The distribution of viable microorganisms in Permo-Triassic rock salt*

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The isolation of viable haloarchaea (halobacteria) has been reported from several subsurface salt deposits of Permo-Triassic age. The study of these unique microorganisms could provide clues for an understanding of long-term dormancy. In addition, the nature and composition of a possible rock salt microbial community would be of interest, when storage of waste materials in caverns, like those produced during solution mining, is considered. However, questions remain as to whether the isolates are actually indigenous to the sediments. We have previously described a novel extremely halophilic archaeon, *Halococcus salifodinae* BIp, which was isolated from Austrian rock salt. Because this species included only one strain, we investigated other microbial isolates from similar sites. Several halococcal strains obtained from Alpine and English salt mines were identical with respect to numerous cellular, physiological and genotypic features; they were thus assigned to the same species. Other halophilic isolates showed remarkable diversity with respect to phenotypic characteristics. Total numbers of colony-forming units (cfu) were up to 1.3×10^5 per kg of dry rock salt from the salt mine at Bad Ischl, Austria. The polymerase chain reaction (PCR), which allows the identification of uncultivated microorganisms, was used here for the first time with dissolved rock salt for the amplification of 16S rRNA genes. Sequences of PCR products indicated the presence of two or more phylotypes with high homology to known haloarchaea. Certain halite samples yielded no or only few microbial isolates and no amplifiable DNA. Thus, the distribution of halobacterial strains appears to vary between different strata. Our results suggest that viable microorganisms, which belong to archaea and, in some cases, to the same species, occur in geographically widely separated evaporites of similar geological age. Furthermore, these data support the notion that haloarchaea from subterranean salt deposits may be the remnants of populations which inhabited ancient hypersaline seas.

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1. INTRODUCTION

Several reports described in the past the isolation of viable microorganisms from ancient halite deposits (for a review see Ref. 1), but only since the definition of archaebacteria (archaea) as the third domain of life (2) was it recognized that the extremely halophilic archaea make up the majority of isolates from rock salt. It has been suggested that the predominantly red-pigmented haloarchaea might be remnants of microbial populations, which inhabited hypersaline brines during paleozoic sedimentations. However, the implication of this claim - a microbial survival time of hundreds of millions of years - was frequently dismissed as unlikely, and the isolates were viewed as modern-day contaminations. Nevertheless, evidence is accumulating for the existence of subsurface microbial communities in various types of sediments, many of which are millions of years old (see 3, 4). Salt sediments are particularly suitable sources for the study of viable ancient microorganisms, since they are considered to be

rather dry, stable environments. For this reason, the tunnels and caverns in salt mines have been or are in use for temporary storage, e.g. of works of art (Altaussee, Austria), and for long-term storage of waste materials, including radioactive wastes. However, the presence of microorganisms could compromise intended storage, particularly if metabolic byproducts, such as acids, gases, or extracellular enzymes, would develop. In fact, cellulose-degrading halophiles from a subsurface formation were described recently (5); such capabilities could pose a problem for packaging materials.

We isolated several halophilic microorganisms from Permo-Triassic salt sediments in England, Austria (6, 7) and Germany (8); some isolates proved to be so similar that they were assigned to the same archaeal species. In addition, strains of this species were found repeatedly in rock salt from the Bad Ischl salt mine (8). We describe here further culturable extreme halophiles from rather large rock salt samples, which provide a representative index of the biodiversity in certain halite deposits, and we show evidence for the presence of uncultivated halophilic archaea from some sites.

2. MATERIAL AND METHODS

2.1. Sites and samples

Rock-salt pieces were from a newly opened tunnel at 500 to 600 m (2. horizon) below the surface in the salt mine of Bad Ischl, Austria. The altitude above sea level was 600 m. Cores were from a horizontal drilling (3 to 12 m) in the salt mine at Berchtesgaden, Germany, at a depth of 550 m, and from a vertical drilling at Altaussee, Austria; altitude above sea level was 850 m, depth of drilling was 110 to 120 m. Several brines were also investigated, such as drippings from a disused cavern from solution mining in Bad Ischl, which had contained brine for about 10 years; samples from the underground salt lakes in the Berchtesgaden and Altaussee salt mines, and from brines produced during solution mining in Berchtesgaden.

