CellPress

Review

Buried Alive: Microbes from Ancient Halite

Salla T. Jaakkola,[1] Janne J. Ravantti,[1] Hanna M. Oksanen,[1] and Dennis H. Bamford[1,*]

Halite is one of the most extreme environments to support life. From the drought of the Atacama Desert to salt deposits up to Permian in age and 2000 meters in burial depth, live microbes have been found. Because halite is geologically stable and impermeable to ground water, the microbes allegedly have a syn-depositional origin, making them the oldest organisms known to live on Earth. Recently, our understanding of the microbial diversity inside halite has broadened, and the first genome sequences of ancient halite-buried microbes are now available. The secrets behind prolonged survival in salt are also starting to be revealed.

Ancient Life Underground

The biosphere extends far below us. Many underground spaces, for example, the deep sea bottom, ground water reserves, and terrestrial **evaporite basins** (see Glossary) are inhabited by microbes. The total number of underground microbes has been estimated to be around $3.8–6.0 \times 10^{30}$ cells, which is around 90% of all the microbial biomass on Earth [1]. However, little is known about these organisms and their overall impact on our planet's ecology.

Hypersaline environments are rich in halophilic microbes (Box 1). Sometimes the halophiles become encased inside precipitating minerals, such as halite. Viable archaea and bacteria have been isolated from halite that is up to 280 Mya old [2–4]. Interestingly, these cells have been suggested to originate from the same time period as the surrounding minerals. Access to these living fossils and their sequences provides an unparalleled opportunity to study microbial evolution and longevity. In this review, we discuss the recent findings regarding the diversity and survival methods of halite-buried microbes, and the significance of studying microbes inside evaporites.

Why Study Ancient Halite-Buried Microbes?

Halite-buried cells are challenged by starvation, accumulation of end metabolites, deleterious mutations, degradation of cellular components, extremely high ion concentrations, and anoxic conditions. Consequently, they must have qualities enabling their survival within evaporite formations, and novel survival strategies could be discovered. Available sequence data from halite-buried microbes is increasing, opening up new possibilities in halophile research. There are several ribosomal 16S sequences from ancient halite [5–9], and the complete genomic sequence of an Early Cretaceous (123 Mya) *Halobacterium* isolate, *Halobacterium hubeiense*, has been recently published [10]. A draft genome for Permo-Triassic (225–280) Mya *Halococcus salifodinae* (GenBank: AOME00000000.1) is also available. The draft genome of *Halosimplex carlsbadense* (GenBank: AOIU00000000.1) from Permian (250 Mya) rock salt was released in 2013, but the strain originates from an unsterilized sample and might not have an ancient origin [11]. These sequences are opening up a new way to study microbial evolution and longevity. However, more of these ancient genomes need to be sequenced before significant results can be obtained.

Trends

The first genome sequences of cultivable halite-buried microbes have become available. Most of the genes are highly homologous to those in contemporary halophiles.

Among cultivated halite-buried microbes, archaea belonging to *Halobacterium* and *Halococcus* are common, whereas bacteria are rare. Ribosomal 16S data has revealed many bacteria and unknown archaea in the sediments.

Some halophilic archaea form miniaturized spore-like cells inside halite.

Polyploidy has been observed in halite-buried and contemporary halophilic archaea. Polyploid microbes are efficient in DNA maintenance, which is beneficial for prolonged survival.

Closely related isolates are found in ancient halite and contemporary hypersaline environments. Microbes in halite are likely to contribute to halophilic surface flora, explaining the similarities.

[1]Institute of Biotechnology and Department of Biosciences, University of Helsinki, Helsinki, Finland

*Correspondence: dennis.bamford@helsinki.fi (D.H. Bamford).

Box 1. Microbes in Hypersaline Environments

Salt lakes and evaporation ponds with salinity close to the point of precipitation house dense populations of halophilic archaea, bacteria, algae, and their viruses [83]. These waters are usually rich in nutrients and reside in warm climates, supporting the rich microbial flora. Archaea of the family Halobacteriaceae dominate, and species of *Salinibacter* and *Dunaliella* are common among bacteria and algae, respectively. The red and pink coloration associated with saline waters is caused by carotenoid pigments in these cells [5,83]. Halophilic microbe populations are also present in dry terrestrial environments, such as salt deserts and halite deposits [5,17].

