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Extremely halophilic archaea and the issue of long-term microbial survival

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Abstract

Halophilic archaebacteria (haloarchaea) thrive in environments with salt concentrations approaching saturation, such as natural brines, the Dead Sea, alkaline salt lakes and marine solar salterns; they have also been isolated from rock salt of great geological age (195–250 million years). An overview of their taxonomy, including novel isolates from rock salt, is presented here; in addition, some of their unique characteristics and physiological adaptations to environments of low water activity are reviewed. The issue of extreme long-term microbial survival is considered and its implications for the search for extraterrestrial life. The development of detection methods for subterranean haloarchaea, which might also be applicable to samples from future missions to space, is presented.

Keywords

Extreme halophiles; Haloarchaea; Life detection; Microbial longevity; Salt mines; Salt sediments; Space missions; Subterranean; Taxonomy of halobacteriaceae

Introduction

The halobacteria are a group of microorganisms with so many unusual features—growth at salt concentrations higher than those used in any food pickling processes, striking pigmentation in red, orange or purple, obligately salt-dependent enzymes, possessors of the first known proton pump, bacteriorhodopsin, which is driven just by sunlight—that early researchers became almost desperate about “the halobacteria’s confusion to biology”, which was the title of a lecture given by Larsen (1973) and which described what was known at the time about that “life in the borderland of physiological possibilities”. While some halobacterial features have turned out to be not completely singular and while the molecular basis for halophilism is being unraveled, there are still many characteristics which are unique, and new ones have been added since—e.g. a square and flat morphology, potential longevity of halophilic microorganisms in salt sediments for millions of years, implications of the discovery of extraterrestrial halite.

Many excellent monographs and books with extensive reviews on halophilic microorganisms exist (Rodriguez-Valera 1988; Javor 1989; Vreeland and Hochstein 1993; Oren 1999, 2002; Ventosa 2004; Gunde-Cimerman et al. 2005). The subjects of this chapter are a brief survey of haloarchaeal taxonomy, a presentation of some of the special properties

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of extreme halophiles, which are not—or only rarely—present in other microorganisms, and a consideration of the questions about their geological age and the possiblity of halophilic life on other planets or moons.

Taxonomy and phylogeny

The extremely halophilic archaea (also called haloarchaea or, traditionally, “halobacteria”) belong to the order Halobacteriales, which contains one family, the *Halobacteriaceae* (Grant et al. 2001); since the publication of Bergey’s Manual of Systematic Bacteriology (2001), which listed 14 recognized haloarchaeal genera, the number has increased to 19 genera, according to the International Committee on Systematics of Prokaryotes (http://www.the-icsp.org). The number of validated species at this time is 57. One taxonomic criterion for the identification and recognition of haloarchaea is the sequence of the 16S rRNA genes; specific signature sequences and signature bases have been described in detail by Kamekura et al. (2004). These authors recommend also the determination of 23S rRNA gene sequences for refined assignment of novel isolates to haloarchaeal genera. The currently recognized genera and species of the family *Halobacteriaceae* are listed in Table 1; shown are also the data bank accession numbers for the 16S rRNA gene sequences, which lead in most cases to the literature for strain isolation and description.

Historically, the composition of membrane polar lipids has long been used as one of the key chemotaxonomic criteria for the differentiation of haloarchaeal genera (Ross et al. 1985; Kamekura and Kates 1999). All haloarchaea examined to date possess ether-linked phosphoglycerides; phosphatidyl glycerol and phosphatidyl glycerol phosphate methyl ester are always present; many strains contain phosphatidyl glycerol sulfate and one or more glycolipids or sulfated glycolipids (Grant et al. 2001); most glycerol ether core lipids contain $C_{20}C_{20}$ (diphytanyl) isoprenoids, although some strains, especially haloalkaliphiles, possesss also $C_{20}C_{25}$ (phytanyl-sesterterpanyl) or $C_{25}C_{25}$ (di-sesterterpanyl) isoprenoid chains. The halobacterial taxonomy based on the polar lipid composition proved remarkably consistent with phylogenetic data deduced from 16S rRNA gene sequence comparisons (Grant et al. 2001). Halobacteria (haloarchaea) are a monophyletic group, with the most distantly related species showing a 16S rRNA gene sequence similarity of 83.2% (Grant et al. 2001). The methanogens, another archaeal group, are their closest relatives, with less than 80% 16S rRNA gene sequence similarity (Olsen et al. 1994). The complete list of required and recommended criteria for the determination and recognition of haloarchaeal species was proposed by Oren et al. (1997). Three genomes of haloarchaea have been sequenced, that of *Halobacterium salinarum* NRC-1 (Ng et al. 2000), *Haloarcula marismortui* (Baliga et al. 2004) and *Natronomonas pharaonis* (Falb et al. 2005).

Morphology, envelopes and inner structures

The principal morphological types of haloarchaea are rods, cocci and irregular pleomorphic forms, which are mostly rather flat cells. A very unusual shape is exhibited by the well known “square bacterium”, which was detected by Walsby (1980, 2005) and belongs to the haloarchaea (Antón et al. 1999): almost perfectly quadratic cells are attached to each other like stamps and can form large thin sheets (Kessel and Cohen 1982); sometimes cell division has occurred, but individual cells have not separated and have grown to sizes of 40 × 40 μm or even larger (Bolhuis 2005). The reason for the occurrence of such sheets is unknown; a proposition is that oxygen diffusion might be facilitated by large surface areas in the notoriously oxygen-depleted brines (Grant et al. 2001); another suggestion is that square cells, which are probably also phototrophic due to the presence of bacteriorhodopsin (see below), would reach the water surface passively, without the expense of energy as required by flagellar movement (Walsby 2005).

