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Astrobiology with haloarchaea from Permo-Triassic rock salt

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Abstract: Several viable halophilic archaebacteria were isolated previously from rock salt of Permo-Triassic age in an Austrian salt mine; one of these strains was the first to be recognized as a novel species from subterranean halite and was designated *Halococcus salifodinae*. The halophilic microorganisms have apparently survived in the salt sediments over extremely long periods of time. Halobacteria could therefore be suitable model organisms for exploring the possibility of long-term survival of microbes on other planets, in particular, since extraterrestrial halite has been detected in meteorites and is assumed to be present in the subsurface ocean on Europa. Our efforts are directed at the identification of the microbial content of ancient rock salt and the development of procedures for the investigation of the halobacterial response to extreme environmental conditions. Using modified culture media, further halophilic strains were isolated from freshly blasted rock salt and bore cores; in addition, growth of several haloarchaea was substantially improved. Molecular methods indicated the presence of at least 12 different 16S rRNA gene species in a sample of Alpine rock salt, but these strains have not been cultured yet. The exploration of Mars is a target of space missions in the 21st century; therefore, testing the survival of haloarchaea under conditions comparable to present-day Mars, using a simulation chamber, was begun. Preliminary results with *Halococcus* and *Halobacterium* species suggested at least tenfold higher survival rates when cells were kept in liquid brines than under dry conditions; staining of cells with the LIVE–DEAD kit, which discriminates between damaged and intact membranes, corroborated these data.

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Introduction

Extremely halophilic bacteria are found in hypersaline environments such as the Dead Sea, the Great Salt Lake, sabkhas and natural or artificial salterns (Javor 1989). They grow optimally in the presence of 2.5–5.2 M NaCl (Kushner & Kamekura 1988) and are generally unable to grow in less than 10% w/v (approximately 1.7 M) salt. They comprise both (eu)bacterial and archaeal genera (Kamekura 1998); the term halobacteria is widely used in a general sense, while members of the haloarchaea (order *Halobacteriales*) have typical archaeal characteristics such as ether-linked lipids. The osmotic pressure of the surrounding brines is balanced by the accumulation of K^+ ions inside their cells. Consequently, the physiology of haloarchaea is specifically adapted to high salt concentrations (Oren 1999). To date, 14 genera of haloarchaea are recognized (Grant *et al.* 2001), reflecting their considerable ecophysiological diversity. From about 20% w/v salt concentration up to halite saturation (approximately 32% w/v), haloarchaea become the dominant microorganisms in evaporating brines (Benlloch *et al.* 1996). They can occur at such high cell densities that they cause brines to turn red. This red colour is caused by carotenoid pigments that protect cells from the harmful effects of ultraviolet light (Shahmohammadi *et al.* 1998). As NaCl starts to precipitate, most of the haloarchaeal cells become trapped inside fluid inclusions, which can constitute 2–6% w/w of freshly harvested solar salt (Lefond 1969). This phenomenon has been observed in solar salterns throughout the world (Norton & Grant 1988; Castanier *et al.* 1999). Haloarchaea have been shown to be motile for several weeks and remain viable inside fluid inclusions for at least 6 months (Norton & Grant 1988). An intriguing question was considered by several scientists – could preservation of viable halobacteria have occurred in ancient halite deposits? Reiser & Tasch (1960) and Dombrowski (1963, 1966) described successful cultivation of bacteria from ancient rock salt (see the review by McGenity *et al.* 2000). In recent years, isolation of haloarchaea from British and Alpine salt sediments (Norton *et al.* 1993; Stan-Lotter *et al.* 1993; Denner *et al.* 1994) and of a halophilic *Bacillus* from the Salado formation of the Midcontinent basin in the USA (Vreeland *et al.* 2000) was reported.

Halococcus salifodinae BIp was the first isolate from a salt deposit that was formally described as a novel species (Denner *et al*. 1994). This strain was isolated from a dry rock salt sample obtained from the Alpine salt mine near Bad Ischl, Austria, from a depth of approximately 650 m below the surface. Based on the stratigraphic position of the horizon, palynological findings and sulphur isotope studies (see Radax *et al*. 2001 and references therein), this salt is believed to have been deposited during the Permian and early Triassic periods (213–286 Ma). Since the species *Halococcus salifodinae* included initially only one strain, additional rock salt samples from the same site were investigated and several halococci, which proved to be identical to *Halococcus salifodinae*, were isolated (Stan-Lotter *et al*. 1999). Moreover, two independently isolated strains, Br3 from solution-mined brine in England and BG2/2 from a salt bore core of Berchtesgaden, Germany, were found to resemble *Halococcus salifodinae* in numerous properties, so that they could be considered as representatives of the same species (Stan-Lotter *et al*. 1999). Thus, it was demonstrated that in geographically separated halite deposits of similar geological age identical species of halococci are present, and that *Halococcus salifodinae* strains were re-isolated from the same site 8 years after the first rock salt samples had been taken. These results supported the notion that the halophilic isolates from subterranean salt deposits may be the remnants of populations that inhabited ancient hypersaline seas. Recently, a second novel species was isolated from rock salt pieces that were obtained 3 days after blasting operations from the same salt mine. This strain differed significantly, according to its 16S rRNA gene sequence and chemotaxonomic characteristics, from known strains belonging to the genus *Halococcus* and was named *Halococcus dombrowskii* (Stan-Lotter *et al*. 2002). Both *Halococcus* strains have not yet been isolated from a surface location. It was also shown, based on polymerase chain reaction (PCR) amplification of 16S rRNA genes, that a broad prokaryotic diversity is apparently present in the salt sediments (Radax *et al*. 2001); most of these strains have not yet been cultured under laboratory conditions.

The apparent longevity of the haloarchaeal isolates in dry salty environments is of interest for astrobiological studies and the search for extraterrestrial life. On Earth, microorganisms were the first life forms to emerge, and were present perhaps as early as 3.8 Ga (Schidlowski 1988, 2001). If Mars and Earth had a similar geological past, as is discussed by Nisbet & Sleep (2001) and Schidlowski (2001), for example, then microbial life, or the remnants of it, could still be present on Mars. Halite has been detected in meteorites, some of which stem from Mars (Gooding 1992; Treiman *et al*. 2000); in addition, a salty ocean is thought to exist under the surface ice cover of the Jovian moon Europa (McCord *et al*. 1998). If halophilic microorganisms survive in dry salt over geological time scales, as our and other studies suggest (Norton *et al*. 1993; Grant *et al*. 1998; McGenity *et al*. 2000), it appears plausible to consider specific searches for halophiles in future extraterrestrial samples. However, it is not known how haloarchaea could remain dormant over extended time periods; they have not been observed to form spores, although resting stages such as cysts may be produced by some representatives (Grant *et al*. 1998). It is also not known whether it is the dry state (in salt crystals) or the embedding in highly saline tiny fluid inclusions, which is necessary for the preservation of haloarchaeal viability. In any case, the environment on Mars is conducive to the stability of hypersaline brines, even at low temperatures and low pressures, since freezing points will be greatly depressed by high concentrations of salt (Litchfield 1998; Wynn-Williams *et al*. 2001), and it was thus speculated that extant halophilic life may exist on Mars (Landis 2001).

A prerequisite for the exploration of halophiles in extraterrestrial locations should be a thorough analysis of the populations of ancient salt sediments on Earth. In order to identify potential microbial signatures in extraterrestrial materials, it is necessary to obtain an extensive record of terrestrial forms of extremophilic life. Therefore, we are developing experimental approaches with the aim of increasing the culturable portion of haloarchaea from Permo-Triassic rock salt. These include the usage of a wide range of growth media and the addition of growth-stimulating factors. We provide data on substantially improved growth conditions for haloarchaea and the initial characterization of several novel strains, which we have obtained. In addition, we expand on the investigation of haloarchaeal biodiversity by molecular methods, which have already led to the recognition of a range of novel haloarchaeal gene sequences (Radax *et al*. 2001).