Adding to the high ionic strength, many hypersaline environments are affected by strong UV radiation, which can cause mutations. Some salt lakes also experience seasonal drought, and may even dry up completely. Halophilic microbes have many adaptations to overcome these challenges. Light-activated membrane proteins, efficient mutation repair systems, high genomic GC%, acidic proteomes, and elevated intracellular potassium ion concentration are typical features of halophilic cells [84,85].

Evaporite minerals, such as halite, sylvite, and gypsum, form by precipitation in saline waters, and tiny liquid inclusions often form inside them. Inclusions in halite are typically of micrometer scale, and their abundance may reach up to 10^{10} inclusions per cm^3 [30]. Sometimes microbes become trapped inside these inclusions, where liquid water allows them to remain viable and wait for the environmental conditions to become more hospitable [56]. The topmost mineral layers at the anoxic bottom of salt lakes are commonly colonized by anaerobic archaea and bacteria, which are referred to as endoevaporitic communities [86].

Glossary

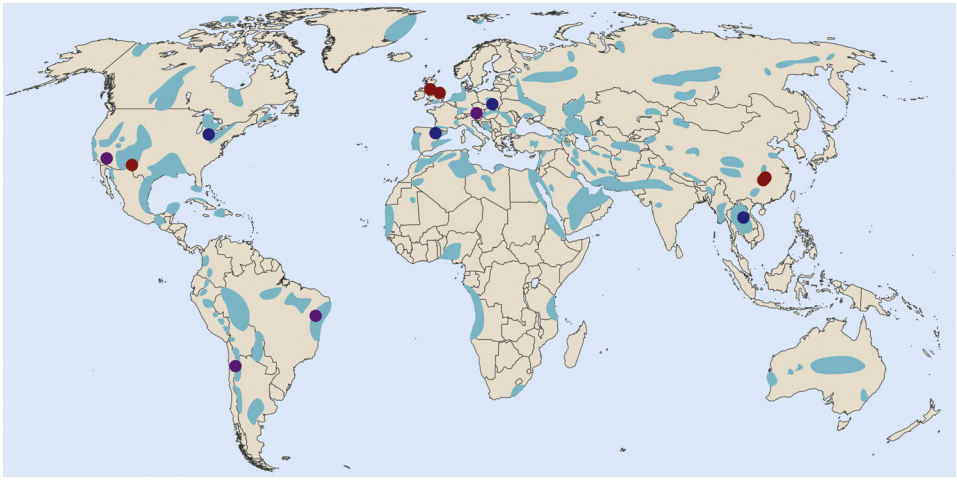
Biomass: the total mass of all living organisms.
Cosmogenic isotope: rare isotopes, such as carbon-14, beryllium-10, and chlorine-36, created by high-energy cosmic rays. These isotopes are produced at a known rate, and can be used for radiological dating.
Endolithic/-evaporitic microbes: microbial cells living inside rocks or evaporitic minerals, such as halite or gypsum.
Evaporite basin: a geological formation of buried minerals that have formed by precipitation.
Halite: a mineral form of sodium chloride, also called rock salt.
kya: thousand years ago, or thousand years of age.
Mya: million years ago, or million years of age.
Necromass: biomass of dead organisms.
Syndepositional or synsedimentary: material that has been deposited simultaneously with sediment formation.