The morphology of non-coccoid haloarchaea can change, dependent on the salt concentration of the environment. With increasing dilution of salt, club-shaped, swollen and bent rods or spheres appear (Mohr and Larsen 1963; Kushner and Bayley 1963). One reason for this behaviour is their cell envelope layer which needs the presence of high concentrations of cations for stability. Rod-shaped and pleomorphic haloarchaeal species possess a surface layer (S-layer), which is composed of a tightly packed hexagonal lattice consisting of one type of glycoprotein (Lechner and Sumper 1987) and is firmly anchored to the plasma membrane by a transmembrane domain; O- and N-glycosylation sites differ from species to species (see Eichler 2003 for a review). The S-layer is thought to have a shape-maintaining function; on lowering the cation concentration, progressively fewer negative charges will be shielded by the positively charge cations, which results in disruption of the envelope layer due to electrostatic repulsion between negative charges on the constituents of the envelope and also of the membranes, with ensuing lysis of cells (Boring et al. 1963). However, other factors play also a role in the process of disintegration, since interference with the biosynthesis of the glycoprotein or its glycosylation produces similarly irregular or spherical cells from rod shaped haloarchaea (Mescher and Strominger 1976). In contrast, a rigid cell wall composed of heteropolysaccharides is present in the coccoid species *Halococcus* and *Natronococcus* (Schleifer et al. 1982; Niemetz et al. 1997). Placing these cells in hypotonic solutions will not cause lysis; cells maintain their viability as was detected by staining with the LIVE/DEAD kit (Leuko et al. 2004, 2005); see also below.

Internal gas vesicles are only produced by prokaryotes; several bacteria and also some halophilic archaea are capable of synthesizing these flotation devices (Walsby 1994). Gas vesicles are filled by diffusion with gases dissolved in the environment; their function is apparently to provide buoyancy and enabling cells to regulate their position in the water. The identification of gas vesicle genes and their regulation is carried out in the laboratories of Pfeifer (Pfeifer et al. 1997; Pfeifer 2004) and DasSarma (Shukla and DasSarma 2004). Walsby (2005) pointed out that without the presence of gas vesicles, which can be detected easily by phase contrast microscopy due to their refractivity, the square haloarchaeal sheets described above would hardly have been recognized as living entities.

Inside the cytoplasm of *Halobacterium salinarum*, fibrillary structures were identified, which apparently consist of a bundle of hollow tubes and were termed “fibrocrystalline bodies” (Cho et al. 1967). Recently, the isolation of these structures was reported and their sensitivity to the drug vincristine (Alba et al. 2001). This feature, together with the appearance of the fibrils, could indicate the presence of a cytoskeleton-like organelle in haloarchaea.

Biochemistry and bioenergetics

Most enzymes from haloarchaea, with only few exceptions, are optimally active and stable in the presence of 3–4 M KCl or NaCl (for reviews see Lanyi 1974; Eisenberg and Wachtel 1987; Oren 2002). The often observed preference of K^+ instead of Na^+ is consistent with the unusual intracellular concentration of potassium ions in haloarchaea, which can be as high as 5 M (Christian and Waltho 1962). The stability and solubility of halophilic proteins under conditions, where non-halophilic proteins would precipitate, was puzzling for early researchers; Reistad (1970) was the first to quantitatively analyse the bulk amino acid composition. She found a high excess—more than 10 mole-%—of acidic amino acids (aspartic and glutamic acid) in proteins from *Halobacterium*. The majority of haloarchaeal proteins have isoelectric points around 4.2 and nearly lack basic proteins; the unique acidity of the proteome from *Halobacterium salinarum* NRC-1 was confirmed by analysis of sequencing data and compared with other microbial proteomes in a clear graphic depiction (Kennedy et al. 2001). Another characteristic feature of halophilic proteins is their low

content of hydrophobic amino acids and accordingly, low extent of hydrophobic interactions within proteins (Lanyi 1974); thus, high salt is needed to maintain those interactions.

Some haloarchaeal proteins were analysed in detail, notably ferredoxin, malate dehydrogenase, dihydrofolate reductase, and showed in X-ray diffraction studies a tight network of acidic residues on the protein surfaces, where water and hydrated K^+ ions are sequestered and form a large number of internal salt bridges (Dym et al. 1995; Frolow et al. 1996; Mevarech et al. 2000; Pieper et al. 1998).

The polar lipids of haloarchaea are used as suitable taxonomic markers (see above); as in polar lipids from all archaea, the core structure is derived from *sn* 2, 3 substituted glycerol, which is a different structure than the polar lipids of most other organisms, where the hydrocarbon chains are connected in a *sn* 1, 2 configuration. Phytanyl (C_{20}) and sesterterpanyl (C_{25}) chains are present, which represent fixed lengths of isoprenoids (Kates 1993; Kamekura 1993; Kates and Kushwaha 1995). An equivalent to the adaptation to temperature changes by varying chains lengths of fatty acids, as is possible for many bacteria, is therefore precluded for haloarchaea. The hydrocarbon chains are generally fully saturated; in one case, *Halorubrum lacusprofundi* from a lake in Antarctica, which grows at the low temperature of 4°C, unsaturated phytenyl chains were found, which might contribute to the regulation of membrane fluidity (Franzmann et al. 1988).