A second goal is the development of procedures for testing the responses of haloarchaea towards environmental extremes, especially Martian conditions. Those types of studies have not yet been performed with any haloarchaea. If most known halophilic microorganisms cannot survive for reasonable times in a simulated Mars environment, then the hypothesis of Martian halophilic life would have to be dismissed. We started to investigate the responses towards environmental extremes with two different halophilic species: (1) *Halobacterium* sp. NRC-1, a rod-shaped haloarchaeon, for which the total genome sequence has been determined (Ng *et al*. 2000); (2) *Halococcus dombrowskii*, a coccoid strain with a sturdy cell wall, which is an isolate from Permo-Triassic rock salt and grows as small aggregates (see below). Initial studies were performed on the survival of cells, changes in morphology and membrane status, following exposure to low temperature, low pressure, extreme dryness, carbon dioxide atmosphere, or combinations of these parameters, as they may be present on Mars or other planetary bodies.

Material and methods

Rock salt samples and isolation of strains

Samples of freshly blasted rock salt at a depth of about 650 m below the surface were obtained from the salt mine near Bad Ischl in Austria, 3 days after blasting operations for the creation of new tunnels. In addition, bore cores were obtained from deep drilling procedures in the salt mine at Altaussee, Austria, which were performed by the mine company (Salinen, Austria) for the evaluation of salt sediments. The bore

cores were 50 mm in diameter and, similar to the rock salt lumps, consisted of 92–95% water soluble material, which was taken as the approximate halite content. If subsequent isolation experiments were intended, freshly drilled salt cores were used, which were transported from the mine tunnels to the laboratory in sterile plastic bags. For additives to growth media, discarded bore cores were used, which were a gift from the salt mine company (Salinen, Austria). The geological setting of the Alpine salt mines has been described previously, as well as the methods for sterile dissolving of the salt pieces and initial steps for culturing of isolates (Stan-Lotter *et al.* 1993; Radax *et al.* 2001). Irish rock salt was obtained from the Carrickfergus area, where bedded halites of Triassic age occur in thicknesses of at least 200 m (Griffith & Wilson 1982).

Haloarchaeal strains used in this work

The following haloarchaeal strains were obtained from the DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany): *Halobacterium salinarum* DSM 3754^T, *Halococcus morrhuae* DSM 1307^T, *Halococcus saccharolyticus* DSM 5350^T, *Halococcus salifodinae* BIp DSM 8989^T and *Halococcus dombrowskii* DSM 14522^T. *Halobacterium* sp. NRC-1 ATCC-700922 was purchased from LGC Teddington Ltd, UK. Haloarchaeal strains, which were isolated during this work, included rod-shaped strains A1, A2, coccoid strains BIHGY10/26, BIHSTY10/19, BIHGY10/26, BIHGY10/25 and pleomorphic strains BIHSTY150/18, BIHGY10/13B, BIHSTY10/14A and BIHGY10/22.

Cultivation of haloarchaea

The medium (M2) for the cultivation of neutrophilic haloarchaea was slightly modified from Hochstein (1987) and contained (in g l^{-1}): NaCl, 200; $MgCl_2 \times 6\ H_2O$, 20; KCl, 2; $CaCl_2 \times 2\ H_2O$, 0.2; yeast extract (Difco), 5; Hycase (casein hydrolysate; Sigma), 5. Some haloarchaea and especially halococci grow slowly and yield only low cell densities when cultured in this medium. Therefore, a variety of media were used, which were supplemented with different substrates or rock salt extracts, as is described below. The pH of the growth media was 7.4, unless indicated otherwise; the cultures were incubated at 37 °C or ambient temperature (21–23 °C). Growth in liquid culture was monitored at 600 nm with a Novaspec II visible spectrophotometer (Amersham Pharmacia, Freiburg, Germany). For growth on solidified media, portions of 100 µl of solutions were streaked on agar plates, containing the respective media. Rock salt of Alpine or Irish origin (Carrickfergus area) was used as an additive following dissolution in neutralized deionized water to obtain a saturated salt solution (approximately 32% dissolved solids at ambient temperature). This solution was sterile-filtered using Stericups (Millipore, 0.22 µm pores). Standard M2 medium was mixed 1:1 with saturated sterile-filtered rock salt solution and then sterilized for 15 min at 121 °C. The rock-salt-supplemented medium was called M2S. For some experiments, rock salt was substituted by commercial sea salt ('instant ocean', Sigma).

The media, which were prepared for attempts to improve recovery of isolates from rock salt contained (in g l^{-1}): NaCl, 200; KCl, 2; $CaCl_2 \times 2\ H_2O$, 0.1; $FeSO_4 \times 7\ H_2O$, 0.01; $MnCl_2 \times 4\ H_2O$, 0.00036; in addition, they contained 10 or 150 mM $MgCl_2$; 0, 1 or 10 g l^{-1} glycerol; 0 or 0.5 g l^{-1} tryptone (Roth); 0 or 0.5 g l^{-1} Hycase (Sigma); 0, 0.1, 0.5 or 1 g l^{-1} yeast extract; 0 or 2 g l^{-1} soluble starch. Agar (20 g l^{-1}) was added for solidification. Inoculated agar plates were incubated at ambient temperature for 4–12 months, and inspected for microbial growth under six-fold magnification. Cell material from 111 strains was transferred to fresh agar plates and purified by re-streaking for four to six times. All further analyses were performed on cell material grown on agar plates. A solidified medium (DSM medium no. 823) with a high content of Mg^{++} (0.578 M) was used for isolation of strains from bore cores; it contained (in g l^{-1}): NaCl, 125; $MgCl_2 \times 6\ H_2O$, 160; K_2SO_4, 5; $CaCl_2 \times 2\ H_2O$, 0.1; yeast extract, 1; casamino acids, 1; starch, 2. Initial growth of colonies was observed following 3 months of incubation at 37 °C. *Halobacterium* sp. NRC-1 was grown in ATCC medium no. 2185, which contained (in g l^{-1}): NaCl, 250; $MgSO_4 \times 7\ H_2O$, 20; trisodium citrate $\times 2\ H_2O$, 3; KCl, 2; tryptone, 5; yeast extract, 3; 0.1 ml of a filter-sterilized trace metal solution (per 200 ml: $ZnSO_4 \times 7\ H_2O$, 1.32 g; $MnSO_4 \times H_2O$, 0.34 g; $Fe(NO_4)(SO_4)_2 \times 6\ H_2O$, 0.78 g; $CuSO_4 \times 5\ H_2O$, 0.14 g) was added per litre. Growth was at 37 °C to an optical density of about 1 or 0.1, respectively. Pigmentation of haloarchaea is light dependent; thus, cells grown in the presence or absence of daylight were used.

Physiological and biochemical tests

The range of salt concentrations that permitted growth was determined by spreading 100 µl of a growing culture on agar plates containing M2 medium supplemented with final NaCl concentrations of 7.5, 10, 12.5, 15, 17.5, 20, 22.5, 25, 27.5 and 30%. Similarly, the requirement for Mg^{++} was tested with M2 agar plates containing final concentrations of 0, 100, 200, 300, 400, 500, 600 and 700 mM $MgCl_2$ in the presence of 15% NaCl. Cultures of strains on agar plates were incubated from 14 to 20 days. The analytical profile index system (API Zym, bioMerieux, Austria) was used for analysis of enzyme activities (Humble *et al.* 1977). Strips were inoculated with a cell suspension in Tris-buffered 3 M NaCl and incubated for up to 24 h. All API tests were performed at least three times.

Whole-cell protein patterns analysis

Sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis (PAGE) of whole-cell proteins was performed as described previously (Stan-Lotter *et al.* 1989, 1993). Briefly, approximately 50 µg of cells (wet weight) per ml were lysed by boiling them in SDS sample buffer (Laemmli 1970) for 10 min, and then centrifuged at 10 000 ***g*** for 15 min, to remove any precipitates. The gel system of Laemmli (1970) was used. Proteins were visualized by staining with Coomassie Blue. Marker proteins from 2.5 to 200 kDa (Mark 12) were from Novex (Vienna, Austria).