Since halite is chemically stable and has a low water permeability and heat conductance, it is used for storing radioactive waste. However, the heat-emitting waste may affect surrounding minerals and biological material and liquid pockets inside them. The risks related to microbial activity have also been considered, especially concerning the microbe-mediated motility of radionuclides [12,13]. The Waste Isolation Pilot Plant in the Salado formation in New Mexico has been shown to house microbes with high phenotypic diversity [14]. The facility is designed to hold various types of waste, including cellulose, which is consumed by various microbes in the sediments [14]. Because metabolically active microbes could cause corrosion of waste containers or build up pressure with gases, it is important to study the risks related to the microbial communities inhabiting the repository sites so that the biological aspect can be taken into account when designing safe solutions for long-term waste-deposition. There is also a possibility of life existing in the subsurface of other planets and celestial bodies [15]. For example, the now-frozen water on Mars may have been liquid in the past, offering a prerequisite for life. There is evidence of evaporitic minerals on the planet [16], and if there ever was life on Mars, remnants of it could now be in slumber under the surface. Spectroscopic techniques have been tested for searching for signs of life in dry halite environments [17], and are intended to be used on Mars as well. Other potential targets for searching for halophilic life in the Solar System are Jupiter's moon Europa and Saturn's moon Enceladus [18,19]. Identifying biological signatures of halophiles in extraterrestrial halite would support the theory of panspermia and an extraterrestrial origin of life [17]. All in all, ancient halite is a fairly unknown habitat, and the overall significance of buried microbes is still uncertain. They might reveal unknown stories from the history of life, perhaps even on other planets, and might help us to understand mechanisms of defying time.

The Age and Origin of Microbes in Halite Sediments

Halite deposits are found all across the world. Microbial isolates and ribosomal 16S sequences have been retrieved from halite sediments in North and South America, Europe, and Eastern Asia (Figure 1 and Table 1). There are two main methods used for halite sampling. First, crystals can be cut or blasted off from freshly exposed mine walls; this is easy and inexpensive. However, these sites have already experienced disturbances. For example, the change in pressure near mine shafts and tunnels may affect fluid movement in halite. Second, drill-core sampling allows us to obtain material from great depths at undisturbed sites, but the costs are high. The drilling fluid might also introduce contaminants into the bore hole, and the outermost layers of the drill cores may become colonized by outside microbes.

To ensure that the isolated cells and extracted DNA originate from ancient material, the halite crystal surfaces need to be sterilized. Methods using ethanol and/or a Bunsen lamp have been



Trends in Microbiology

Figure 1. Ancient Halite Used for Microbiological Studies. Global halite deposits are shown in turquoise, modified from Kozary *et al*. [87]. Sampling sites are also indicated where viable cells were isolated (red dots), multiplying ribosomal 16S sequences were found (blue dots), or both (purple dots).

used up to 2001 [6], but should be considered obsolete. Chemical sterilization methods have been developed by Rosenzweig *et al*. [20], Gramain *et al.* [5], and Sankaranarayanan *et al*. [21], the latter two concentrating on DNA extraction from halite. Direct extraction of the inclusion brine by a microdrill has also been employed [2,22], as well as cutting off the outer layers of surface-sterilized crystals [10,23].

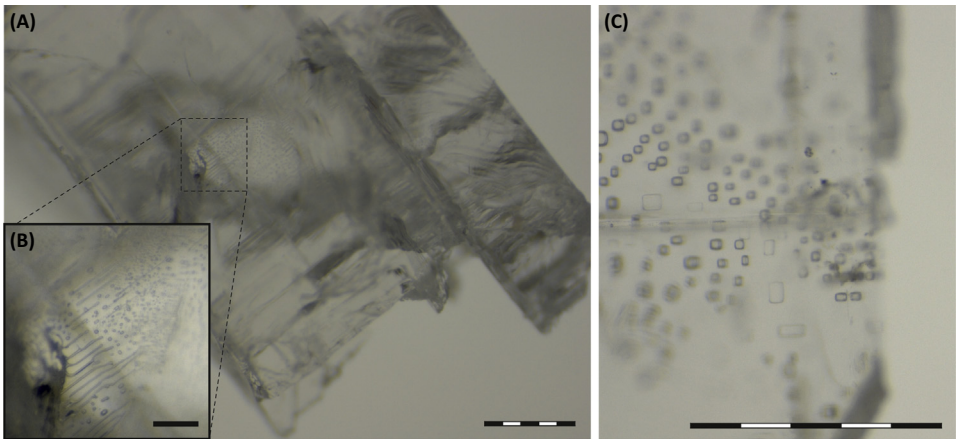
Age estimates of some evaporite formations are based on fossil and lithological data only [24], but radiometric methods give more precise results. In the best case, the isolation sample itself is dated [10]. Because halite is usually poor in fossils and datable isotopes, surrounding minerals are often dated instead. Zircons are fairly ubiquitous in sediments, and can be dated by measuring uranium and lead isotope ratios [25]. Cosmogenic isotopes of beryllium or chlorine can reveal when the halite surface has last been exposed [26]. Argon or potassium–argon dating is used for evaporitic minerals such as polyhalite or langbeinite, which are often associated with halite. Because argon has a half-life of 1.3 billion years, these methods are applicable to the oldest rocks and sediments. Argon dating of polyhalite has been used for the dating of Alpine salt formations and the Salado formation, where microbes have been isolated (Figure 1, Table 1) [27,28]. Halite can also be dated optically, although this technique is not commonly used [29]. Energy from ionizing radiation in the mineral reveals the last time it has been exposed to sunlight.