The intense red, pink or purple pigmentation of haloarchaea is due to carotenoids in their membranes and to the C_{50} compound bacterioruberin or its derivates (Kamekura 1993). These non-polar lipids provide a protection against the strong sunlight which is usually characteristic for the natural environments of haloarchaea, and they may act as membrane reinforcers (Kamekura 1993). The retinal containing protein bacteriorhodopsin is present in some species of haloarchaea (Javor 1989) and has been thoroughly studied, because it is a comparatively simple light driven proton pump, capable of producing a pH gradient across the membrane, which is used for the production of ATP; this was first demonstrated in a famous reconstitution experiment by Racker and Stockenius (1974) and opened the way for modern bioenergetics and Peter Mitchell's chemiosmotic theory. For recent reviews on bacteriorhodopsin and related proteins see Lanyi and Varo (1995), Varo (2000), Essen (2002) and Lanyi (2005). Bacteriorhodopsin is not an obligately halophilic protein, but functions well in the absence of salt. The membrane ATP-synthase of several haloarchaea was isolated and showed structural similarities to the V-type ATPases of plants, animals and some bacteria (Ihara et al. 1991; Stan-Lotter et al. 1991; Steinert et al. 1995), but enzyme activity was also affected by compounds which are inhibitors of the F-type ATPase/ATP-synthase (Hochstein 1992; Hochstein and Lawson 1993). Only the haloarchaeal ATPase moiety has been isolated so far, as well as the membrane-embedded proteolipid, but not the whole ATP synthesizing complex (Ihara et al. 1997). Haloarchaea use protons as coupling ions between their respiratory chains and ATP synthases; this applies also to *Natronomonas pharaonis*, which was rather unexpected, since alkaliphiles had generally been thought to use sodium ions instead (Falb et al. 2005).

Genetics

The genome of *Halobacterium salinarum* strain NRC-1 was the first halobacterial genome to be completely sequenced (Ng et al. 2000); in 2004 and 2005, the genome sequences of *Haloarcula marismortui* and *Natronomonas pharaonis*, respectively, were published (Baliga et al. 2004; Falb et al. 2005). The genome of *Halobacterium salinarum* NRC-1 consists of a total of 2,571,010 base pairs, which are organised in one large chromosome and two plasmids called pNRC100 (191,346 bp) and pNRC200 (365,425 bp); several smaller minor plasmids may be present, which are deletion derivatives of pNRC100. The plasmids have

been called minichromosomes or megaplasmids in earlier work, since it was recognized that halobacterial DNA consists of several fractions; they account for 11–36% of the total DNA (Joshi et al. 1963; Pfeifer 1988) and are found in many members of the *Halobacteriaceae* (Gutierrez et al. 1986). The genome of *Halobacterium salinarum* strain NRC-1 contains a total of 91 insertion sequences, which had been identified and classified previously (Charlebois and DasSarma 1995). The insertion sequences are highly mobile elements and are responsible for the high frequency of spontaneous mutations (Charlebois 1999; Pfeifer et al. 1997). The genomes of *Haloferax volcanii* and *Haloferax mediterranei* contain much less insertion sequences and are therefore more stable (Lopez-Garcia et al. 1995).

The genome of *Haloarcula marismortui* is almost twice the size of that of *Halobacterium salinarum* NRC-1—a total of 4,274,642 bp were reported, which are organised into nine replicons (Baliga et al. 2004). The largest replicon was called chromosome I and consists of 3,131,724 bp. Comparisons of the genome sequences of the two haloarchaea revealed interesting additional capabilities for survival in *Haloarcula marismortui*, such as extra signal transducers, numerous environmental response regulators and extra pathways for amino acid synthesis; the number of identified insertion elements was about 40 (Baliga et al. 2004). The genome of *Natronomonas pharaonis* shows certain similarities to that of *Halobacterium salinarum* NRC-1, including nearly identical transposases, but much less insertion elements, which are often present in multiple copies (a total of about 40). Its size is 2,595,221 bp and it contains one plasmid of 130,989 bp and a second multicopy plasmid of 23,486 bp. High similarities to components of the signal transduction systems for chemo- and phototaxis of *Halobacterium salinarum* were detected, albeit with extensive shuffling of domains; coping with the extreme high pH of its environment appears to be supported by different types of glycoproteins in *Natronomonas pharaonis*, which make up a more complex envelope that the usual S-layer of many haloarchaea (Falb et al. 2005).

Extremely halophilic archaea possess, like other archaea, a range of eukaryote-like features such as multisubunit RNA polymerases, homologues to eukaryotic transcription factors, TATA-box promoters; these features make them distinct from eubacteria at the molecular level (Ng et al. 2000; Kennedy et al. 2001). The transcription of proteins in dependence of medium salinity has begun to be investigated with *Haloferax volcanii*; different salt concentrations triggered a differential transcription (Ferrer et al. 1996); differences in gene expression were identified with restriction patterns from genomic DNA, which originated from cells grown at 12% or 20% NaCl (Juez et al. 1990).