PCR amplification of 16S rRNA gene fragments

Archaeal 16S rRNA gene fragments were amplified by PCR with archaea-specific oligonucleotide primers. Two different primer pairs were used: primers Arch21F (forward) and Arch958R (reverse, DeLong 1992) served to amplify fragments of approximately 920 bp from environmental rock salt DNA, during screening for insert-containing plasmids (see below), and from colonies recovered from agar plates. The primer pair Arch21F and 1525R (McGenity *et al*. 1998) was used for the amplification of a larger fragment (approximately 1470 bp) of the 16S rRNA gene of some isolates. Typical PCR reaction mixtures (50 µl volume) contained 25 pmol of each forward and reverse primers; 12.5 nmol deoxy nucleotides; 1.5 mM $MgCl_2$; 1 unit Taq DNA polymerase; appropriately diluted PCR buffer; and approximately 0.1–1 ng template DNA. Positive controls containing genomic haloarchaeal DNA and negative controls that contained genomic DNA of *Escherichia coli*, no template DNA, or no polymerase, respectively, were included each time. PCR reactions were performed in a programmable thermal cycler. The PCR protocol for the primer pair Arch21F/Arch958R was as follows: initial denaturation at 94 °C for 5 min; 35 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 1 min and extension at 72 °C for 1 min; final extension at 72 °C for 5 min. The PCR protocol for the primer pair Arch21F/1525R differed from the above protocol with respect to annealing temperature (53 °C) and extension time (1.5 min). In some experiments with environmental rock salt DNA, only 25 cycles were performed and the final extension was omitted (Radax *et al*. 2001).

DNA sequencing and phylogenetic analysis of sequences

DNA sequences of purified PCR products from isolates or plasmid DNA from clones were obtained 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), as recommended by the manufacturer. Sequencing primers were described in Radax *et al*. (2001) and Stan-Lotter *et al*. (2002). Searches for the closest relatives of the complete insert sequences obtained in this study were performed using the Fasta3 (Pearson & Lipman 1988) web interface from Genbank. Based on a subset of aligned archaeal sequences obtained from the Ribosomal Database Project II (RDP), release 8 (Maidak *et al*. 2000), an alignment was created, which 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 obtained in this study were fitted in the haloarchaeal alignment with Clustal X (Thompson *et al*. 1997). Reconstruction of phylogenies was performed with corrected (Jukes & Cantor 1969) distance-matrix, maximum-likelihood and maximum-parsimony methods, using programs of the PHYLIP package, version 3.5.1c (Felsenstein 1993) and Clustal X. In the case of sequences obtained during the analysis of environmental rock salt DNA, the potential presence of chimeric sequences was examined with the CHIMERA_CHECK program available through the RDP (see Maidak *et al*. 2000), and also by looking for taxa, which changed positions in neighbour-joining trees based on sequences of 250 nucleotides from either the 5′ or the 3′ end (Radax *et al*. 2001).

Purification of DNA from dissolved rock salt

DNA was recovered from dissolved rock salt by two different methods. The first method (here referred to as a filter-based method) was described in detail in Radax *et al*. (2001); briefly, the dissolved rock salt was filtered through a 0.22 µm pore size membrane filter to collect bacteria, the filter was treated with buffers containing SDS and lysozyme in the presence of acid-washed glass beads, boiled and treated with proteinase K. Then a thermal shock was applied by first keeping samples at −70 °C and subsequently boiling them. DNA was extracted with phenol-chloroform, precipitated with ammonium acetate and ethanol, and purified further with a commercially available kit (Gene Clean II, Bio101, Vista, CA). In the second DNA extraction method (centrifugation-based method), dissolved rock salt was centrifuged at 500 ***g*** for 10 min, the pellet was washed several times with a high-salt buffer (4 M NaCl, 50 mM Tris. HCl, pH 7.4), and all supernatants were centrifuged at 12 000 ***g*** for 5 min. All resulting pellets were taken up in a buffer containing 1% SDS, 100 mM EDTA and 50 mM Tris. HCl, pH 7.6, treated with lysozyme and proteinase K, and DNA was extracted with phenol/chloroform. After digestion with RNase A, all DNA-containing fractions were precipitated with ammonium acetate/ethanol and pooled. A more detailed description will be given elsewhere.

Generation and analysis of clone libraries

Ribosomal RNA genes contained in rock salt DNA samples, which were prepared by the filter-based and the centrifugation-based purification method (see above), were analysed independently as follows: PCR products were obtained, using the primer pair Arch21F/Arch958R; following separation by agarose gel electrophoresis; bands of the expected size (approximately 920 bp) were excised and purified with the Nucleo-Trap kit (Macherey and Nagel, Düren, Germany). Since the PCR products consisted of DNA molecules of similar sizes, but presumably different sequences, the molecules were separated by cloning. For this purpose, six purified PCR products from each DNA preparation were pooled, approximately 30 µg of DNA were ligated into vector pGEM-T, and transformed into competent *E. coli* JM109 cells (both from Promega). Transformants were screened by blue–white selection on Luria Bertani agar plates treated with X- Gal/IPTG and containing 100 µg ml^{-1} ampicillin, picked and screened for the presence of the desired insert by PCR with the primer pair Arch21F/Arch958R, using a small amount of each picked colony as a template. Plasmid DNA of 54 colonies with confirmed inserts ('PCR-positive') from DNA prepared by the centrifugation-based method was purified with the GFX Micro Plasmid Prep. Kit (Amersham

Pharmacia) and subjected to partial sequencing with primer U519R (Munson *et al*. 1997). Full sequences (917–919 bp) of representative clones were obtained (see Radax *et al*. 2001). PCR-amplified inserts from 123 PCR-positive clones from DNA samples prepared by the filter-based method were digested with restriction endonuclease DdeI (Promega), followed by separation of restriction fragments by PAGE and ethidium bromide staining. Partial sequences of at least 25% of the members of each group, which exhibited similar restriction patterns, were obtained. Representative clones were selected, based on restriction patterns and partial sequences, and their inserts sequenced. All full-length insert sequences were then subjected to phylogenetic analysis as described above.

Exposure of haloarchaea to physical extremes

Cultures of *Halococcus dombrowskii* and *Halobacterium* sp. NRC-1 were each grown in liquid media (see above) to an $OD_{600\,nm}$ of 0.9–1.0 or approximately 0.1. Cells were centrifuged in portions of 250 µl at 2500 ***g*** for 5 min at ambient temperature. Supernatants were discarded and the pellets were suspended in 250 µl of a buffer containing 50 mM Tris-HCl, pH 8.0, 4 M NaCl (TN) or, in addition, 80 mM $MgCl_2$ (TNM). Aliquots of cell suspensions were kept at −70 °C for up to 7 days in a laboratory deep freezer, or freeze-dried in a commercial lyophilizer (Labconco; the minimum pressure in this chamber was about 33×10^{-3} mbar); residual moisture of samples after 24–48 h of freeze-drying is estimated as 2–3% (Labconco manual). Samples were transported in a vacuum desiccator to the Mars Simulation chamber (Fig. 1), which is described below, and were exposed to an atmosphere of 6 mbar, 98% of carbon dioxide, and an average temperature of −60 °C for about 6 h in a liquid-nitrogen-cooled chamber. Four to five aliquots of each type of sample were used for the exposure experiments. Following treatments, cells were plated on solidified growth medium and incubated at 37 °C for several days (*Halobacterium* sp. NRC-1) or up to 3 weeks (*Halococcus dombrowskii*). Viable cells were estimated from colony-forming units (cfus) from four to ten agar plates per sample. Statistical analysis of these data as well as those from experiments described in the next paragraph was performed with the independent samples T-test from SPSS for Windows, Release 10.0.0, 1999, Chicago (SPSS Inc.).