Halite-buried microbes are not necessarily as old as the mineral, which can dissolve and recrystallize. Recrystallization of solid halite may also occur due to pressure, but only cells inside primary crystals are guaranteed to be synsedimentary. In primary halite, liquid inclusions are usually small, numerous, and often aligned along the crystal faces (Figure 2) [30,31]. Secondary crystals tend to be clear with larger and irregular inclusions [30,31]. Gases inside inclusions dissolve at the temperature present at inclusion formation [32]. The temperatures indicate whether the inclusions have formed near the surface or after being buried [33,34]. Because sea water ion ratios have changed over geological eras, the ion ratios of the inclusion brine work as indicators of the inclusion age. Environmental scanning electron microscope X-ray energy dispersive analysis technique has been used to measure these ratios to prove the ancient origin of isolates from buried halite [34].

Table 1. Isolated Microbes and Ribosomal 16S Sequences from Surface Sterilized Ancient Halite

Genus	Domain[a]	Location	Age[b]	Refs
Isolates				
Haloarcula	A	Winsford mine, UK	195–225 Mya	[24]
Halobacterium	A	Altaussee, Austria Bad Ischl, Austria Death Valley, USA Salar Grande, Chile Sergipe Basin, Brazil Qianjiang depression, China Winsford mine, UK Yunying mine, China	225–280 Mya 225–280 Mya 97 kya 2 Mya 112–121 Mya 123 Mya 195–225 Mya 40 Mya	[2,3,5,10,22–24,88]
Halococcus	A	Bad Ischl, Austria Berchtesgaden, Germany	225–280 Mya 225–280 Mya	[49,50,88,89]
Halolamina	A	Yunying mine, China	40 Mya	[23]
Halorubrum	A	Death Valley, USA	22–34 kya	[35]
Haloterrigena	A	Death Valley, USA	22–34 kya	[35]
Natronobacterium	A	Sergipe Basin, Brazil	112–121 Mya	[90]
Natronomonas	A	Death Valley, USA	22–34 kya	[35]
Oceanobacillus	B	Yunying mine, China	34–49 Mya	[33]
Virgibacillus	B	Salado formation, USA	250 Mya	[2]
Unidentified	A	Boulby mine, UK	225–270 Mya	[24]
Ribosomal 16S Sequences				
Acinetobacter	B	Khorat plateau, Thailand	65–96 Mya	[8]
Burkholderia	B	Khorat plateau, Thailand Wieliczka mine, Poland Wyandotte, USA	65–96 Mya 11–16 Mya 415–425 Mya	[8]
Haloarcula	A	Remolinos, Spain Death Valley, USA	23 Mya 22–34 kya	[7,9]
Halobacterium	A	Alpine region, Austria/Germany Death Valley, USA Michigan Basin, USA Salar Grande, Chile Sergipe Basin, Brazil Wieliczka mine, Poland	225–280 Mya 22–34 kya 419 Mya 2 Mya 112–121 Mya 11–15.8 Mya	[5–9]
Halobiforma	A	Death Valley, USA	22–34 kya	[9]
Halomicrobium	A	Death Valley, USA	22–34 kya	[9]
Halonotius	A	Death Valley, USA	22–34 kya	[9]
Halorhabdus	A	Death Valley, USA	22–34 kya	[9]
Halorubrum	A	Remolinos, Spain Death Valley, USA	23 Mya 22–34 kya	[7,9]
Halosimplex	A	Death Valley, USA	22–34 kya	[9]
Natronomonas	A	Death Valley, USA	22–34 kya	[9]
Pseudomonas	B	Khorat plateau, Thailand	65–96 Mya	[8]
Stenotrophomonas	B	Wieliczka mine, Poland	11–16 Mya	[8]
Unidentified	A	Alpine region, Austria/Germany Death Valley, USA Michigan Basin, USA Sergipe Basin, Brazil	225–280 Mya 22–34 kya 419 Mya 112–121 Mya	[6,7,9]