Viable haloarchaea in rock salt

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 million cubic kilometers of salt were deposited in the late Permian and early Triassic periods alone (ca. 240–280 million years ago; Zharkov 1981). The continental land masses were concentrated and formed the supercontinent Pangaea. Salt sediments developed in large basins, which were connected to the open oceans by narrow channels. The paleoclimate was warm and arid in a wide belt around the equator, causing large scale evaporation. About 100 million years ago, Pangaea started to break up; the continents were displaced to the North, and folding of new mountain ranges such as the Alps and Carpathians was underway (Einsele 1992). As a result of these movements driven by plate tectonics, huge salt deposits are found today mainly in the Northern regions of the continents (Fig. 1), e.g. in Siberia, Northern and Central Europe (Zechstein series), South-Eastern Europe (Alps and Carpathian mountains), and the Midcontinent basin in North America (Zharkov 1981).

The formation of most of the Alpine salt sediments and the Zechstein deposits is dated to the Late Permian period, while some Alpine deposits are dated to the Early Triassic period (see also Radax et al. 2001 for a detailed description). No significant salt sedimentation had occurred after that period in the pre-Alpidic regions. This is different from some other salt evaporites in Europe; for instance, waters from the receding Tethys sea in the Eastern parts of Eurasia caused salt sedimentation well into the Miocene (about 20 million years ago). The stratigraphic positions of the evaporites, together with the determinations of sulfur isotope ratios, are indicators for the geological age (Holser and Kaplan 1966). In addition, pollen grains or spores from extinct plants in the sediments, which are often well preserved and exhibit distinct morphological features, have been examined (Klaus 1974). Both methods confirmed the Permo-Triassic origin of the Alpine and also of the Zechstein salt sediments. The Alpine salt deposits are located today at altitudes between 500 and 1200 m; their thickness is between 200 and 500 m, although some deeply buried layers were estimated to be up to 1000 m. The layers of clay and limestone prevented the washing-out of the salt during the heavy precipitation during the ice ages. Many of the salt deposits in Europe have been mined for centuries, and newly opened mine tunnels and shafts, as well as deep drilling operations provide opportunities for obtaining rock salt samples. Figure 2 shows freshly drilled bore cores, which were used for our investigations.

Dombrowski (1963) and Reiser and Tasch (1960) were the first to describe viable microorganisms, which were isolated from ancient rock salt, in the 1960s (reviewed by McGenity et al. 2000). More recently, Norton et al. (1993) classified isolates from British salt mines of Permian and Triassic age as species of *Haloarcula, Halorubrum, Halobacterium* and a variety of new types on the basis of polar lipid composition, and Gemmell et al. (1998) investigated the evolution of the *Haloarcula* representatives by comparing 16S rRNA gene sequences with those of surface isolates. Vreeland et al. (1998) isolated halophilic bacteria, some of them probably haloarchaea, from the Permian Salado formation in the midcontinent basin in the USA, and from brines close to that formation.

Our group isolated from Permian rock salt, which was collected from the salt mine in Bad Ischl, Austria, numerous colonies with intense pigmentation (Fig. 3), which indicated the presence of carotenoids and bacterioruberin. One isolate was a coccus, growing in clusters, which was designated strain BIp (Stan-Lotter et al. 1993). Based upon polyphasic taxonomic data, the strain was recognized as a novel species and named *Halococcus salifodinae* (Denner et al. 1994). This was the first isolate from ancient rock salt, which was formally classified and deposited in several international culture collections. Two independently isolated strains, Br3 (from solution-mined brine in Cheshire, England) and BG2/2 (from a bore core from the mine of Berchtesgaden, Germany) resembled *Halococcus salifodinae* BIp in many properties; in addition, rock salt samples were obtained eight years later from the same site and several halococci were recovered from these samples, which proved to be identical to strain BIp (Stan-Lotter et al. 1999). The data suggested that viable haloarchaea, which belong to the same species, occur in geographically separated evaporites of similar geological age. Another halococcal isolate from the Bad Ischl salt formation, which differed from the previously described strains, was subsequently identified as a novel species and named *Halococcus dombrowskii* (Stan-Lotter et al. 2002). *Halococcus salifodinae* and *Halococcus dombrowskii* have so far not been found in any hypersaline surface waters, or any location other than salt mines. Several non-coccoid strains were later obtained from a freshly drilled bore core at the salt mine in Altaussee, Austria (about 40 km distance from Bad Ischl), which were similar in their 16S rRNA sequence to *Halobacterium salinarum* NRC-1; however, other properties were different and consequently, a novel species was created, *Halobacterium noricense* (Gruber et al. 2004). Table 2 contains a list of the formally classified isolates from alpine rock salt and a strain from a British salt mine. Figure 4 shows the relationships of the haloarchaeal isolates from Permo-Triassic rock salt to several

haloarchaeal type species in the form of a phylogenetic tree, based on 16S rRNA gene sequence data.

