12 Halophilic Microorganisms

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Concentrated salt solutions like salt or soda lakes, coastal lagoons or man-made salterns, inhabited by only a few forms of higher life, are dominated by prokaryotic microorganisms. Global salt deposits show that evaporation of marine salt water and the development of hypersaline habitats is an ongoing process for millions of years and providing ample time for the evolution of specialized halophilic Bacteria and Archaea. Halophiles, which require more than 0.5 M NaCl for optimal growth [1], have developed two different basic mechanisms of osmoregulatory solute accumulation to cope with ionic strength and the considerable water stress. These mechanisms allow halophiles to proliferate in saturated salt solutions and to survive entrapment in salt rock. The latter was proven by the isolation of viable halophilic Archaea from several subsurface salt deposits of Permo-Triassic age. If halophilic prokaryotes on Earth can remain in viable states for long periods of time, then it is reasonable to consider, under similar extraterrestrial environments, the existence of extraterrestrial organisms. This becomes all the more plausible, considering that halite has been found in several extraterrestrial materials. Here we consider the different mechanisms of osmoadaptation, the environment of halophiles, especially of subterranean halophilic isolates, and the relevance of microbial survival in high saline environments to astrobiology.

12.1 Adaptation to Saline Environments

Adding a solute like NaCl to water will lead to changes in the characteristics of the solvent water's freezing and boiling points as well as vapor and osmotic pressures. These changes are caused by the decrease of the water's chemical potential μ_W, which can be expressed as:

$$\Delta\mu_W = \Delta H_W - T\Delta S_W \qquad (12.1)$$

where ΔH is the change in enthalpy (the heat of reaction), T (K) is temperature, and ΔS is the degree of randomness (change in entropy).

According to Sweeney and Beuchat [2] the second term of the equation above ($T\Delta S_w$) is dominating and the decrease of the chemical potential largely depends on the change in entropy of the water. This is explained by the interference of salt with the ordered water structure, thereby increasing the randomness of the solvent molecules. ΔS_w, the entropy of the solvent, will therefore be positive resulting in a reduction of

the water potential μ_w. A non-adapted organism exposed to a saline environment must cope with its cytoplasmic water having a higher chemical potential than the water of the surrounding environment. Water always flows from a high to low chemical potential until the potential gradient is abolished. Thus, the cytoplasm which is surrounded by a cytoplasmic membrane freely permeable to water, will lose its cytoplasmic water resulting in cell shrinkage. The reduction in cell volume is mainly caused by the loss of free water (approx. 80% of total water in a fully hydrated cell), while the bound water level remains unchanged [3]. This results in the cessation of growth, possibly due to molecular crowding, and thus, reduced diffusion rates of proteins and metabolites. In order to gain sufficient free water and to maintain an osmotic equilibrium across the membrane, the cell has to reduce the chemical potential of the cytoplasmic free water. Two principle mechanisms have evolved on Earth to lower the chemical potential of cell water, allowing an osmotic adaptation of microorganisms: "salt-in-cytoplasm mechanism" and organic-osmolyte mechanism.

1. Salt-in-cytoplasm strategy: Organisms following this strategy adapt the interior protein chemistry of the cell to high salt concentration. The thermodynamic adjustment of the cell can be achieved by raising the salt concentration in the cytoplasm.
2. Organic-osmolyte strategy: Whereby organisms keep the cytoplasm, to a large extent, free of NaCl and the design of the cell's interior remains basically unchanged. The chemical potential of the cell water is mainly reduced by an accumulation of uncharged, highly water-soluble, organic solutes.

12.1.1 Salt-in-Cytoplasm Mechanism

The "salt-in-cytoplasm mechanism", first discovered in *Halobacteria*, is considered the typical archaeal strategy of osmoadaptation. Fermenting Bacteria, acetogenic anaerobes (*Haloanaerobium*, *Halobacteroides*, *Sporohalobacter*, *Acetohalobium*), and sulfate reducers are now known to employ this strategy as well [4]. Despite the abundance of NaCl in the typical haloarchaeal environment, halophilic Archaea keep the cytoplasm relatively free of sodium. Instead, potassium accumulates in the cell (as shown for *Haloferax volcanii* through an energy-dependent potassium uptake system) and together with its counter ion Cl^-, K^+ can be found in molar concentrations in the cytoplasm. Because the K^+ concentration inside the cell is 100 times higher than in the surrounding environment, a part of the proton motive force must be used to maintain the ion gradient. In this energetic respect, the situation in halophilic anaerobic Bacteria is thought to be different; there is evidence that these organisms invest as little as possible in the maintenance of ion gradients. Measurements of the ion composition of exponentially growing cells of *Haloanaerobium praevalens* show that K^+ is the dominating cation, but that Na^+ levels are also relatively high. Cells entering the stationary phase eventually replace K^+ for Na^+ [5]. Analysis of *Haloanaerobium acetoethylicum* even suggest that Na^+ could be the main cation in stationary cells as well as in exponentially growing cells [6].