Experiments in the Mars Simulation Chamber

The cryogenic vacuum chamber at the Space Research Institute of the Austrian Academy of Sciences was used. This chamber (shown in Fig. 1) is a cylindrical receptacle made of stainless steel with an inner diameter of 40 cm and a height of 40 cm. The interior (copper cold shroud along the walls and copper bottom plate) can be cooled by a liquid nitrogen circuit. The attached pumping system (rotary vane pre-pump for evacuation down to about 1 mbar and turbomolecular pump for the high-vacuum range) allows it to reach a pressure of about 10^{-5} mbar if no major outgassing materials are placed inside the chamber. In order to establish and maintain atmospheric conditions similar to the present-day Mars surface (CO_2 composition; about 6 mbar gas pressure) the evacuated chamber is connected to a commercial CO_2 gas bottle via a special valve that allows one to regulate the gas pressure inside the chamber in small steps. In this pressure regime the turbomolecular pump is switched off and the system operates with the pre-pump only. A more detailed description of the simulation chamber can be found in Kömle *et al*. (1996) and Kargl (1998). The experimental setup is shown in Fig. 1 (inset). The specimen to be subjected to the Martian conditions were loosely placed on the bottom of the cooling plate and several temperature sensors for monitoring the temperature at interesting locations were installed. Subsequently the chamber was closed, evacuated and cooled down by the attached liquid nitrogen circuit. The temperature of the samples decreased steadily during the experiment, from room temperature down to about −60 °C. After the initial evacuation and cooling phase, a CO_2 atmosphere was established inside the chamber at a pressure of 6 mbar and maintained for approximately 5 h. After completion of the experiment the chamber was opened and the specimen was sealed in an evacuated container for further investigation.

Fig. 1. Mars simulation chamber at the Space Research Institute of the Austrian Academy of Science, Graz. The chamber (foreground) is cooled by a liquid nitrogen circuit. In the white containers behind the chamber the liquid nitrogen is stored; it is driven through copper tubes by the pressure that is provided by gaseous nitrogen from the tank visible in the background; the chamber atmosphere is produced from gaseous N_2 and CO_2. Inset: chamber without a lid, containing a rack with 96 samples in small plastic tubes.

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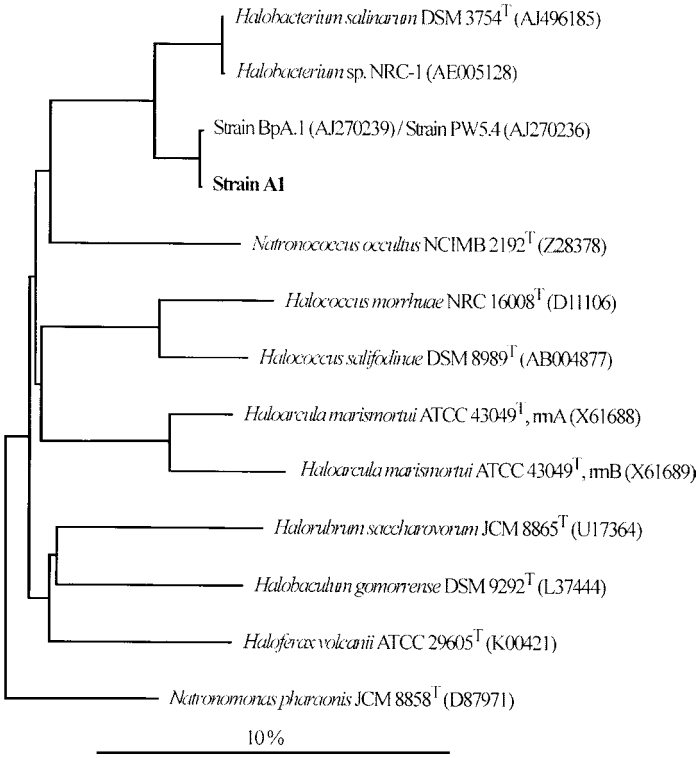

Fig. 2. Dendrogram showing the phylogenetic relationship of the haloarchaeal rock salt isolate strain A1 to several described genera of the *Halobacteriaceae*. The tree is based on an alignment of 16S rRNA gene sequences. Sequence accession numbers are in brackets. The bar represents a 10% sequence difference.

Pulsed field gel electrophoresis of haloarchaeal DNA

The DNA structure was investigated by pulsed field gel electrophoresis (PFGE), modified after Hackett (1995). The PFGE run was performed in a CHEF-DR III system (Bio-Rad, Vienna, Austria). Running conditions were a field angle of 120°, voltage gradient of 6 V cm^{-1}, switch times of 60–120 s, running time of 24 h; the gel contained 1% PFGE-agarose (BioRad). The DNA size marker contained chromosomes from *Saccharomyces cerevisiae* (BioRad).

Other methods

Unstained cells were observed with a Zeiss Axioskop microscope using phase contrast. Following staining with the LIVE–DEAD kit (Molecular Probes, Eugene, OR), cells were examined by fluorescence microscopy (Zeiss Axioskop, excitation by ultraviolet light) with appropriate filter sets (red and green, respectively). The kit consists of two dye substances and allows discrimination between damaged or intact cell membranes, which is reflected in the appearance of red or green fluorescence. The red colour indicates the entrance of the dye propidium iodide (damaged membrane), whereas the green colour, owing to overall staining with SYTO 9, indicates non-entry (intact membranes). Cells were prepared for electron microscopy (EM) similarly as described previously (Denner *et al.* 1994), except that sputter-coating with approximately 2 nm Pt was used and samples were viewed in a Hitachi S-900 scanning electron microscope (SEM) operated at 2.0 kV. The electron microscopical work was carried out by Chris Frethem, University of Minnesota, USA.

Results

Novel haloarchaeal isolates

From freshly drilled bore cores of rock salt from the salt mine in Altaussee, Austria, from a depth of about 600 m below the surface two small light red pigmented colonies were obtained on agar plates, following incubation for several months. The isolates were purified further by re-streaking of the colonies and were designated strains A1 and A2. Examination by phase contrast microscopy revealed very small, motile short rods. Optimum growth occurred at NaCl concentrations ranging from 15–17.5% NaCl; no growth was observed below 12.5% NaCl and above 22.5% NaCl. The optimum Mg^{++} concentration was 500–700 mM, in the presence of 15% NaCl. Sequences (1473 bases) of the 16S rRNA genes of strains A1 and A2 were identical. The phylogenetic position of strain A1 is shown in Fig. 2. Comparison of the sequence with members of the family Halobacteriaceae placed strain A1 into the genus *Halobacterium*. The greatest similarity to characterized species of *Halobacterium* as deduced from this analysis was to *Halobacterium* sp. NRC-1 and *Halobacterium salinarum* (97.1% identity to each). The sequences showed more than 99.8% similarity to some uncultured archaeal clones from rock salt, e.g. A175 (Radax *et al.* 2001) and 99.7% similarity to culturable

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haloarchaeal isolates from ancient salt deposits in England (strain BpA.1 from the Winsford salt mine brine) and Poland (strain PW5.4 from the Wieliczka salt mine brine; see McGenity *et al.* 2000). SDS PAGE of whole-cell protein patterns revealed complete identity of strains A1 and A2, but numerous differences with respect to *Halobacterium salinarum* and *Halobacterium* sp. NRC-1 (data not shown). The presence of the following enzymes was detected in strains A1 and A2: esterase (C4), valine arylamidase and cystine arylamidase; these enzymes were not found in the strains *Halobacterium salinarum* and *Halobacterium* sp. NRC-1, but the latter possessed alkaline phosphatase, acid phosphatase and naphtol-AS-BI-phosphohydrolase, which were absent in strains A1 and A2. Comparison of DNA fragments by PFGE (see the Methods section), which were produced from restriction digests, revealed identical patterns of strains A1 and A2, which differed from those of other haloarchaeal strains (data not shown). For the formal description of strain A1 as a novel species, a full characterization according to the international guidelines (Oren *et al.* 1997) is necessary, which is in progress in our laboratory.