Another example of an isolate from ancient sediments is a single rod-shaped *Halobacterium* strain from a 97,000-year-old salt formation in the USA (Mormile et al. 2003); the isolate was deemed to resemble *Halobacterium salinarum* NRC-1. The microbial content of ancient rock salt is generally low - estimates range from 1–2 cells/kg of salt from a British mine (Norton et al. 1993) to 1.3×10^5 colony forming units (CFUs) per kg of alpine rock salt (Stan-Lotter et al. 2000); nevertheless, the reports showed that viable haloarchaeal isolates were obtained reproducibly by several groups around the world. The data support the hypothesis that the halophilic isolates from subterranean salt deposits may be the remnants of populations which once inhabited ancient hypersaline seas; in addition, they provide strong evidence against the notion that the recovered strains could be the result of laboratory contamination, since the isolates were obtained independently from different locations.

Analysis of dissolved alpine rock salt with molecular methods was also performed by extracting DNA and subsequent amplification and sequencing of 16S rRNA genes. The results provided evidence for the occurrence of numerous haloarchaea, which have not yet been cultured (Radax et al. 2001; Fish et al. 2002). Similarities of these 16S rDNA gene sequences were less than 90–95% to known sequences in about 37% of approximately 170 analysed clones (Radax et al. 2001; Stan-Lotter et al. 2004); the remaining clone sequences were 98–99% similar to isolates from rock salts of various ages (McGenity et al. 2000) and to known haloarchaeal genera. These data suggested the presence of a very diverse microbial community in ancient rock salt.

How old are the cells from rock salt?

The salt sediments are thought to have been deposited about 280–240 million years ago; while there is no direct proof that viable haloarchaea have been entrapped in rock salt since its deposition, it would also be difficult to prove the opposite, namely that masses of diverse microorganisms entered the evaporites in recent times (see also McGenity et al. 2000). Especially for the Alpine deposits, an influx of meteoric waters containing microorganisms seems rather improbable, because these sediments have been folded up and located at altitudes of 1,000 m or higher for at least the last 100 million years (Einsele 1992), covered by impermeable layers of clay and carbonates (see Radax et al. 2001). In addition, a special property of most haloarchaea (except halococci) is their quick lysis when they are suspended in pure water; therefore, they would not survive for very long in salt-free aquatic environments. If a Permo-Triassic age is postulated for the haloarchaeal isolates, then it becomes necessary to explain the biological mechanisms for such extreme longevity. Grant et al. (1998) discussed several possibilities, such as the formation of resting stages other than spores—since haloarchaea are not known to form spores—or the maintenance of cellular functions with traces of carbon and energy sources within the salt, which would imply an almost infinitely slow metabolism.

What seemed rather inconceivable just a few years ago, is now being considered more seriously: vast numbers of microbes were detected repeatedly in subterranean locations; they may have stayed there for centuries or even millenia, since they were literally found almost everywhere in great depths (the current record is 5,278 m for thermophilic anaerobes; see Pedersen, 2000). Their occurrence is apparently only limited, apart from nutritional support, by the increase in temperature with depth. However, at this time there are no methods available to prove directly a great prokaryotic age, whether it be a bacterium or a haloarchaeon. The mass of an average prokaryotic cell is only about 10^{-12} g (picograms); it is composed of about 3,000 different biomolecules, which are present at femtogram levels or

less; therefore, no current dating procedures can be applied. Perhaps suitable methods for application to single prokaryotic cells will be available in the future. After all, single atoms can be visualized in the laboratory by element-selective electron spectroscopy (Suenaga et al. 2000), and it is conceivable that certain isotopes may be identifed in a similar way from ancient material.

Astrobiology

Mars is a planet where the presence of salts has been demonstrated. Evidence for halite was found in the SNC meteorites (Gooding 1992); their Martian origin has been confirmed independently by several groups (Treiman et al. 2000; Rieder et al. 2004). Elements from Martian soil and rocks, which were recently determined with the alpha-particle X-ray spectrometer, include Na, Mg, Cl, Br (Rieder et al. 2004). Therefore, saturated salt solutions, which would possess greatly depressed freezing points, could be envisaged on Mars. They may not be present as standing pools, but rather could occur in small pore spaces between mineral grains, as suggested by Landis (2001). The apparent longevity of haloarchaeal strains in dry salty environments is of interest for astrobiological studies and the search for life on Mars. On Earth, microorganisms were the first life forms to emerge and were present perhaps as early as 3.8 billion years ago (Schidlowski 1988, 2001). If Mars and Earth had a similar geological past (Schidlowski 2001; Nisbet and Sleep 2001), then microbial life, or the remnants of it, could still be present on Mars. Since halophilic microorganisms appear to survive in dry salt over geological time scales, as our and other studies suggested (Norton et al. 1993; Grant et al. 1998; McGenity et al. 2000), it appears plausible to include specific searches for halophiles in the exploration of extraterrestrial samples or environments. If such searches are planned, several issues should be considered, e.g.

– where are microorganisms located in salt sediments—in fluid inclusions, between grains?

– how can their presence be recognized with some certainty?

– what is the survival potential of microorganisms from salt, under terrestrial and under Martian conditions?

Some answers to these issues can be obtained with the investigation of natural salt samples, e.g. with drill cores and salt lumps from subsurface sediments; some issues would best be approached with laboratory studies, e.g. by embedding halophilic microorganisms in salt crystals, under various settings, in a simulation of extraterrestrial and/or Martian conditions.