The effect of the accumulation of potassium and/or sodium in the cytoplasm is that the cytoplasm is exposed to an increased ionic strength. To adapt the enzymatic machinery to an ionic cytoplasm, proteins of halophilic anaerobic Bacteria and halophilic

Archaea contain an excess of acidic amino acids over basic residues [7]. This leads to a predominance of charged amino acids on the surface of enzymes and ribosomes which is thought to stabilize the hydration shell of the molecule when in high ionic surroundings. In low saline environments, the excess of negatively charged ions will destabilize the molecule's structure, due to repulsion when the shielding cations are removed [8]. This mechanism explains the fact that organisms employing the salt in cytoplasm strategy display a relatively narrow adaptation and their growth is restricted to high saline environments [9]. However, in habitats with saturated salt concentrations, halophilic Archaea outcompete organic-osmolyte producers, proving members of the "salt-in-cytoplasm mechanism" as *extreme* halophiles

12.1.2 Organic Osmolyte Mechanism

The organic osmolyte mechanism is widespread among Bacteria and Eukarya and also present in some methanogenic Archaea [10, 11]. In response to an osmotic stress, these organisms mainly accumulate organic compounds like sugars, polyols, amino acids and/or amino acid derivatives either by *de novo* synthesis or by uptake from the surrounding environment. These non-ionic, highly water-soluble compounds do not disturb the metabolism, even at high cytoplasmic concentrations and are thus aptly named *compatible solutes* [12]. Halophilic cells using compatible solutes can basically preserve the same enzymatic machinery as non-halophiles, needing only minor adjustments in their interior proteins (i.e. ribosomal proteins), which are slightly more acidic than the cytoplasmic proteins in *Escherichia coli* [13]. Halophiles employing the organic-osmolyte mechanism are more flexible than organisms employing the "salt-in-cytoplasm strategy" because even though they display wide salt tolerance, they can also grow in low salt environments.

12.1.2.1 Stress Protection by Compatible Solutes

In addition to their function of maintaining an osmotic equilibrium across the cell membrane, compatible solutes are effective stabilizers of proteins and even whole cells. They can act as protectors against heat, desiccation, freezing and thawing, and denaturants such as urea and salt [14, 15]. The reason, why these organic compounds are compatible with the metabolism and can even act as stabilizers of labile biological structures, is explained at the molecular level by the preferential exclusion model. According to this theory, compatible solutes are absent from protein surfaces due to the structural dense water bound to the protein. Compatible solutes show a preference for the less dense free water fraction in the cytoplasmic surrounding. They stabilize the two water fractions by fitting into the lattice of the free water and allowing for the formation of hydration clusters. As a consequence, unfolding and denaturation of proteins become thermodynamically unfavorable (reinforcement of hydrophobic effect). This explains, why organisms adapted to other low water-potential environments take advantage of the beneficial properties of compatible solutes. It is not surprising that cyanobacterial species found in deserts accumulate the compatible solute trehalose to compensate for the deleterious effects of desiccation [16].

12.1.2.2 Compatible Solutes of Halophiles and Their Synthetic Pathways

Halophilic Bacteria and Archaea of the organic-osmolyte mechanism synthesize *de novo* nitrogen-containing compounds as their major compatible solutes. In true halophiles analyzed so far the most predominant solutes are the amino acid-derivatives glycine-betaine and ectoine [17]. Sugars like trehalose or sucrose, which are common in a wide range of microorganisms, and necessary for osmoadaptation, rarely exceed cytoplasmic concentrations of 500 mM and are typically present in organisms of restricted salt tolerance.