2.2. Isolation of microorganisms

The isolation and description of *Hc. salifodinae* was reported previously (6-8). In the work presented here similar procedures were used and are described briefly as follows: rock-salt pieces of 500 to 600 g (94 to 99 % halite) were surface-sterilized by flaming; sterile water was added in small portions until the pieces were dissolved. The salt solution was left to become saturated with respect to salt at ambient temperature (20 to 22°C). Bore cores (weight 180 to 200 g) were treated in the same way. Strains were grown in M2 medium, which contained per liter 200 g of NaCl, 5 g of yeast extract, 5 g of casamino acids, 12.1 g of Tris, 20 g of $MgCl_2$ x 6 H_20, 2 g of KCl, 0.2 g of $CaCl_2$ x 2 H_20; the pH was adjusted to 7.4 with HCl. For solid media, 20 g of agar were added per liter. Aliquots of 1 to 3 ml of the rock salt solutions were spread on M2 agar plates immediately following dissolution and incubated at 37 to 39°C. The presence of airborne microorganisms was tested by depositing 100 m^3 air onto a standard agar plate of 90 mm diameter containing M2 agar, using an air sampler (MAS 100; biomerieux). Other methods, including physiological and biochemical tests, determination of G+C contents, whole-cell protein electrophoresis and electron microscopy were performed as described previously (8).

2.3. Extraction of DNA, amplification of 16S rDNA and sequencing

Extraction of DNA from dissolved rock salt and subsequent PCR amplification of 16S rDNA were performed as described by Benlloch et al. (9), except that biomass was collected by passing 30 to 200 ml of the sample solution through 0.22 µm pore size filters. For PCR, the archaea-specific primers Arch21F and Arch958R and bacteria-specific primers Eubac27F and 1492R (10) were used. Positive controls containing whole cells of *Haloferax mediterranei* or *Escherichia coli*, respectively, and negative controls (omitting DNA) were included. Partial nucleotide sequences were determined from the 16S rDNA gene mixture by automated dideoxynucleotide methods with the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction kit, using an ABI PRISM 310 Genetic Analyzer (both from Perkin-Elmer); the same primers as above were used, in addition to primers HaloR6 (5′-GGGCCGTTACCCCACC-3′) and HaloF3 (5′-GCGGTAATACCGGCAG-3′) for the corresponding regions of the antiparallel DNA strand; these are homologous to positions 716-738 and 1199-1179, respectively, of the Ribosomal Data Base alignment, release 7.0 (11). Similarity percentages of sequences were calculated manually.

3. RESULTS

3.1. Cultivated microorganisms

Halococcus salifodinae was isolated from a salt mine in Austria and was first described by us in 1994 (6), with strain BIp representing the type strain. Subsequently it was found that independently isolated strains Br3 (from Cheshire, England) and BG2/2 (from Berchtesgaden, Germany) resembled *Hc. salifodinae* BIp in cellular and colonial morphology. *Hc. salifodinae* BIp grows in tetrads or larger clusters; this was also observed for strains BG2/2 and Br3 (Fig. 1). Cell diameter is approximately 1 µm. All strains were then analyzed in detail (8) according to the chemotaxonomic standards for haloarchaea (12). They were found to possess identical 16S rRNA gene sequences, very similar whole-cell protein patterns, which were different from those of other *Halococcus* species, similar G+C contents (62-63 mol %) and identical sequences in a 108 base-pair insertion in their 5S rRNA gene. Other similarities included composition and relative abundances of polar lipids, antibiotic susceptibility, enzymatic activities and Fourier-transform infrared spectra. Thus, their assignment to the same species, *Hc. salifodinae*, is justified. In addition, more very similar strains were isolated repeatedly from the same site in Bad Ischl, e.g. strains N1 and H2, which are also described in (8), were obtained eight years later than strain BIp; their morphological features, 16S rRNA genes, whole