Towards life detection methods

We considered the location and survival of haloarchaea in artificially produced halite and the potential effects of dissolution of salt crystals on embedded haloarchaea. We used analysis of single cells, which was mainly based on the labeling with fluorescent dyes (Leuko et al. 2004; Stan-Lotter et al. 2006) contained in the LIVE/DEAD® *Bac*Light bacterial viability kit (referred to as LIVE/DEAD kit) from Molecular Probes. The kit consists of two nucleic acid stains: SYTO 9, which penetrates most membranes freely, and propidium iodide, which is highly charged and normally cell-impermeant; it will, however, penetrate damaged membranes. Simultaneous application of both dyes can be used for enumeration of active cells: green fluorescence indicates viable cells with an intact membrane, whereas dead cells, due to a compromised membrane, show red fluorescence (Haugland 2002).

It is not known if the microorganisms in sediments survive while embedded in dry salt crystals, or in highly saline fluid inclusions, which occur in halite (Roedder 1984). So far,

only one example of a single viable haloarchaeon in a fluid inclusion of a natural salt sample exists: a *Halobacterium* species was isolated by using a micro-drill device and subsequently cultured (Mormile et al. 2003). Norton and Grant (1988) noted the preferential enclosure of halobacteria in fluid inclusions upon formation of crystals. We stained haloarchaeal cells with the LIVE/DEAD kit prior to embedding in salt and prepared salt crystals by drying the cellular suspensions (Fendrihan and Stan-Lotter 2004). Figure 5 shows an example of stained *Halobacterium* cells which were examined 2–3 days, following their embedding in artificial halite. At low magnification, the bright green fluorescence of stained haloarchaea was outlining well the morphology of the characteristic rectangular fluid inclusions of halite (Fig. 5, left panel). At higher magnifications, individual cells became visible (Fig. 5, right panel). The data suggested that the localization of halobacterial cells in the salt was probably exclusively in fluid inclusions of artificial halite, which formed during desiccation, and which are present also in natural halite (Roedder 1984; Mormile et al. 2003).

The recovery of viable cells, which were embedded in artificial halite for various times and under different environmental conditions, is being investigated. Figure 6 shows *Halobacterium salinarum* NRC-1 cells, which were carefully resuspended, following storage in halite for 2 weeks at 37°C, and subsequently stained with the LIVE/DEAD dyes. The majority of cells had intact membranes, as indicated by the green fluorescence; viability was confirmed by plating cell suspensions on solidified medium and determination of CFUs.

Conclusions and outlook

1. Viable haloarchaea were isolated from ancient rock salt; since extraterrestrial halite was detected, a search for halophiles on Mars or in extraterrestrial samples might be plausible. For this purpose, in situ tests, which reveal the presence of cellular entities and provide intense signals, should be considered.
2. Detection of single, preferably viable cells in environmental samples should be improved and developed further. Background signals should be avoided, or their source be clearly identified; studies with different minerals, which are to be expected on Mars, should be done.
3. Embedding studies of haloarchaea in halite should be carried out under Martian or space conditions and the response of haloarchaea to low pressure and a carbon dioxide atmosphere should be examined.
4. The dormancy status of microorganism should be better clarified, preferably on a genetic basis. For this goal, proteomic studies of haloarchaea should be done, which are expected to show differences between protein patterns of actively growing cells and dormant cells, e.g. starved cells, embedded in halite.

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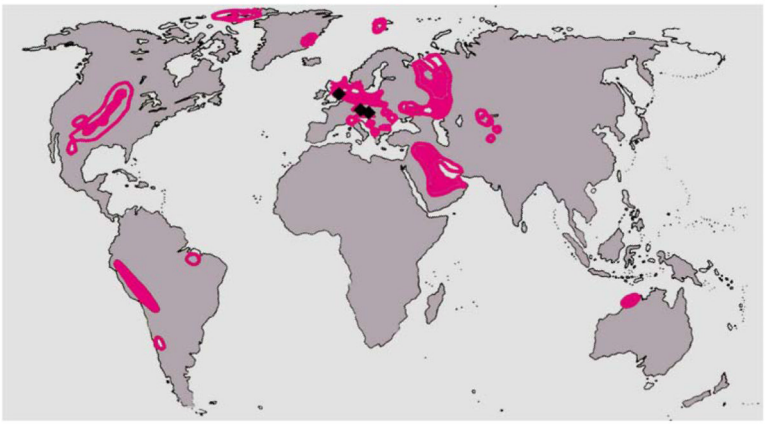

Fig. 1.
Distribution of known and presumed Permian salt sediments (modified from Javor, 1989). Black diamonds indicate locations of rock salt samples (Zechstein deposit in England; alpine deposits in Germany and Austria)

Fig. 2.
Drilled bore cores from the salt mine in Altaussee, Austria, obtained from about 500 m below surface. Pink portions represent halite; greyish portions contain mostly anhydrite and some clay

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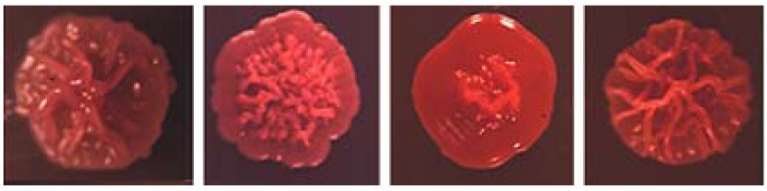