On seven different high salt media, which contained 0.1% yeast extract in combination with glycerol (0–1%), tryptone (0–0.05%) or starch (0–0.2%), respectively, growth of pink and red haloarchaeal colonies was observed, following up to 1 year of incubation. Pink colonies consisted of halococci-like strains, and red colonies consisted of pleomorphic cells, including small rods, irregularly shaped cells and flat discs. Further analysis revealed that coccoid strains (e.g. isolates BIHSTY10/19, BIHGY10/26 and BIHGY10/25) showed identical restriction patterns of amplified 16S rRNA genes (Fig. 3, lanes 1–3), which differed distinctly from those of the pleomorphic strains BIHGY10/13B, BIHSTY10/14A and BIHGY10/22 (Fig. 3, lanes 4–6). Sequence analysis of 16S rRNA genes showed a close relationship of the coccoid strains to *Halococcus dombrowskii*, and of the pleomorphic strains to one cluster of previously obtained sequences from rock salt (Radax *et al.* 2001). SDS PAGE of whole-cell proteins indicated different patterns within the coccoid and pleomorphic groups, which suggested that they belong to different species, despite their identical 16S rRNA gene sequences (data not shown). The phylogenetic relationship of two representatives of these latter novel isolates (BIHSTY150/18 and BIHGY10/26) to other haloarchaea is shown in Fig. 5.

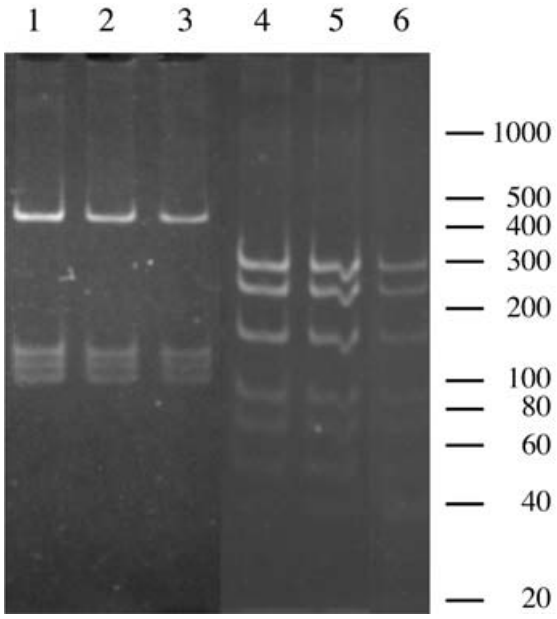

Fig. 3. Differentiation between halococci and non-halococci by cleavage patterns from 16S rRNA gene fragments. PCR products (see the Methods section) were digested with the restriction enzyme DdeI, fragments were separated on an 8% acrylamide gel and visualized with ethidium bromide. Sizes of DNA marker molecules (in base pairs) are indicated to the right. Lanes 1, 2, 3, coccoid strains BIHSTY10/19, BIHGY10/26 and BIHGY10/25; lanes 4, 5,

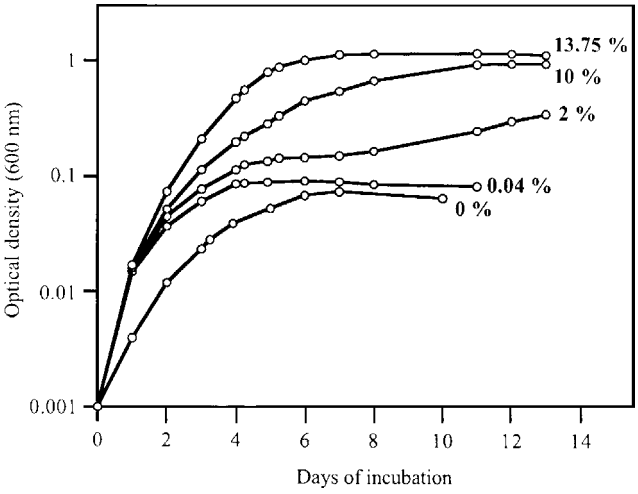

Fig. 4. Effect of increasing concentrations of Alpine rock salt on the growth of *Halococcus dombrowskii*. Growth curves following up to 14 days of incubation at 37 °C are shown; growth medium contained saturated solutions of sterilized Alpine rock salt in concentrations (v/v) of 13.75, 10, 2, 0.04 and 0%, which were added to standard complex medium for neutrophilic haloarchaea (Hochstein 1987).

Improved haloarchaeal growth

Cultures of the halococcal strains *Halococcus morrhuae*, *Halococcus salifodinae*, *Halococcus saccharolyticus* and *Halococcus dombrowskii*, when grown in M2S medium, had lower doubling times (e.g. approximately 14 h, instead of 30 h) and reached a greater turbidity ($OD_{600\,nm}$ up to 2) compared with cultures grown in M2 medium. For further studies of the apparent growth stimulating factor, increasing amounts of sterile filtered Alpine rock salt were added to an equal volume of M2 medium. Higher concentrations of rock salt caused a decrease in generation time and an increase in optical density of cultures of *Halococcus dombrowskii* (Fig. 4). The slowest growth and lowest turbidity were observed when cultures were grown in standard M2 medium (see Fig. 4). An increase

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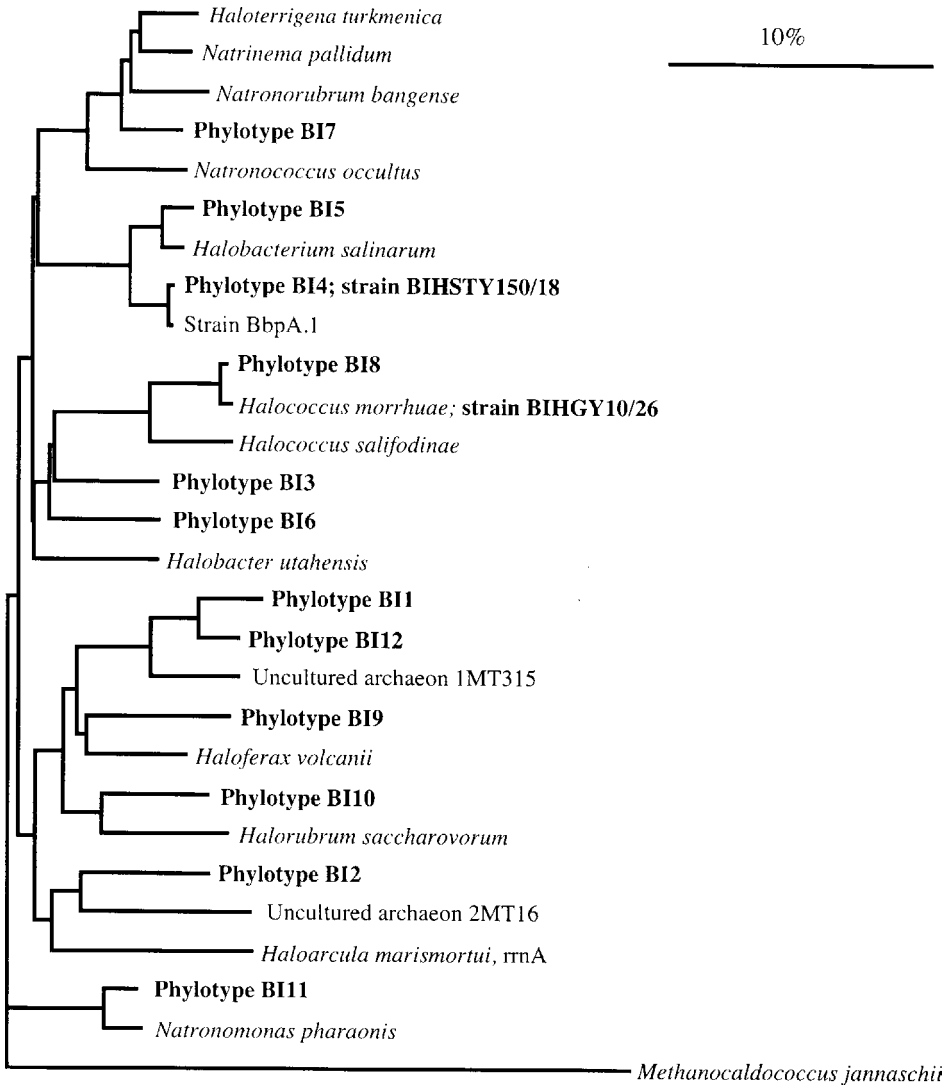