In contrast, glycine-betaine and ectoine, which are energetically cheaper to synthesize, maintain suitable cell buoyancy and accumulate well above 1 M. Glycine-betaine, a typical product of halophilic phototrophic Bacteria [17], has also been found in a range of halophilic methanogenic Archaea [18, 19]. In cyanobacteria glycine-betaine is most likely synthesized via the serine/ethanol-amine pathway with choline as an intermediate [20]. In the *Ectothiorhodospira* species the biosynthesis proceeds via the direct methylation of glycine [21]. A similar pathway is proposed for methanogenic Archaea [22]. Among aerobic chemoheterotrophic Bacteria the ability to synthesize betaine is rare. Heterotrophic halophiles belonging to the Proteobacteria and Firmicutes synthesize predominantly the aspartate-derivative ectoine as their main solute [23]. The biosynthesis of ectoine proceeds via aspartic-semialdehyde, diaminobutyric acid and Nγ-acetyl-diaminobutyric acid [24]. The genes encoding the enzymes of this pathway have been isolated and sequenced [25, 26], and their regulation is under investigation [27].

12.1.2.3 Compatible Solute Transport and Osmosensing

Halophilic microorganisms do not rely entirely upon *de novo* synthesis of solutes. They are able to take up solutes or precursors from the surrounding environment, if available [28-30]. To allow for this uptake, these microorganisms are equipped with a set of different transporters, which are osmotically regulated at the level of expression and/or transport activity. These transporters facilitate a far more economical accumulation of compatible solutes. Non-halophiles, unable to synthesize nitrogen-containing compatible solutes, can switch to this energetically favorable method of osmoadaptation thus, gaining a certain degree of salt tolerance [31, 32]. In halophiles, such transport systems may have originally been intended as a means to recover compatible solutes leaking out of the cytoplasm (due to the steep solute gradient across the membrane) which would have otherwise been lost to the environment. Solute producers lacking a functional transporter would lose a significant amount of solutes to the medium [33] and thus, this explains why halophiles must also have transporters specifically for their own synthesized compatible solutes [34].

Osmoregulated compatible solute transporters have been studied mainly in *E. coli*, *Corynebacterium glutamicum*, *Bacillus subtilis* and some halotolerant microorganisms [35]. The uptake systems of these organisms are either high affinity binding protein-dependent ABC-transporter like ProU (*E. coli*), OpuA and OpuC (both *B. subtilis*) or, secondary transporters consisting of a single transmembrane protein. The ABC-systems comprise a periplasmic substrate binding domain, a transmembrane unit and a cytoplasmic protein, which fuels the transport through ATP-hydrolysis. The secondary

transporters are either members of the major facilitator family (MFS; i.e. ProP (*E. coli*) and OusA *(E. chrysanthemi*), respectively) or the sodium/solute symporter superfamily (SSSS; i.e. BetP, EctP, OpuD) [36, 37].

The only compatible solute uptake system of a halophilic bacterium, characterized at the molecular level, is the transporter for ectoine accumulation Tea from *Halomonas elongata* [34]. Tea is not related to any osmoregulated transporter known so far, but is a member of a novel type of secondary transporters called tripartite ATP-independent periplasmic transporters (TRAP-T) [38]. TRAP transporters are binding protein-dependent secondary uptake systems consisting of a substrate binding protein and two transmembrane spanning units. Tea is only the second TRAP transporter described at the molecular level and the first osmoregulated transporter of this family. The affinity of Tea for ectoine is high (K_s= 25 µM) and the transporter's design may be intended to combine this advantage with a high transport rate as known from other secondary transporters.

Since osmoregulated transporters are exposed to both the high saline environment and the cytoplasm, it was hypothesized that these systems would also function as sensors for osmotic changes. This was proven to be true for the secondary solute transporter ProP from *E. coli* [39]. Reconstitution experiments in proteoliposoms showed ProP to be a stand-alone osmosensor, able to regulate its own activity in response to osmotic stress. In the cellular background, however, ProP is also influenced by other components like the regulatory protein ProQ, which is responsible for fine-tuning the transporter's activity [40]. Successful reconstitution experiments have also been carried out using the solute uptake system BetP from *C. glutamicum* demonstrating that this transporter acts as an osmosensor as well [41]. Due to their function as transporters and sensors, systems like ProP or BetP exert an important influence on osmoadaptation of the cell e.g., osmoregulatory processes like compatible solute synthesis will be shut down, while osmoregulatory transporters are active. This implies that these systems must be integrated in signal transduction with the cell's metabolism. Whether osmoregulated transporters also take on the function as transducers in signal transmission is still to be resolved.