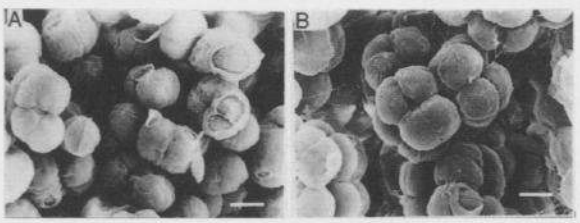

Fig.1. Scanning electron micrographs of *Hc. salifodinae* strains BG2/2 (A) and Br3 (B), grown in liquid culture. Bar: 1 µm.

cell protein patterns and phenotypical properties were identical to those of *Hc. salifodinae*. Thus it can be concluded that in geographically separated halite deposits, which are of similar age, identical species of halococci are present.

We isolated numerous other halophilic microorganisms from dissolved rock salt from the 2. horizon (Bad Ischl), all of which were pigmented. Fig. 2 shows a few typical examples. The different shapes and structures of the colonies reflect differences in morphology, growth rates and surfaces of isolates. Growth on agar plates was generally slow, taking 4 to 8 weeks, before colonies were visible, and occasionally up to 4 or 5 months. None of the isolates could grow when the NaCl concentration was less than 15%.

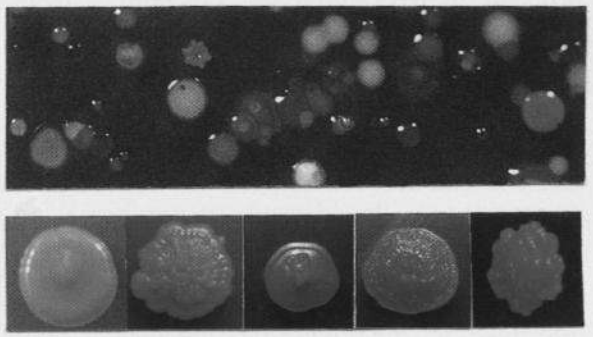

Fig. 2. Colonies (clones 1-4) of representative isolates from rock salt (2. horizon), grown on M2 agar for 3-4 months; clone 5 is a type strain (*Hc. morrhuae*). Top: colonies from cavern fluid on M2 agar.

The numbers of colony forming units (cfu) on M2 agar in samples from the 2. horizon were 130 ± 20 per g of dry rock salt (about 1.3×10^5 per kg). From microscopic observation, whole-cell protein electrophoresis patterns and preliminary physiological and sequence information, the isolates shown in Fig. 2 were tentatively assigned to the archaeal genera *Halococcus* (clones 1, 3), *Haloarcula* (clone 2), and *Halorubrum* (clone 4). While we have not characterized all isolates, we found about 50 different types of colonies on M2 agar plates, which suggests the presence of at least this number of different extremely halophilic microorganisms in the rock salt. From some other salt samples, much lower numbers were obtained; for instance, drilling cores from Altaussee or Berchtesgaden produced only about 10 – 20 cfu per g of dry salt.

Brines from various locations in salt mines, however, yielded profuse growth; Fig. 2, top, shows the culturable community from the remaining liquid in an old cavern in the Bad Ischl salt mine. This microbial population is

mixed with respect to pigmentation and colony types, suggesting the presence of both haloarchaea and bacteria. The total cell count from this sample was 10^5 to 10^6 cfu per ml, when using M2 agar medium. Similar cell numbers were found with samples from underground salt lakes, although the diversity on M2 plates was distinctly different, being dominated by whitish-pink pigmented colonies (haloarchaea of the *Haloferax*-type); in contrast, raw brine from solution mining had a cell count of about 3 x 10^2 cfu per ml; most of those colonies were pigmented red (data not shown).