Fig. 5. Dendrogram showing the phylogenetic relationships, based on 16S rRNA gene sequences, of two novel rock salt isolates (BIHSTY150/18 and BIHGY10/26) and 12 uncultured phylotypes (BI1–BI12), which are described in this work, to a set of known haloarchaeal sequences, including several type strains and uncultured haloarchaea. The sequence of the archaeon *Methanocaldococcus jannaschii* was used as an outgroup.

in cell mass, following harvesting by centrifugation, was observed, concomitant with the increase in turbidity; for instance, 11 cultures of *Halococcus dombrowskii*, *Halococcus salifodinae* and *Halococcus morrhuae* yielded cell masses of 1.9, 1.6 and 1.8 g, when grown in M2 medium, but 4.6, 3.3 and 3.2 g, respectively, when grown in M2S medium. The growth stimulating effect by some unknown heat stable factor was detected initially when Alpine rock salt was used as an additive to media; similar effects were observed with Irish rock salt from Carrickfergus and also with a commercial sea salt, called 'instant ocean'.

Uncultured phylotypes (*16S rRNA gene species*)

The classical methods of enrichment procedures and isolation of novel strains suffer from the fact that, owing to heterogenous physiological needs, only a fraction of the microorganisms present in an environment can be cultured on nearly any customary medium (Amann *et al.* 1995). This conclusion resulted from the analysis of environmental DNA, which revealed the presence of large numbers of unexpected and often novel gene sequences. Environmental DNA from Permo-Triassic rock salt from Bad Ischl, Austria, was prepared and was submitted to amplification of 16S rRNA genes with haloarchaeal primers. The fragments contained in the PCR products were subsequently separated by standard cloning methods and sequenced. Their analysis suggested the presence of at least 12 different 16S rRNA gene species, which were designated phylotypes BI1–BI12. Their relationship to known haloarchaeal species as well as some hitherto uncultured clones is shown in Fig. 5. None of the phylotypes was identical to sequences from databases. Two phylotypes, BI4 and BI8, corresponded to pleomorphic (strain BIHSTY150/18; 100% sequence similarity) and coccoid (strain BIHGY10/26; 99.6% sequence similarity)

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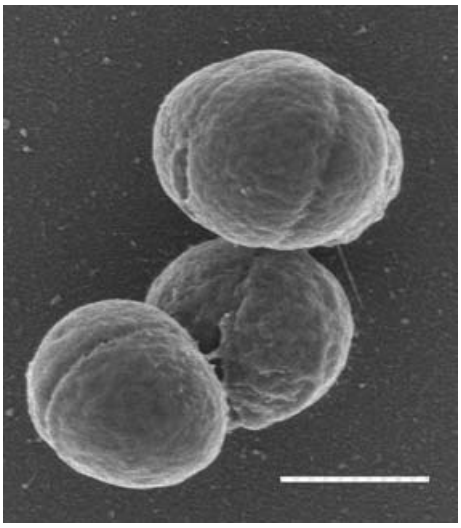

Fig. 6. High-resolution field emission scanning electron micrograph of *Halococcus dombrowskii* grown in liquid culture. The bar represents 860 nm.

cultured isolates, respectively. Seven phylotypes represented novel gene species, which were less than 97% similar to known sequences. Three phylotypes possessed 97–98% similarity to their closest cultured or uncultured relatives. Currently, similarities of 97%, or less, in 16S rRNA gene sequences are considered to indicate delineation of a species in most cases (Stackebrandt & Goebel 1994).

Survival at deep temperatures, low pressures and in a Martian simulation chamber

The conditions obtainable in the Mars simulation chamber at the Austrian Academy of Sciences, Graz, were: temperature down to $-100\,^{\circ}C$, pressure of about 6–8 mbar and an atmosphere of approximately 98% CO_2. Temperatures on Mars range between $-100\,^{\circ}C$ and $-5\,^{\circ}C$, but sometimes with values up to $+17\,^{\circ}C$ (Mancinelli 1998), therefore an average value of $-60\,^{\circ}C$ was chosen. Besides the well-characterized strain *Halobacterium* sp. NRC-1, *Halococcus dombrowskii* was used for survival experiments. This haloarchaeon grows in small aggregates (Fig. 6), which make it amenable for quantitative studies, and, like all known halococci, possesses a sturdy envelope (Denner *et al*. 1994), in contrast to *Halobacterium* species. Cell numbers of untreated *Halobacterium* sp. NRC-1 and *Halococcus dombrowskii*, grown in the presence or absence of daylight, ranged between 10^8 and 10^9 cells ml^{-1} culture for both species, determined as colony-forming units. Besides in simulated Martian conditions, viability of haloarchaeal cells was examined following exposure at $-70\,^{\circ}C$ in a deep freezer and lyophilization of cells in a commercial freeze-dryer, where a pressure of approximately 33×10^{-3} mbar can be maintained.

Survival of *Halococcus dombrowskii* following exposure for 6 h to simulated Martian conditions is shown in Fig. 7. The highest number of survivors, about 10% of the initial cells (sample 4, 1.45×10^8 cfu ml^{-1}; sample 5, 9.91×10^7 cfu ml^{-1}) was observed in cell suspensions, which had been kept liquid before and during the exposure experiments. Significantly fewer survivors, about 1%, remained in samples, which had been freeze-dried (Fig. 7, sample 2, 3.4×10^7 cfu ml^{-1}; sample 3, 1.16×10^7 cfu ml^{-1}); it is likely that freeze-drying by itself leads to a reduction of viable halococcal cells, as was noticed in separate experiments (data not shown). An apparent small protective effect by Mg^{++} ions was observed, since cells suspended in TNM buffer instead of TN buffer contained a higher portion of survivors (Fig. 7, samples 2 and 4, versus samples 3 and 5). Post-exposure staining of cells of *Halococcus dombrowskii* with the LIVE–DEAD kit and observation by fluorescence microscopy revealed about 30% 'dead' cells (with damaged membranes) and about 70% viable cells, when about 15 microscopic view fields were examined; although this result is only semi-quantitative, it can be taken as corroboration of the viable counts. No morphological changes were observed with post-exposure *Halococcus dombrowskii* cells.

Deep-freezing for 48 h of a culture of *Halobacterium* sp. NRC-1, which contained 1.2×10^9 cfu ml^{-1}, resulted in a recovery of 4.4×10^6, 1.3×10^8 and 3.0×10^8 cfu ml^{-1}, when cells had been suspended in TN buffer, or TN buffer supplemented with 80 mM Mg^{++} or 80 mM Ca^{++}, respectively. Freeze-drying of *Halobacterium* sp. NRC-1 cells (3×10^9 cfu ml^{-1}) for 24 h at approximately 33×10^{-3} mbar in the presence of glycerol resulted in recovery of viable counts between 5.3×10^6 and 8.8×10^6, when the glycerol concentration had been 2–4%. PFGE analysis of several freeze-dried samples indicated the loss of intact genomic DNA in samples, which had contained 0% and 1% glycerol during freeze-drying, whereas concentrations of 2–4% glycerol apparently provided protection against DNA breakage, since more intact genomic DNA was present (gels not shown). Cells of *Halobacterium* sp. NRC-1 were also subjected to simulated Martian conditions. Samples were placed either as liquids or following freeze-drying in the chamber; two different cell concentrations were tested ($OD_{600\,nm}$ of 1.0 and 0.1, respectively). The results following the determination of surviving cells are shown in Fig. 8. The liquid samples contained approximately 1.3×10^6 cfu ml^{-1} following exposure, which represented about 0.1% survivors (lanes 2–4). Lyophilized samples contained only between 100–200 cfu ml^{-1} (lanes 5, 6), or sometimes no cfus (not shown). The supplementation of TN buffer with 80 mM Mg^{++} (TNM) during exposure to Martian conditions had a marginal effect on the survival of *Halobacterium* sp. NRC-1 under the conditions used here. PFGE patterns indicated the presence of intact genomic DNA, concomitant with the presence of viable cells (samples 1–6 from Fig. 8); DNA of non-viable cells was visible as smear, suggesting strand breakage (data not shown). Staining of *Halobacterium* sp. NRC-1 cells with the LIVE–DEAD kit revealed similar ratios of apparently damaged to undamaged membranes as noticed for *Halococcus dombrowskii* (see above); no morphological changes of *Halobacterium* sp. NRC-1 were observed following the treatments described here; however,