12.2 Saline Terrestrial Environments and Their Inhabitants

The major habitats of halophilic microorganisms are (i) salt waters (salt lakes, brines, ponds) and (ii) soils. In the latter, the matrix potential of the soil adds to the water stress caused by high salt concentrations. High saline waters originate either by seawater condensation (thalassohaline) or by evaporation of inland surface water (athalassohaline). The salt composition of thalassohaline waters resembles that of seawater with NaCl as the main constituent. Athalassohaline lakes can differ in their ion composition from seawater derived lakes. Some athalassohaline waters have a very high concentration of divalent cations (for example, the Dead Sea with the main cation Mg^{2+} instead of Na^{+}), while others are free of magnesium and calcium due to the presence of high levels of carbonate. Increased carbonate concentrations lead to the formation of soda lakes, which have pH-values well above 10 (for example, the Wadi Natrun in Egypt). Microflora have been found in all of the above types of saline waters, indicat-

ing that halophilic microorganisms tolerate high salinity and can adapt to different stressors like high pH or extreme temperatures. Cold salt lakes, like the well-studied Organic Lake in the east Antarctic Vestfold region, are of interest, since they are thought to perhaps resemble the extraterrestrial environments on the Jovian moon Europa (see below).

The Organic Lake ecosystem contains salt concentrations of between 0.8 and 21% and an anoxic layer below a depth of 4 to 5 m [42]. Eukaryotic algae of the genus *Dunaliella* are found in this ecosystem, but no multicellular organisms have been detected. Procaryotes, including, moderately halophilic chemoheterotrophic Bacteria and many strains belonging to genera including *Halomonas* and *Flavobacterium* have also been isolated from the lake. Strains of *Halomonas subglaciescola*, upon further analysis, displayed a broad salt tolerance from 0.5 to 20% and were able to grow at temperatures below 0 °C.

Often overlooked and ignored saline environments are the subsurface salt deposits. These sites are of specific importance to research on extraterrestrial life, since the isolation of viable halophiles has been reported from ancient terrestrial subsurface salt environments. It has also been suggested that organisms on other planets may have survived in the planet's subsurface environment (i.e. Mars, see below). It is therefore of interest to examine in greater detail the characteristics of terrestrial subsurface salt deposits and the organisms isolated from these sites.

12.2.1 Distribution and Dating of Ancient Salt Sediments

During several periods in the Earth‘s history, immense 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. 245 to 280 million years ago; [43]). These salt sediments formed, during the existence of the supercontinent Pangaea (Permian) or the earlier Gondwanaland (Cambrian and Devonian), in large basins which were connected to the open oceans by narrow channels. Warm temperatures and an arid climate prevailed around the paleoequator, where the land masses were concentrated, causing large scale evaporation. About 100 million years ago, fragmentation of Pangaea took place, the continents were displaced to the North, and mountain ranges such as the Alps and Carpathians folded up, due to plate tectonics [44]. As a result of this shifting, the huge salt deposits, some of them up to 1200 m in thickness, are found today predominantly in the Northern regions of the continents, e.g., in Siberia, Canada (Mackenzie basin), Northern and Central Europe (Zechstein series), South-Eastern Europe (Alps and Carpathian mountains), and the Midcontinent basin in North America [43].

In contrast to other sediments, salt deposits are nearly devoid of macroscopic fossils, on which an age determination can be based. Instead, palynological and isotope studies, in addition to stratigraphical information are used. Klaus [45, 46] detected in dissolved rock salt, from numerous samples of Alpine deposits, plant spores from extinct species, which exhibited well preserved morphological features. The spores *Pityosporites*, *Gigantosporites* and others that were detected, are characteristic for the Permian period and can be clearly distinguished from *Triadosporites* species which are found in Triassic evaporites. The formation of most of these Alpine salt sediments and

the Zechstein deposits were dated to the Upper Permian period, while some Alpine deposits were dated to the Triassic period.

Sulfur isotope ratios (expressed as $\delta^{34}S$) are used for evaporites, which contain sulfates of marine origin [47]. Worldwide results from samples of doubtless stratigraphical relationships showed an extremely low $\delta^{34}S$ value for evaporites of Permian age (+8 to +12‰), and a higher $\delta^{34}S$ value (+20 to +27‰) for those of Triassic origin. Using sulfur isotope ratios from numerous anhydrite and gypsum samples, Pak and Schauberger [48] could confirm a Permian or a Triassic age for many of the alpine salt deposits ($\delta^{34}S$ values of +10 or +25/27‰, respectively). Similarly, the Zechstein series of deposits were confirmed to be of Permian origin.