3.2. Uncultivated microorganisms

PCR amplification of diagnostic molecules, such as the 16S rRNA genes, and subsequent sequencing of cloned products are increasingly used for an evaluation of microbial diversity in various environments. This technique obviates culturing of microorganisms and has permitted the detection of novel and unexpected phylogenetic groups, e.g. in ocean samples (10, 13). We applied PCR amplification to dissolved rock salt and used archaeal as well as bacterial primers (Fig. 3). Amplification products were obtained with both primers; however, the amplified archaeal rDNA was much more prominent (Fig. 3, lane 3) than bacterial rDNA (Fig. 3, lane 6). The archaeal PCR product was purified and sequenced. A mixture of at least two haloarchaeal 16S rDNA genes was present (Fig. 4). Sequences from positions 56 to 132 (*Hc. morrhuae* numbering) were largely homologous to all haloarchaeal rDNAs; they were different from bacterial rDNA (*E. coli*; Fig. 4) and also

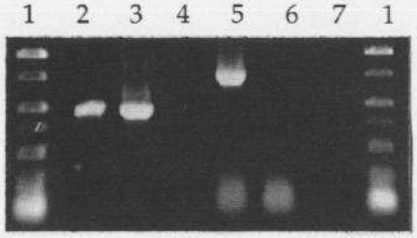

Fig. 3. Amplification of 16S rDNA from pure cultures and DNA extracted from rock salt. Lanes 2-4: archaeal primers; lanes 5-7: bacterial primers. Reactions contained the following DNA templates: archaeal DNA, *Hf. mediterranei* (lane 2), extracted DNA from dissolved rock salt from the 2. horizon (lanes 3 and 6), negative control, no DNA (lanes 4 and 7); bacterial DNA, *E. coli* (lane 5). Lane 1 contains a PCR marker; fragment sizes (in base pairs) are from top: 2000, 1500, 1000, 750, 500, 300, 150. Gels were stained with ethidium bromide.

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E.coli   GTCGAACGGTAACAGGAAGAAGCTTGCTTCTTTGCTGACGAGTGGCGGACGGGTGAGTAATGTCTGGGA-AACTGCCTGATGGAGGGGGATAACTACTG
Mc.jann  GTCAAGGG--------GCTCCCTTCGGGGAGC--------ACCGGCGCACGGCTCAGTAACACGTGGCTAACCTACCCTCGGGTGGGGGATAACCTCGG
Hr.sacc  GTCGCACG----------AGTTCAGACT----------CGTGGCGAATAGCTCAGTAACACGTGGCCAAACTACCCTTCGGAGCTCCATACCCTCGG
Hb.sali  GTTGTGCG----------GGTTTAGACC----------CGCAGCGGAAAGCTCAGTAACACGTGGCCAAGCTACCCTGTGGACGGGAATACTCTCGG
Hb.halo  GTTGTGCG----------GGTTTAGACC----------CGCAGCGGAAAGCTCAGTAACACGTGGCCAAGCTACCCTGTGGACGGGAATACTCTCGG
Hf.medi  GTTGCACG----------AGTTCACACT----------CGTGGCGAAAAGCTCAGTAACACGTGGCCAAACTACCCTACAGAGAACGATAACCTCGG
Hf.volc  GTTGCACG----------AGTTCATACT----------CGTGGCGAAAAGCTCAGTAACACGTGGCCAAACTACCCTACAGAGAACGATAACCTCGG
Ha.marA  GTCGCACG----------GGCTTAGACC----------CGTGGCATATAGCTCAGTAACACGTGGCCAAACTACCCTACAGACCGCGATAACCTCGG
Ha.marB  GTTGCACG----------AGTTTAGACT----------CGTAGCATATAGCTCAGTAACACGTGGCCAAACTACCCTACAGACCGCAATAACCTCGG
Ha.sinB  GTTGCACG----------AGTTTAGACT----------CGTAGCATATAGCTCAGTAACACGTGGCCAAACTACCCTACAGACCGCGATAACCTCGG
Ha.sinA  GTTGCACG----------AGTTTAGACT----------CGTAGCATATAGCTCAGTAACACGTGGCCAAACTACCCTACAGACCGCGATAACCTCGG
Hc.morr  GTTGTACG----------GGTTTAGACC----------CGTAGCAAATAGCTCCGTAACACGTGGCCAAACTACCCTCTGGACCGGGATATCCTCGG
Hc.sali  GTCGCACG----------GGCTCAGACC----------CGTGGCGAATAGCTCCGTAACACGTGGTCAAACTACCCTCTGGACTGGGATACCCTCGG
2ndhor   GTYGTGCG----------RGTTYAGACY----------CGYRGCGKAWAGCTCAGTAACACGTGGCCAAACTACCCTGYRGAYGGGAAYAAYCTCGG
         ** x  x*           x o x xx           xx  **  * x*o*o ****oooo*** xo*x**o**oo   *x     * *  oo*o*
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Fig. 4. Alignment of a 16S rDNA region from *E. coli* (positions 61 to 158), *Methanococcus jannaschii* (positions 57 to 139) and members of *Halobacteriaceae* with the sequenced PCR product from DNA extracts (2. horizon). Organisms and accession numbers are (from top): *E. coli*, V00348; *Mc. jannaschii*, U67517; *Halorubrum saccharovorum*, U17364; *Halobacterium salinarium*, X92978; *Halobacterium halobium*, M11583; *Haloferax mediterranei*, D11107; *Haloferax volcanii*, K00421; *Haloarcula marismortui*, rRNA gene A, X61688; *Haloarcula marismortui*, rRNA gene B, X61689; *Haloarcula sinaiiensis* rRNA gene B, D14130; *Haloarcula sinaiiensis* rRNA gene A, D14129; *Halococcus morrhuae*; D11106; *Halococcus salifodinae*, Z28387; 2ndhor, PCR product of DNA isolated from rock salt, this work. Residues which are identical in all sequences (*), in all haloarchaeal sequences (x), in all archaeal sequences (o), and gaps (-) are indicated. Y, C or T; R, A or G; W, A or T; K, G or T.