[a]A, Archaea; B, Bacteria.
[b]kya, thousand years ago, or thousand years of age; Mya, million years ago, or million years of age.



Trends in Microbiology

Figure 2. Liquid Inclusions in a 40 Mya Halite Crystal. Bars represent 0.5 mm in (A) and (C), and 0.1 mm in (B). Figure from Jaakkola *et al.* [23], reprinted by permission from the journal *PLoS ONE*.

Cells inside halite can be detected by *in situ* microscopy, proving their endoevaporitic origin [35,36]. In one study, 20% of liquid inclusions in recently formed halite contained cells, 1% of inclusions in halite 10 000–58 000 years old contained cells, and none of the inclusions contained cells in halite 100 000 years old [36]. In another study, some cells were detected in halite 100 000 years old as well, but it was a rare occurrence, whereas all crystals 10 000–34 000 years old contained cells [35]. Biomolecules, such as carotenoids, peptides, and nucleic acids, can be identified by Raman spectroscopy [37]. This technique has been used to detect halophile pigments in halite up to 1.44 million years old [38]. Because the molecules themselves may be preserved in halite (Box 2), spectroscopic data cannot distinguish between living and dead cells. However, it can be used to identify potential samples for microbe isolation.

Acyclic isoprenes from archaeal membranes make up most of the organic matter in hypersaline basins [39]. Because haloarchaeal membranes contain only diether lipids, the ratio of archaeol to caldarchaeol (ACE index) can be used to estimate the variation of salinity in paleoenvironments [40,41]. Another indicator of paleosalinity is a low pristane–phytane ratio [42]. Pristane and phytane are derived from phytol, which is a common degradation product of chlorophyll. Under the anoxic conditions of the salt lake subsurface, formation of phytane is favored, and ratios below 0.5 are typically detected [42]. Gammacerane is abundant in hypersaline and other stratified waters [43]. C_{35} hopanes are common, and *n*-alkanes with an even number of carbons are preferred [44]. Biomarkers also provide the oldest evidence of halophilic archaea. Neoproterozoic (542–1000 Mya) evaporites in central Australia [45] and Proterozoic (85–1730 Mya) evaporites in Northern China contain indigenous biomarkers from haloarchaea [45].

Biomarker studies suffer from hydrocarbon contamination from drilling fluids and airborne petroleum pollutants, and the contaminating molecules may diffuse inside porous samples [46]. The distribution of a biomarker from the inside to the exterior of the sample rock can be used to rule out contaminants since diffused molecules tend to have a higher concentration on the outside [45]. Hydrocarbons inside liquid inclusions provide contaminant-free biomarkers [47].

Microbial Diversity in Ancient Halite

Deposited halite is not rich in species. The number of species is estimated to be in the range of dozens [44]. Isolates from ancient halite represent archaea from only eight known genera, while nine archaeal genera were found from metagenomic studies (Table 1). Several ribosomal 16S

Box 2. Biomarkers in Halite

Even microbes leave fossils behind [91]. However, single-cell fossils are rare, and only crude structural features are preserved. Microbial populations of the past may also be identified from molecular fossils. The hydrocarbon skeletons of lipids can remain intact for over a billion years inside sediments, serving as biomarkers [92]. Hypersaline basins are important reservoirs of crude oil and natural gas. Under high pressure, organic matter forms solid and highly insoluble mass called kerogen, from which oil and gas are derived. When searching for fossil fuels, the biomarkers help with distinguishing oil-producing and gas-producing kerogen from each other, dating the source rock, and estimating source rock thermal maturity [92]. Biomarkers from different types of organism can also reveal environmental conditions of the past.