12.2.2 Subterranean Halophilic Microorganisms and Their Relation to Surface Halophiles

Dombrowski [49, 50] and Reiser and Tasch [51] were the first to describe viable microorganisms which were isolated from rock salt. Other reports, one as early as 1935, demonstrated bacteria-like rods in dissolved rock salt residues and thin sections of rock salt, but recovery of these viable microorganisms was either not tried or not successful [52, 53].

Dombrowski´s [49, 50] enrichments from rock salt samples of Zechstein (Northern Germany) and Precambrian (Siberia) origins, when introduced to a salt-saturated medium, yielded strains resembling *Bacillus circulans*, and another isolate, called *Pseudomonas halocrenea*. The latter was subsequently shown to be identical to *Pseudomonas aeruginosa* and thus, has to be considered a contaminant.

Tasch and coworkers [51, 54] described diplococci which were obtained from rock salt of Permian age from Kansas (Carey mine, Hutchison) and Bibo et al. [55] were able to isolate viable halophilic rods and diplococci from Zechstein salt (Neuhof near Fulda, Germany).

It is not always clear in the results of these researchers whether the isolates consisted of halophilic Archaea, since red or pink pigmentation was not mentioned by Dombrowski [49, 50] and Bibo et al. [55]. However, the papers by Tasch [54] and Nehrkorn [56], which critically evaluated Dombrowki´s work, contain references to pigmentation. After 1991, publications on this topic consistently include descriptions of red or pink pigmentation of isolates, e.g., from subterranean brines of a Permian basin [57] or from rock salt of Permo-Triassic age [58, 59]. From these reports it appears that haloarchaea are the prevailing types of viable halophilic microorganisms in rock salt. To some extent, their prevalence may be due to the methods of enrichment, which favor selection of mainly aerobic, neutrophilic, heterotrophic and reasonably fast growing halophiles. It is highly likely that there exists many more haloarchaeal genera in rock salt than detected, as can be deduced from molecular analyses (see below). These other genera quite possibly have simply not been brought to growth yet. In contrast, extremely halophilic Bacteria of mostly white or yellowish appearance were recovered from brine pools, brine injection fluids, and only rarely from rock salt [58, 55].

Norton et al. [58] classified isolates from two British salt mines (Winsford, Chesire, and Boulby, Cleveland) of Permian and Triassic age as *Haloarcula* and *Halo-*

bacterium species on the basis of polar lipid composition. Their strain 54R was identified as a close relative of *Halobacterium* (now *Halorubrum*) *saccharovorum* by analysis of its 16S rRNA sequence [62], whole cell protein composition and presence of a V-type ATPase [60]. However, DNA:DNA hybridisation showed only 48% homology [58]. Phylogenetic analysis of 16S rRNA sequences confirmed the relationship of several isolates from British rock salt to *Haloarcula* and *Halorubrum*, and showed also that 16S rRNA genes, of which *Haloarcula* species possess at least two, did not differ significantly from those of present-day isolates [63].

Halococcus salifodinae was isolated from Permian rock salt in Austria and was described by us as a novel species [59] with strain BIp representing the type strain. Subsequently, it was found that independently isolated strains Br3 (from Cheshire, England; isolated by W. D. Grant and C. F. Norton in 1989) and BG2/2 (from Berchtesgaden, Germany; isolated by K. O. Stetter in 1988) resembled *H. salifodinae* BIp in colonial morphology and partial 16S rRNA sequences. *H. salifodinae* BIp grows in tetrads or larger clusters. This growth pattern was also observed in the strains BG2/2 and Br3 (see Fig. 12.1).