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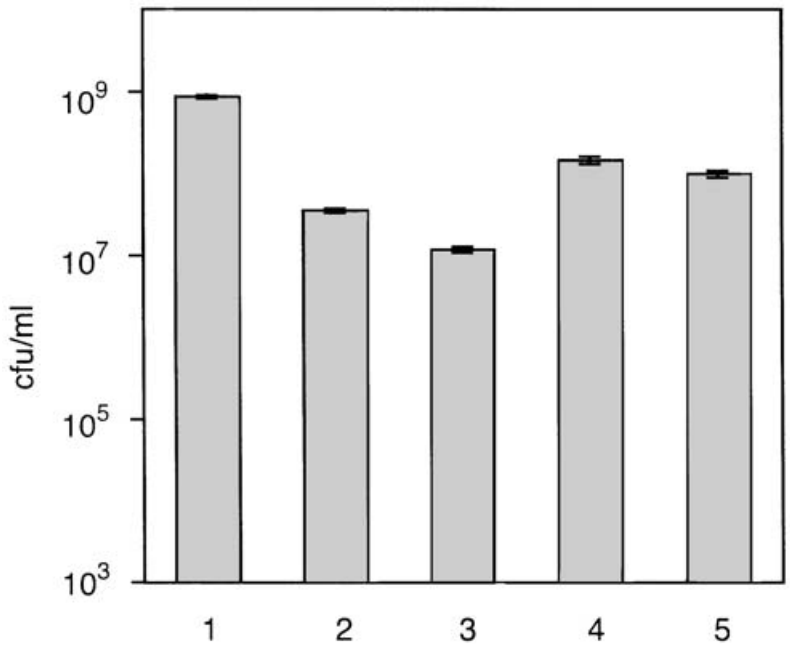

Fig. 7. Survival of *Halococcus dombrowskii* following exposure to a simulated Martian atmosphere. $OD_{600\,nm}$ of all samples was 0.9. Column 1, untreated control; columns 2, 3: samples were lyophilized before exposure and suspended in TNM (2) or TN (3) buffer, while in the Mars simulation chamber; columns 4, 5: samples were kept liquid before exposure and suspended in TNM (4) or TN (5) buffer. Values represent the mean of four determinations, except for column 1 (ten determinations); error bars indicate standard deviations. Columns 2–5 were significantly different ($P < 0.0001$), compared with the untreated control.

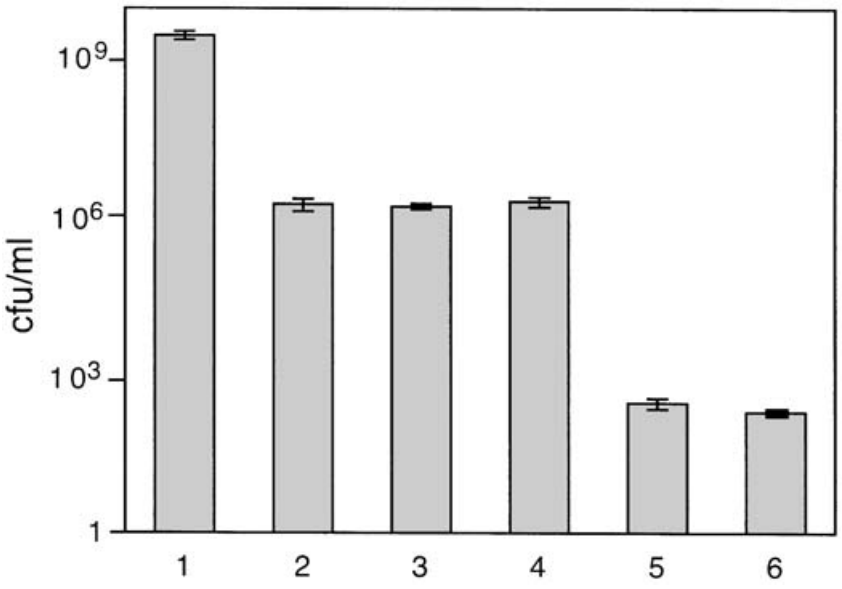

Fig. 8. Survival of *Halobacterium* sp. NRC-1 following exposure to a simulated Martian atmosphere. Sample preparation and analysis was similar as described in the legend to Fig. 7. $OD_{600\,nm}$ was 1.0 (samples 1, 2, 4, 6) or 0.1 (samples 3, 5) respectively. Column 1, untreated control; columns 2–4: samples were kept liquid before exposure and suspended in TN (2, 3) or TNM (4) buffer while in the Mars simulation chamber; columns 5, 6: samples were lyophilized before exposure and suspended in TN (5) or TNM (6) buffer. Values represent the mean of four determinations, except for column 1 (eight determinations); error bars indicate standard deviations. Columns 2–6 were significantly different ($P < 0.0001$), compared with the untreated control.

following heat treatment, cells of *Halobacterium* sp. NRC-1 were changing from rods to globules, which suggested the capacity to alter morphology in response to the environment (Weidler & Leuko, unpublished observations).

Discussion

Several new insights are emerging from our results, which could have an impact on the planning of research strategies

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for the exploration of Mars and other celestial bodies. We considered here mainly two issues: first, the viable haloarchaeal content of ancient halite deposits, which comprises culturable as well as uncultured species, and secondly, initial studies on specific responses of two species of haloarchaea to extreme environmental conditions, similar to those thought to occur on present-day Mars.

The hypersaline brines, which gave rise to the ancient salt deposits, can be viewed as early environments for haloarchaea and other halophilic microorganisms. During several periods in the Earth's history, extensive sedimentation of halite and some other minerals from hypersaline seas took place. An estimated 1.3×10^6 km^3 of salt were deposited in the late Permian and early Triassic periods alone (Zharkov 1981). The continental land masses during those times were concentrated around the paleoequator and formed the supercontinent Pangaea; the climate was arid and warm. Salt sediments developed in large basins, which were connected to the open oceans by narrow channels. About 100 Maago, fragmentation of Pangaea was beginning; the continents were displaced to the north, and folding of new mountain ranges such as the Alps and Carpathians was taking place (Einsele 1992). The haloarchaeal isolates, which are found today in halite from British, Polish and Alpine salt mines, are similar in their properties, including strong pigmentation, to isolates from hypersaline lakes and evaporation ponds from the warmer zones of the Earth. It is thus reasonable to consider the salt mine strains as remnants of the populations that once inhabited the brines during Pangaean times. The evidence for this assumption has been considered in a recent review (McGenity *et al*. 2000).