Typical bacterial lipids have ester-linked fatty acyl chains (Figure I). Instead of ester-linked fatty acyl chains, archaeal lipids have ether-linked branched isoprenoid chains. Archaea have both bilayer-forming diether lipids, such as archaeol, and monolayer-forming tetraether lipids, such as caldarchaeol (Figure I). The tetraether lipids are common in thermophilic archaea, but are not synthetized by halophilic archaea. Crenarchaeol, present in crenarchaeal membranes, has isoprenoid chains with carbon rings.

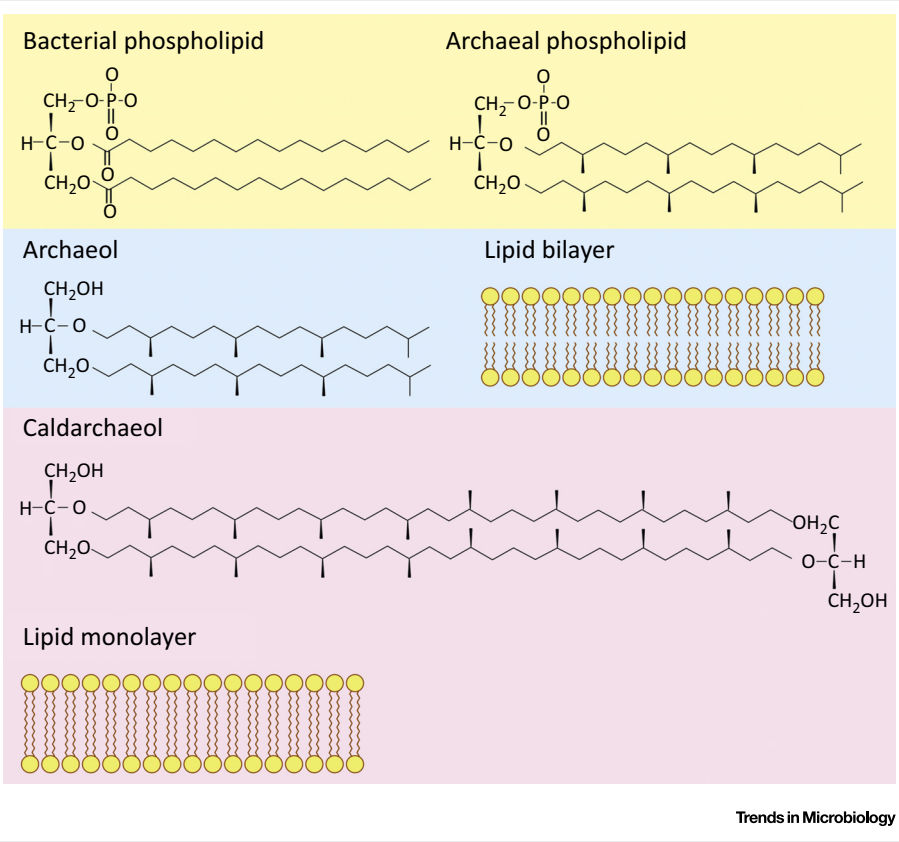


Figure I. Examples of Lipids in Archaea and Bacteria. Examples of phospholipids of bacteria and archaea are shown on the yellow background. Structures of archaeol and caldarchaeol are shown with their corresponding membrane types on blue and pink backgrounds, respectively.

sequences from ancient halite also reveal archaea and bacteria of unknown genera (Table 1, Figure 3) [6,7,9,44]. Some species and genera might exist only in burial sites, or are best represented in underground habitats. For example, *Hsx. carlsbadense* from deposited halite used to be the only representative of its genus, until two novel species of *Halosimplex* were isolated from a salted seaweed product in 2014 [11,48].