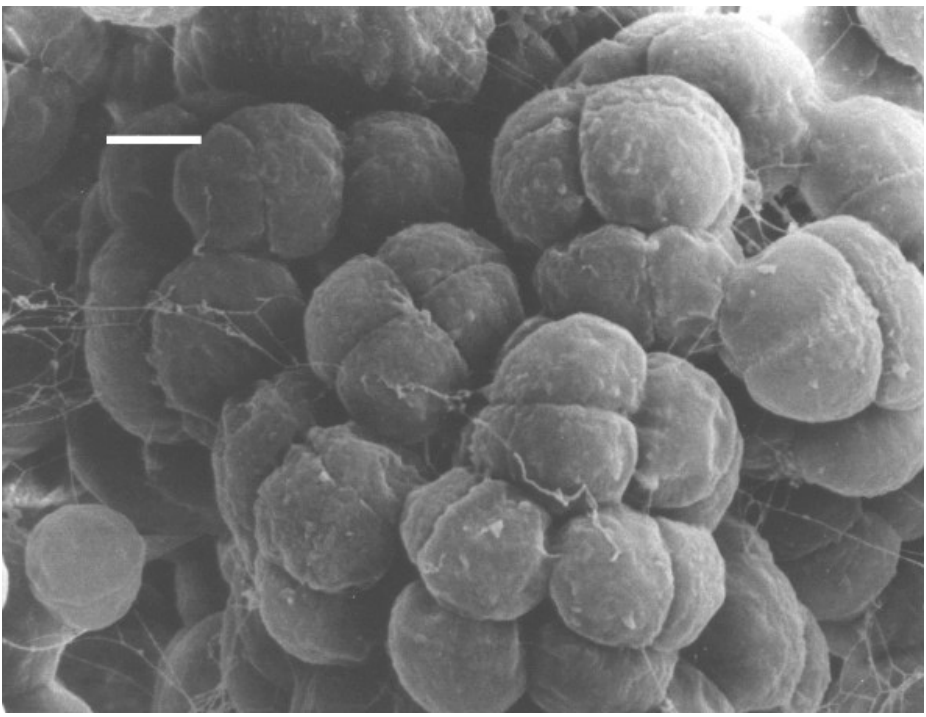

Fig.12.1 Scanning electron micrograph of *Halococcus salifodinae* Br3 (DSM 13046), grown in liquid culture medium [59]. Bar represents 1 micrometer. Photograph by Dr. G. Wanner.

Because *H. salifodinae* BIp included initially only one strain, we obtained more salt samples from the same site and recovered eight years after the original isolation several

halococci, which proved to be identical to *H. salifodinae*. The detailed analysis of these isolates and the strains Br3 and BG2/2, which includes complete 16S rRNA sequences, G+C contents, sequences in a 108 base-pair insertion in the 5S rRNA gene, composition and relative abundance of polar lipids, antibiotic susceptibility, enzymatic activities and Fourier-transform infrared spectra, has been described [64]. This led to the conclusion that all isolates belong to one species, *H. salifodinae*. Thus, we demonstrated that in geographically separated halite deposits of similar geological age, identical species of halococci are present.

H. salifodinae has not yet been found in any hypersaline surface waters. While it is too early for a definite conclusion about its true native environment, it is tempting to speculate that it might be a marker organism for ancient salt deposits from certain geological periods.

12.2.3 Uncultivated Phylotypes

PCR amplification of diagnostic molecules, such as the 16S rRNA genes, and subsequent sequencing of cloned products are increasingly used for an evaluation of microbial diversity in various environments. This technique obviates culturing of microorganisms and has permitted the detection of novel and unexpected phylogenetic groups, e.g., in ocean samples [65, 66]. We used PCR with DNA prepared from dissolved rock salt (from Bad Ischl, Austria) for the amplification of 16S rRNA genes, which yielded at least two partial sequences with strong homology to haloarchaeal genes [61]. Further DNA sequences derived from about 60 cloned PCR products indicated the presence of five clusters of distinct phylotypes. Similarity values of three clusters to known 16S rRNA gene sequences were below 95%, suggesting the presence of several uncultivated novel haloarchaeal taxa [67].

In other rock salt samples (drilling cores from Altaussee, Austria, and Berchtesgaden, Germany) none, or only very few culturable cells were found, and no amplifiable DNA was detected. It is likely that these salt samples did not contain sufficient cellular material for the DNA extraction procedure modified from Benlloch et al. [68], which involves some precipitation steps. The apparent presence of uncultivated haloarchaea in only certain rock salt samples confirmed our impression that the distribution of halobacterial strains appears to vary between different strata (see below).

12.2.4 Distribution, Origin and Dispersal of Haloarchaea

The total number of colony-forming units (cfu) on complex solid medium with 20% NaCl was approximately 1.3×10^5 per kg of dry rock salt, from the salt mine at Bad Ischl, Austria [61]. Culturable strains exhibited differences in pigmentation, cellular and colonial morphology, whole cell protein patterns and other phenotypical characteristics (Fig. 12.2). The results from PCR amplification of 16S rRNA genes corroborated the presence of haloarchaeal diversity. Thus, the halophile microbial community in rock salt appears almost comparable to that of salt lakes in various parts of the world [69].