Fig. 3.
Colonies of haloarchaeal cells isolated from alpine rock salt, following growth for 3–4 months on agar with 20% NaCl. Diamenter of individual colonies is about 1 cm

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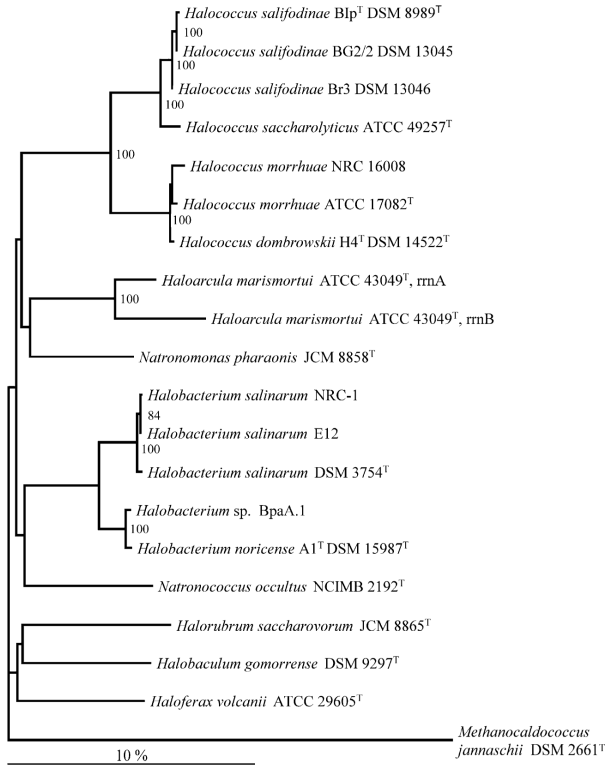

Fig. 4.

Phylogenetic tree based on 16S rRNA gene sequence data indicating the relationship of haloarchaeal species isolated from Permo-Triassic rock salt with established haloarchaeal type species. Catalogue numbers are indicated following type species names. Sequences were aligned with Clustal X (Thompson et al. 1997) and 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). The tree was constructed using the neighbor-joining method (Saitou and Nei 1987). Bootstrap values greater than 70% are indicated at nodes. Scale bar represents 10% sequence difference. *Methanocaldococcus jannaschii* was used as an outgroup

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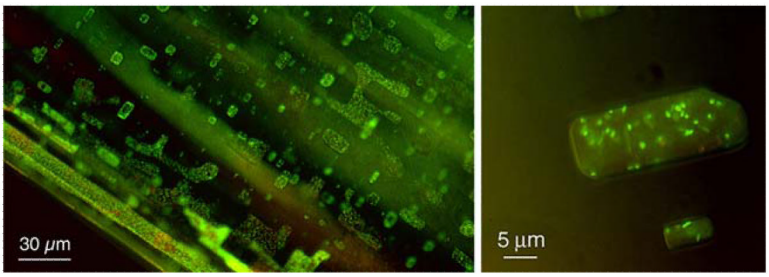

Fig. 5.

Localisation of pre-stained haloarchaea in fluid inclusions. Cells were stained with the LIVE/DEAD *Bac*Light kit prior to embedding in artificial halite. Low (left panel) and high (right panel) magnification of *Halobacterium salinarum* NRC-1 cells, trapped in fluid inclusions for about 2 days. Cells were observed with a Zeiss Axioskope fluorescence microscope

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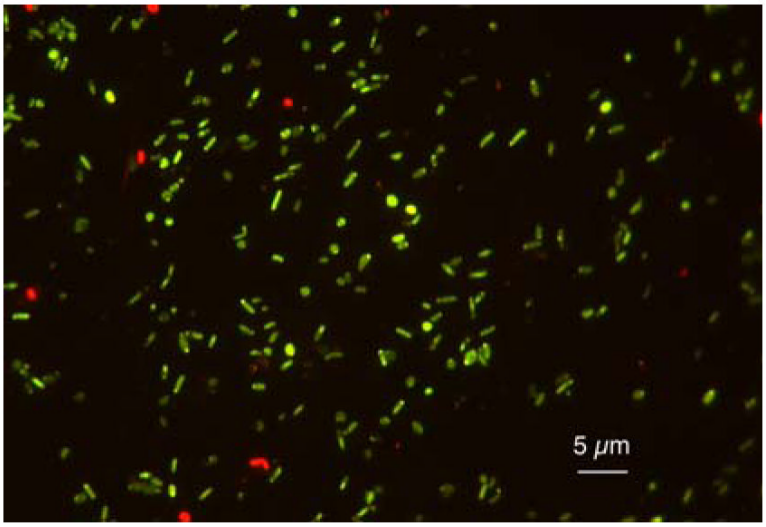

Fig. 6.