Halobacterium cells and sequences are common in halite sediments around the world (Table 1). Two closely related species, *Halobacterium hubeiense* and *Halobacterium noricense* originate

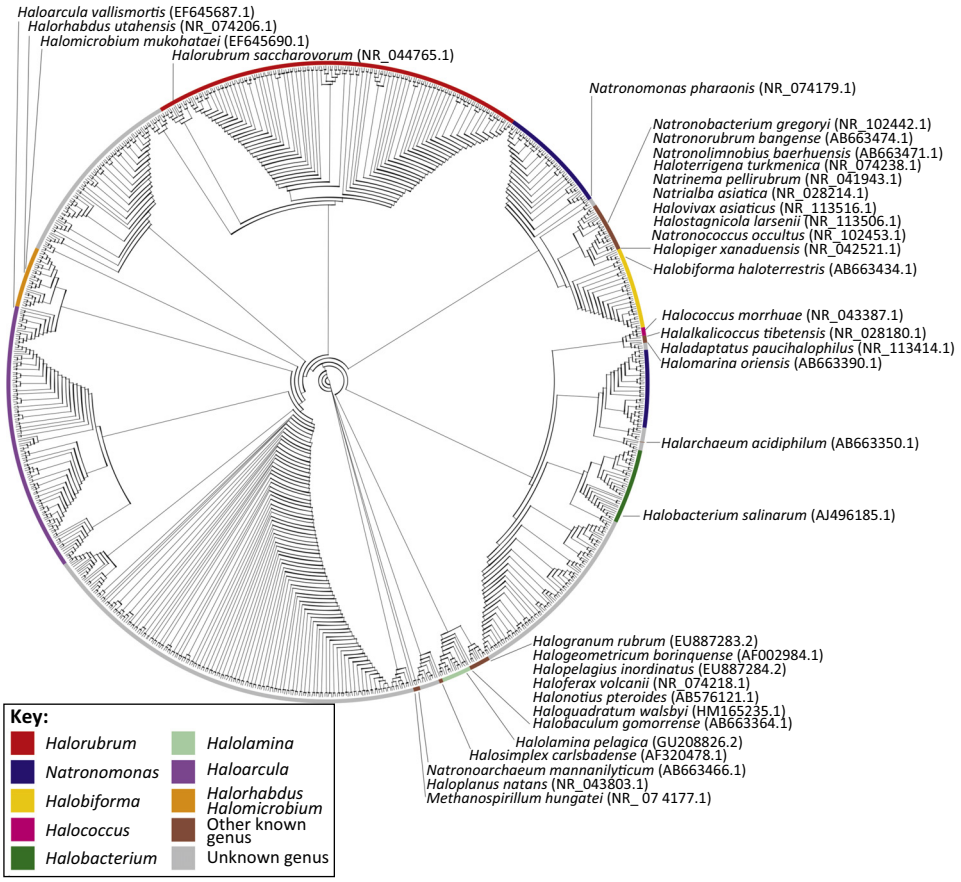


Figure 3. Maximum Likelihood Tree of Archaeal Ribosomal 16S Sequences from Ancient Halite (Table 1). Type strains of haloarchaeal genera (indicated in the picture) were used as references. The genera indicated by colors have members with at least 97% ribosomal 16S sequence similarity to the sequences from halite. The tree was constructed by MEGA6 using the Tamura-Nei model, and the bootstrap consensus tree is inferred from 1000 replicates [93]. Sequences shorter than 400 bp were discarded for proper alignment.

from Early Cretaceous and Permo-Triassic halite, respectively [3,10]. *Hbt. noricense* has also been detected in other ancient and contemporary hypersaline environments [5]. *Halobacterium* cells recover faster than other common halophiles from entombment inside laboratory-grown halite, showing that it is well adapted for temporary halite-entombment [5]. Surprisingly few *Halobacterium* sequences were identified from Death Valley halite compared to other halite burial sites [9]. Either the prehistoric conditions in this area have favored genera other than *Halobacterium*, or this anomaly might result from primer selection.

Halococci are coccoid halophilic archaea that are able to withstand great fluctuations in salinity due to their rigid cell wall structures. Strains of *Halococcus salifodinae* and *Halococcus dombrowskii* have been isolated from different locations in the Permian Zechstein Sea (251–258 Mya) region in Europe, suggesting that halococci were abundant in this prehistoric sea [49,50].