From some other rock salt samples, however, much lower numbers were obtained;

for example, drilling cores from Altaussee or Berchtesgaden produced only about 10 cfu per g of dry salt [61]. Norton et al. [58] obtained on average one viable enrichment per 0.5 kg of rock salt from British salt mines. In contrast, Vreeland et al. [70] reported up to 10^4 cfu per g of rock salt from the Salado formation in New Mexico, USA.

These data suggest that the microbial content of salt sediments may vary greatly between sites. The reasons for the differences are at present unknown, but they could be due to geological (high local pressures due to tectonics, and therefore high temperatures) or perhaps biological (e.g., presence of halophilic phages or other agents, which would decimate populations) factors.

While there is no direct proof that viable microorganisms in rock salt have been entrapped since the time of their deposition, it would also be difficult to prove the opposite, namely that large numbers of extremely halophilic Archaea, or Bacteria, entered salt deposits in recent times. An influx of liquids containing microorganisms is rather improbable for the Alpine deposits in particular, since the ancient evaporites are now folded up to heights of 1000 to 2000 m, and the salt layers are located on average between 400 and 1200 m above sea level. Migration of microorganisms into the halite would require transportation by meteoric water, a source that is not known to contain extreme halophiles. In addition, layers of dolomite, limestone, marl, clay and other rocks cover alpine salt deposits. Most of these layers are water-impermeable, and have contributed to the preservation of the salt deposits during tectonics and thrust over geological times and the heavy precipitation during the ice ages.

The finding of identical strains of *H. salifodinae* in Alpine and British salt sediments could be explained by a continuous Permian hypersaline sea in Europe, which was inhabited by haloarchaea, and which gave rise to evaporites with trapped microorganisms.

H. saccharovorum was isolated from a saltern near San Francisco [71]; the similar strain 54R was obtained from Permian rock salt in England. The distance across the densely packed land masses in paleozoic times was only about 8500 km between the

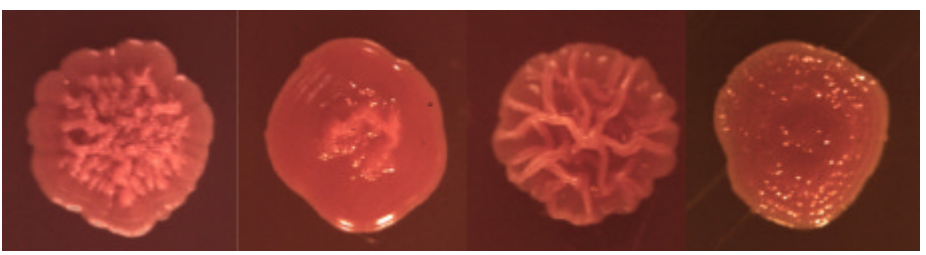

Fig.12.2 Colonies of several haloarchaeal isolates from Austrian Permo-Triassic rock salt, grown on agar plates containing nutrients and 20% NaCl.

two sites, with the huge mid-continent evaporitic basin in between [43]. It is not inconceivable that dispersal of halophiles, perhaps enclosed in crystals, did occur across the land, since wind-blown halite, together with Saharan sand, was found in England in 1984 (see [62]).

12.2.5 Long-Term Survival

Morita provides in his book [72] a history of the recognition by several microbiologists of a "cryptobiotic", "dormant", "latent" or "moribund" state of microorganisms, where no signs of a normal metabolism could be recognized, yet from which they were able to return to a physiologically active state. Such microorganisms are thought of being in a state of starvation, which could persist for long periods of time. Starvation responses of several bacterial genera have been studied in the laboratory; these include miniaturization of cells, protein degradation, reduction in endogenous respiration rate, and others (reviewed in [73]). No similar studies have yet been made with haloarchaea; thus, only suggestions for possible mechanisms of long-term survival can be considered here.

Our hypothesis is that the halophilic isolates from rock salt are the remnants of populations, which inhabited the ancient brines and were included in salt crystals upon evaporation. Whether they persisted in the dry salt sediments or in tiny fluid inclusions, which are present to various extents in rock salt [74], is not clear. At least in some Zechstein salt samples no fluid inclusions were detected in thin polished sections of 15 µm thickness [75]; microscopic inspection of several alpine salt samples has so far failed to reveal inclusions (Stan-Lotter, unpublished data). The argument for the entrapment of halophilic Archaea in fluid inclusions is based on laboratory experiments, where salt crystals with embedded Archaea were produced [76], and from which viable haloarchaea could be cultivated after at least 6 months. Similar fluid inclusions in salt sediments could contain potential energy sources, at least during the early stages of entrapment, as pointed out by McGenity et al. [62]. Later, migration of fluid inclusions could have taken place with dissolution and re-crystallization on a small scale, which could presumably transport halophiles to new energy sources.