Although there were several reports of halophilic microbial isolates from halite deposits, only a few strains have been studied in detail, and as yet only six (five independently isolated strains of *Halococcus salifodinae* and one strain of *Halococcus dombrowskii*) were deposited in publicly available culture collections (Denner *et al*. 1994; Stan-Lotter *et al*. 1999, 2002). The knowledge of the prokaryotic content of rock salt is still very incomplete, mainly because the classical procedures of microbial enrichment suffer from the notorious phenomenon of the 'great plate count anomaly' (Staley & Konopka 1985), since only a fraction of the existing community can be cultured in the currently used types of nutrient media (Amann *et al*. 1995). Using extraction of DNA, following filtration of dissolved rock salt, and amplification of genes by PCR, we had previously obtained evidence for the presence of numerous novel haloarchaeal 16S rRNA genes in Permo-Triassic salt, some of which were similar to those of known haloarchaea, and some corresponded to cultured haloarchaeal isolates from ancient salt deposits in other parts of Europe (Radax *et al*. 2001). In this work, we used improved methods for cultivation of isolates, including the addition of a range of potential substrates and sterilized rock salt to growth media, and an improved method for the preparation of DNA from dissolved rock salt. These approaches turned out to be fruitful since a wide variety of culturable halophilic strains could be collected from agar plates, including a potentially novel rod-shaped species (see Fig. 2), and, in addition, a range of novel haloarchaeal gene sequences was identified in rock salt. Initial incubation times for the visualization of culturable haloarchaea were often extensive, such that growth of small colonies took sometimes up to a year, as has been observed previously (Stan-Lotter *et al*. 1999; Radax *et al*. 2001); this extended lag phase could perhaps be ascribed to haloarchaeal dormancy states. Characterization of the isolates indicated similarities to halococci, rod-shaped haloarchaea of the *Halobacterium* type (Grant *et al*. 2001) and pleomorphic forms. A rapid distinction between halococci and pleomorphic forms was provided by restriction digests of 16S rDNA and separation of the resulting fragments (Fig. 3). The improved preparation method for DNA from dissolved rock salt and subsequent amplification of 16S rRNA genes yielded at least 12 novel phylotypes, which were different from previously described cultured as well as uncultured haloarchaea (Fig. 5). Taken together, these results suggested that there is a microbial community of halophilic archaea present in rock salt, which comprises a much wider biodiversity than previously expected.

During the course of this work it was discovered that sterilized rock salt greatly stimulates growth of some haloarchaea (Fig. 4) and leads to higher cell yields. When attempting to grow fastidious microorganisms from a complex environment in the laboratory, it is common practice to add factors from that environment to aid enrichment. For cultivation of extremely halophilic microorganisms, dissolved rock salt (Gillow *et al*. 1999) or extracts from halobacteria (Wais 1988) have been added to media. In many cases, stimulation of growth is due to organic molecules, such as certain amino acids or vitamins; however, with dissolved rock salt, the effect is probably caused by inorganic ions, since organic carbon is generally lacking in rock salt. We are in the process of exploring this phenomenon further, since we believe that its resolution could be useful for future cultivation studies with intra- or even extraterrestrial halite samples.

The isolation of viable terrestrial microorganisms from rock salt of great geological age makes it intriguing to consider the existence of similar extraterrestrial forms of life, particularly since halite was found in several meteorites (Zolensky *et al*. 1999; Whitby *et al*. 2000), including some, which are known to stem from Mars (Gooding 1992; Treimann *et al*. 2000). When extraterrestrial halite material is available for examination, a comprehensive knowledge of terrestrial halophilic microorganisms should have been established, including their potential variations in morphologies, occurrence of nanocells or dormant forms, growth characteristics including extreme lag phases, and molecular properties, which can be examined with ease and precision. It is evident from the data presented here, as well as from those of others (Munson *et al*. 1997; Eder *et al*. 1999; Bowman *et al*. 2000; Cytryn *et al*. 2000; Benlloch *et al*. 2002) that the picture of the diversity of haloarchaea in their natural environments is as of yet quite incomplete, and this may also be true for halophilic members of the eubacteria

(Benlloch *et al*. 2002), which were not considered in this study. The issue is also of crucial general importance, since it touches on the problems which will have to be faced when future data from extraterrestrial materials will be evaluated: in the event that positive signs for microbial life are found, it has to be decided whether these are from novel microorganisms, which are not present on Earth, or microorganisms which are like the ones on Earth, but had not been detected previously, or microorganisms which were transported from Earth into space environments, e.g. by forward contamination.

Several authors have pointed out the stability of liquid hypersaline brines on Mars and speculated on the possibility of extant halophilic life forms in those environments (Fredrickson *et al*. 1997; Litchfield 1998; Landis 2001; Wynn-Williams *et al*. 2001). Assuming carbon-based extraterrestrial life, it is therefore of interest to find out whether terrestrial halophilic microorganisms would be capable of survival and possibly even propagation under Martian conditions. We started a first exploration of exposure conditions and survival estimations of haloarchaea; it should be borne in mind that these experiments were performed with laboratory-grown pure cultures of high densities, which is different from the mixed populations that are present in various hypersaline natural environments. In addition, the haloarchaea investigated here were dried, frozen, freeze-dried or starved (by suspending them in buffer) 'artificially' by exposing them within a short time interval to the respective extremes. This was also different from the situation in most natural settings, where the various microbial survival mechanisms and strategies for dormancy known to date (for a compilation see, for example, Deming & Baross 2000) can develop in a gradual manner over time. Thus, the interpretation of our results is also preliminary, since almost no experimental data are available for haloarchaea in extreme conditions such as those which were used here. The viable cell numbers (expressed as cfu) of haloarchaea following exposure to a Martian-like atmosphere for 6 h were about 10%, or less, of the original culture for *Halococcus dombrowskii*, and about 0.1%, or less, for *Halobacterium* sp. NRC-1, when cells were suspended in saline buffer during exposure. These are, in real numbers, 1–10 million cells per ml of a culture that contained originally 100–1000 million of cells. Similar survival values were obtained following exposure of cells to $-70\,^{\circ}C$ in normal atmosphere; however, much lower survival rates (0.0001–0.001%) – sometimes no survivors – were observed, when cells had been freeze-dried. Glycerol in concentrations of 2–4% provided distinct protection during freeze-drying, which was evident as higher survival rates (0.01–0.1%) and fewer DNA strand breaks, as judged from the presence of intact genomic DNA (data not shown). Glycerol is known as a compatible solute, which can provide osmotic stability to cells; it is produced in natural saline environments by the alga *Dunaliella* and other eukaryotic microorganisms (see Oren 2002 for a review).

Our preliminary results suggested that halococci are somewhat more resistant to extremes of environments – by approximately a factor of 10 under the conditions tested here – than rod-shaped haloarchaea of the *Halobacterium* type. This could possibly be due to the sturdy cell envelope of halococci (Denner *et al*. 1994), in contrast to the thin, flexible cellular surface layers of *Halobacterium* species. Both types of haloarchaea have been found in rock salt; in addition, the genera *Halorubrum* and *Haloarcula* were isolated from ancient salt sediments (McGenity *et al*. 2000). It would be desirable to investigate the responses of all of these genera to the Martian environment, separately as well as in combination, in order to delineate common survival strategies.

We could show that the LIVE–DEAD kit can be used successfully for haloarchaea at high salt concentrations, although it was developed for use at low ionic strength; this suggested that it could serve as a convenient discriminating tool for the rapid estimation of viability of cells, following exposure to various environmental extremes. The high sensitivity of this method allows the detection of single cells and the determination of their morphology, and could probably be directly applicable for the examination of terrestrial and extraterrestrial samples.

The initial studies of the responses of microorganisms to Martian environmental conditions should be greatly expanded, in order to help planning of missions and also judging the forward contamination potential of spacecraft (see Rummel 2001). The possible protective influences of compatible solutes and cations on the survival of haloarchaea and the extent of damage of their DNA should be investigated. In addition, the examination of proliferation of haloarchaea, if any, under Martian conditions should be studied. Besides low temperature, low pressure and a Martian atmosphere, ultraviolet radiation is an important parameter, which affects microbial life (Cockell *et al*. 2000), the influences of which should be investigated using appropriately designed Martian simulation chambers; these type of studies will be carried out in the coming years by the Space Research Institute at the Austrian Academy of Sciences.

Acknowledgements

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