Epifluorescence microscopy of *Halobacterium salinarum* NRC-1 cells. Cultures were grown in complex medium, embedded in salt crystals for 14 days at 37°C, resuspended in a salt buffer and stained with the LIVE/DEAD *Bac*Light kit. Pictures were taken on a Aristoplan fluorescence microscope. Green fluorescence indicates intact membranes and thus viable cells, red fluorescence indicates non-viable cells

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Table 1

The family *Halobacteriaceae*. Genera, species and accession numbers (Source: http://www.the-icsp.org and *Epub Int J Syst Evol Microbiol*)

Genus	Species	Accession number
Genus I. *Halobacterium*	*Halobacterium salinarum* (type species)	AJ496185
	Halobacterium noricense	AJ548827
Genus II. *Haloarcula*	*Haloarcula vallismortis* (type species)	D50851
	Haloarcula marismortui	X61688 (rmA), X61689 (rrnB)
	Haloarcula hispanica	U68541
	Haloarcula japonica	D28872
	Haloarcula argentinensis	D50849
	Haloarcula quadrata	AB10964
Genus III. *Halobaculum*	*Halobaculum gomorrense* (type species)	L37444
Genus IV. *Halococcus*	*Halococcus morrhuae* (type species)	D11106
	Halococcus saccharolyticus	AB004876
	Halococcus salifodinae	AB004877
	Halococcus dombrowskii	AJ420376
Genus V. *Haloferax*	*Haloferax volcanii* (type species)	K00421
	Haloferax gibbonsii	D13378
	Haloferax denitrificans	D14128
	Haloferax mediterranei	D11107
	Haloferax alexandrinus	AB037474
	Haloferax lucentensis	AB081732
	Haloferax sulfurifontis	AY458601
Genus VI. *Halogeometricum*	*Halogeometricum borinquense* (type species)	AF002984
Genus VII. *Halorhabdus*	*Halorhabdus utahensis* (type species)	AF071880
Genus VIII. *Halorubrum*	*Halorubrum saccharovorum* (type species)	U17364
	Halorubrum sodomense	D13379
	Halorubrum lacusprofundi	X82170
	Halorubrum coriense	S70839
	Halorubrum distributum	D63572
	Halorubrum vacuolatum	D87972
	Halorubrum trapanicum	X82168

Genus	Species	Accession number
	Halorubrum tebenquichense	AJ276887
	Halorubrum terrestre	AB090169
	Halorubrum xinjiangense	AY510707
	Halorubrum alkaliphilum	AY510708
Genus IX. *Haloterrigena*	*Haloterrigena turkmenica* (type species)	AB004878
	Haloterrigena thermotolerans	AF115478
Genus X. *Natrialba*	*Natrialba asiatica* (type species)	D14123
	Natrialba magadii	X72495
	Natrialba taiwanensis	D14124
	Natrialba aegyptiaca	AF251941
	Natrialba hulunbeirensis	AF262026
	Natrialba chachannaoensis	AJ003193
Genus XI. *Natrinema*	*Natrinema pellirubrum* (type species)	AJ002947.
	Natrinema pallidum	AJ002949
	Natrinema versiforme	AB023426
	Natrinema altunense	AY277583
Genus XII. *Natronobacterium*	*Natronobacterium gregoryi* (type species)	D87970
Genus XIII. *Natronococcus*	*Natronococcus occultus* (type species)	Z28378
	Natronococcus amylolyticus	D43628
Genus XIV. *Natronomonas*	*Natronomonas pharaonis* (type species)	D87971
Genus XV. *Natronorubrum*	*Natronorubrum bangense* (type species)	Y14028
	Natronorubrum tibetense	AB005656
Genus *Halomicrobium*	*Halomicrobium mukohataei* (type species)	D50850
Genus *Halobiforma*	*Halobiforma haloterrestris* (type species)	AF333760
	Halobiforma nitratireducens	AB045012
	Halobiforma lacisalsi	AY277582
Genus *Halosimplex*	*Halosimplex carlsbadense* (type species) (three 16S rDNA sequences were reported)	AF320478
		AF320479
		AF320480
Genus *Halalkalicoccus*	*Halalkalicoccus tibetensis* (type species)	AF435112
Genus, “*Halovivax*”	*Halovivax asiaticus* (type species)	AM039978

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Genus	Species	Accession number
	Castillo et al. (2006)	

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Table 2

Haloarchaeal isolates from Permo-Triassic rock salt and salt mine brine

Organism, strain	Type strain (^{T}), catalogue numbers	Origin	Reference
Halococcus salifodinae Bip	DSM8989^T	Rock salt (lumps), Bad Ischl, Austria	Denner et al. (1994)
	ATCC51437^T		
	JCM9578^T		
Halococcus salifodinae BG2/2	DSM13045	Salt drill core, Berchtesgaden, Germany	Stan-Lotter et al. (1999)
Halococcus salifodinae Br3	DSM13046	Brine in salt mine, Cheshire, England	Stan-Lotter et al. (1999)
Halococcus salifodinae N1	DSM13070	Rock salt (lumps), Bad Ischl, Austria	Stan-Lotter et al. (1999)
Halococcus salifodinae H2	DSM13071	Rock salt (lumps), Bad Ischl, Austria	Stan-Lotter et al. (1999)
Halococcus dombrowskii H4	DSM14522^T	Rock salt (lumps), Bad Ischl, Austria	Stan-Lotter et al. (2002)
	ATCC BAA-364^T		
	NCIMB13803^T		
Halobacterium noricense A1	DSM15987^T	Salt drill core, Altaussee, Austria	Gruber et al. (2004)
	ATCC BAA-852^T		
	NCIMB13967^T		