Some further possibilities for the longevity of halophiles have been discussed by Grant et al. [63], such as the formation of resting stages or cyst-like structures, which were described for extremely halophilic Archaea from soil, for instance *Halobacterium distributum* and *Natronomonas* (formerly *Natronobacterium*) *pharaonis* [77]. Spore production has not yet been found in any halophilic Archaea, but certain thallus-like structures from coccoid haloarchaeal strains [78] are known, which apparently occur in natural environments and are lost during culturing in the laboratory. It is not sure, if such forms may be involved in long term survival. Still, these findings suggest that the potential for morphological changes, dependent on environmental conditions, appears to be present also in haloarchaea.

There is at present no method, which would allow the determination of the age of a single prokaryotic cell, since its biomass is only about 1 picogram and its composition comprises more than 3000 different molecules, putting the task beyond any procedural sensitivity obtainable to date. Most scientists, therefore, agree with the suggestions by Kennedy et al. [79] and Grant et al. [63], who demand repeated culturing from similar environments (see also Chap. 4, Horneck et al.), and would not consider claims of single isolates [80] from ancient materials as proof for antiqueness, even if procedures were used, which are customary in the food industry for reducing the possibility of contamination [80].

12.3 Relevance to Astrobiology

The discovery of nanofossils in the carbon-containing meteorite ALH 84001 [81] was interpreted as evidence for Martian life, which is thought to resemble terrestrial microbial life. Although there is still much controversy about the true significance of the fossilized structures (e.g., [82]), possible contamination by terrestrial polycyclic aromatic hydrocarbons [83] and issues relating to the composition and inclusions of the meteorite, no evidence currently exists to completely refute the hypothesis of Martian traces of life.

In fact, several features of the discovery have strongly influenced and focussed our thoughts on what to look for, in terms of traces of life on Mars, namely: microbial life of a similar morphology of terrestrial microbes, albeit perhaps of smaller sizes, with similar building blocks [81], and an occurrence in the subsurface environment of the planet. Stevens [84] and others [85] suggested that life may have survived in the subsurface of Mars. Thus, terrestrial salt sediments of great geological age are an eminently suitable analog to perceived Martian lakebeds.

Mars and Earth share a similar geological history; they may have possessed similar early environments and thus, it is imaginable that life arose on both of these planets. If evolution followed a similar course to that on Earth, and if it proceeded at a similar rate, one could postulate that halophiles appeared early on Mars, as was suggested by Fredrickson et al. [86]. Numerous features on the Martian surface suggest the possibility of past erosion by liquid water. Recently obtained high-resolution views of the Martian surface (6 m per pixel), taken by a Mars orbiter camera [87], have led to the proposal that there is evidence for liquid water on Mars.

Traces of halite were detected in the SNC meteorites, which stem from Mars [88, 89]. Also, on the Jovian moon Europa salts were detected [90] and the Galileo spacecraft collected evidence that support the existence of a liquid ocean on Europa. Galileo's onboard magnetometer, which measures magnetic fields, detected fluctuations that are consistent with the magnetic effects of currents flowing in a salty ocean.

Recently, even macroscopic crystals of extraterrestrial halite, together with traces of sylvite (KCl) and water inclusions, were found in the Monahans meteorite, which fell in Texas in 1998 [91]. The age of this meteorite was estimated by Sr/Rb dating to 4.7 ±0.2 billion years. It is remarkable that the contents of the liquid inclusions of this meteorite - NaCl, KCl and H_2O - are also important ingredients for growth of halophilic Archaea and Bacteria.

Lastly, the issue of forward contamination should be considered, since NASA plans to select sites on Mars which will be probed for the presence of extant or extinct life. Samples from these sites will be returned to Earth for further investigation. Recovery and identification of novel microscopic life forms should take into consideration the potential presence and extreme long-term survival of terrestrial microbes.

A deeper understanding of the practical issues, constraints and limits of prokaryotic survival under extreme conditions can be developed from the study of halophilic Bacteria and Archaea.

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