from another archaeal rDNA (*Methanococcus jannaschii*, Fig. 4). It is remarkable, that all ambiguous bases (denoted by Y, R, W or K) of the 2. horizon sequence did not differ from haloarchaeal bases, whichever nucleotide was considered. A second sequence from positions 502 to 925 was also highly homologous to those of haloarchaea (not shown); counting different nucleotides, it was 93 to 96 % similar to the sequences from *Hc. morrhuae* or *Hr. saccharovorum*. Although these data are preliminary, since only partial 16S rDNA sequences were determined and PCR products had not been separated by cloning, they strongly suggested the presence of at least two species in dissolved rock salt, which belong to the haloarchaea.

4. DISCUSSION

4.1. Microbial content of salt deposits

Two methods were applied to the analysis of microbial biodiversity in rock salt samples from halite deposits and in brine samples from mine areas, culture- and non-culture (PCR-) based techniques. Both are designed such as to provide a picture of the natural microbial community, without prior enrichment of organisms. Results obtained with either method indicated the presence of a sizable community of extremely halophilic microorganisms, most of them haloarchaea, in the investigated sites. Culturable microorganisms exhibited differences in pigmentation, cell sizes and shapes, whole-cell protein patterns and other phenotypical characteristics. Thus the diversity of the viable rock salt flora appeared to approach that of halophile communities in salt lakes in various parts of the world (14). The possibility of airborne contamination of rock salt samples can be ruled out, because we tested air samples in various places in the mines, and no haloarchaeal colonies were found. The minimum number of extremely halophilic colony forming units (cfu) was about 130 per g of salt from the 2. horizon in the salt mine near Bad Ischl. If this is typical of the microbial distribution in salt, then only about 40 cfu would be present per ml of dissolved rock salt. On standard agar plates, often only 100 µl or less is applied. If in addition growth is slow – in the order of months – such plates would likely be discounted. These factors may have contributed to the opinion of some workers, who viewed halite as sterile (see citations in Ref. 5, 15).

