999; Stan-Lotter et al. 1999, 2000), the sequences reported here were novel and the procedural control, consisting of *Hf. mediterranei* DSM 1411^T, yielded its expected sequence without a single substitution.

The isolation of viable terrestrial microorganisms from rock salt of great geologic age makes it intriguing to consider the existence of similar extraterrestrial forms of life, particularly because halite has been found in several meteorites (Zolensky et al. 1999; Whitby et al. 2000) and evidence exists for a salty ocean on the Jovian moon Europa (McCord et al. 1998). If these lines of research are to be pursued, and if extraterrestrial halite material becomes available for examination, comprehensive knowledge of terrestrial halophilic microorganisms must be established. It is evident from the data presented here, as well as from those of other reports (Munson et al. 1997; Eder et al. 1999; Bowman et al. 2000; Cytryn et al. 2000), that the picture of the diversity of Haloarchaea is as yet quite incomplete.

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