A predominant haloarchaeon in several sites was *Hc. salifodinae;* based on extensive chemotaxonomic and molecular characterization, strains of this species were identified independently in samples from Alpine rock salt and from solution-mined brine, derived from Northwich halite in the UK, which is of similar geological age (8, 16). *Hc. salifodinae* has so far not been isolated from a surface hypersaline environment; while it is too early for a definite conclusion about its true native environment, it is tempting to speculate that it might be a marker organism for ancient salt deposits from a certain geological period.

The presence of uncultivated cells in dissolved rock salt was demonstrated by amplification of 16S rRNA genes, which yielded at least two partial sequences with strong homology to haloarchaeal sequences. In some rock salt samples, such as drilling cores, no or only very few culturable cells were found, and no amplifiable DNA. It is likely that these salt samples contained too little material for the DNA extraction procedure, since there are precipitating steps, which might not work with only a few molecules. It is worth exploring the reasons behind the failures to grow cultures and to obtain detectable DNA. Perhaps they can be ascribed to geological reasons – e.g. raising of temperature beyond biological extremes during tectonic movements, as has been suggested (17), or to biological reasons, e.g. lack of nutrients.

4.2. Age of microorganisms in salt

The age of the Austrian rock salt deposits is known from palynological and isotope studies (17) and was determined for most formations as Upper Permian, with some originating from the Triassic. While there is as yet no direct proof that viable microorganisms in rock salt have been entrapped since the time of their deposition, it would also be difficult to prove the opposite, namely that masses of extremely halophilic archaea, or bacteria, entered salt deposits in recent times. An influx of liquids containing microorganisms is rather improbable, especially for the Alpine deposits, since the evaporites, which originated from the ingression of the Tethys ocean, are now folded up to heights of 1000 to 2000 m, and the salt layers are located on average between 400 and 1000 m above sea level. A migration of microorganisms into the halite would require transportation by meteoric water, a source which is

not known to contain extreme halophiles. Besides, the Alpine salt deposits are covered by layers of dolomite, limestone, marl, clay and other rocks; most of them are water-impermeable, and thus have contributed to the preservation of the salt deposits during tectonics and thrust over geological times.

If a Permo-Triassic age is postulated for the microorganisms from halite, it becomes necessary to explain the biological mechanisms for such longevity. Some possibilities have been discussed by Grant et al. (1), such as the presence of trace amounts of energy and carbon sources, which may be sufficient for maintenance, or the formation of resting stages, such as cysts. Our laboratories are currently engaged in trying to approach these problems experimentally.

The repeated isolation of viable halophilic microorganisms from different salt deposits indicates the presence of an indigenous flora in this environment in different parts of the world. Since some of the organisms are very similar to each other, and all of them resemble species in extant hypersaline surface waters, the conclusion is justified that the microorganisms from rock salt could be considered remnants of populations which once inhabited hypersaline seas.

4.3. Practical aspects.

The requirements for safe permanent underground storage of (nuclear or other) wastes are to ensure the stability of the storage system, and to prevent the release of hazardous amounts of waste to the biosphere (18). In this connection it is not the origin of the microorganisms, which is important, but whether they will be metabolically active. From the data presented here it can be concluded that ancient salt sediments are not devoid of life; on the contrary, they do themselves represent a biosphere, although one that is limited in species diversity, and mostly in a dormant state. However, if conditions are favorable, multiplication will set in readily. Just adding water, as in solution mining, appears sufficient to start microbial growth. Although the temperature of about 8°C in most Alpine salt mines is well below the optimum for haloarchaea (near 40°C), and the nutrient content is probably low, they can grow over time to high densities, as was apparent from the cavern sample. While the microbial content may be different in different sites, slow growth appears to be characteristic for all isolates. Long-term studies to examine the presence of microorganisms may thus be advisable, if a given salt deposit is intended for storage purposes; however, molecular methods, such as the PCR amplification described here, would provide much faster results, at least in some cases, with respect to the detection of halophilic archaea and bacteria in halite.

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