It is common that only a fraction of microbial diversity detected by sequence-based methods is revealed by culturing. Two bacterial isolates, *Virgibacillus* sp. 2-9-3 and *Oceanobacillus* 3-4, have been obtained from ancient salt [2,33], but ribosomal 16S sequences suggest a greater

abundance (Table 1). Eukaryotes seem to exist in the sediments only as cellular remains, such as those of microalgae *Dunaliella* that have been found inside 100 kya halite [51]. Anaerobic endoevaporitic microbes are common in modern saline lakes, but there are no reports of strict anaerobes in ancient halite, although species of *Virgibacillus* and many haloarchaea may grow anaerobically.

Screening for viruses from this environment has been attempted without success [10]. Having no way to counteract DNA and protein degradation, free virions are unlikely to remain infective. Proviruses instead could potentially survive within halite-buried cells, and they are known to exist in subseafloor sediments at least up to the depth of 320 meters [52].

Some hypersaline lakes have a very low pH. Microscopy of acid-precipitated halite has revealed entrapped cells in both modern and Permian crystals [53,54]. Structures called 'hairy blobs' are unique to halite and gypsum from extremely acidic hypersaline environments with a pH of 3 or lower [55]. They are suspected to consist of acidophilic sulfur-oxidizing microbes, which are organized on sulfate crystal surfaces [55]. Since most hairy blobs reside outside of liquid inclusions, the cells are likely to be dead [55]. It seems likely that only certain species are able to survive the harsh conditions in halite, and the observed diversity does not reveal the full microbial diversity present at the prehistoric environments. However, the differences between microbial communities in different halite deposits may reflect the conditions in the ancient hypersaline waters.

Factors Contributing to Longevity

Halite-encasing protects cells from desiccation and radiation, enabling life even in dry deserts [17]. It also allows salt-lake microbes to survive when the salinity rises or the lake dries up completely [56]. Nonetheless, in laboratory experiments only 1–10% of *Halobacterium* cells survive entrapment in salt [57,58]. Some bacteria, including *Virgibacillus* sp. 2-9-3 [2], sporulate under unfavorable conditions, usually triggered by nutrient deficiency. The maximum age for bacterial spores has been estimated to be around 109–250 million years, which is around the same age as the oldest microbial isolates from ancient halite [59,60]. However, it is not known if sporulation occurs inside halite. Archaea do not sporulate, but might form resting cells adapted to starvation survival.

Small spherical cells of *Halobacterium salinarum*, *Hbt. noricense*, and *Haloferax mediterranei* have been observed in laboratory-grown salt crystals and elevated ionic concentrations [58,61]. The spheres form by division of rod-shaped cells, and have a reduced ATP content and a prolonged lag phase, thus resembling bacterial spores [58,61]. Miniaturization of the cells increases their surface-to-volume ratio, enabling more efficient nutrient uptake. However, because salt lakes are usually rich in nutrients, and the division occurs in high osmolarity, it seems to be an adaptation to increased osmotic pressure instead of starvation. Miniaturized cells have also been detected within ancient halite and ice-core samples [36,62], and atypically spherical *Halobacterium* cells were isolated from 40 Mya halite [23]. In one study, however, only long, thin cells of *Hbt. salinarum* were observed inside laboratory-grown halite [57].

The buried cells could be metabolically active. Ongoing metabolism has been observed in cyanobacteria inside halite 10 months after entombment [63], and microbes inside permafrost show evidence of DNA replication and repair [64,65]. The scarce energy is most likely used for cell maintenance instead of growth and replication. Microbial activity *in situ* has not been studied in buried halite, but the subsurface prokaryote turnover rate has been estimated to be around 1000–2000 years [1], and the necromass turnover rate in subseafloor sediments around 100 000 years [66]. As liquid inclusions in halite form small closed systems, the metabolic activity rates are likely to be even lower.

Starvation survival is essential inside the inclusions. *Hsx. carlsbadense*, isolated from unsterilized Permian halite, can grow only on glycerol supplemented with acetate or pyruvate [11]. Glycerol produced by microalgae *Dunaliella* is an important source of carbon in hypersaline environments, and viable microbes in halite have been shown to co-occur with *Dunaliella* cell remains [51,67]. The percentage of surviving cells may actually depend on the amount of dead ones providing sustenance for the remaining population. In the subseafloor, only ~4% of the amino acid carbon is in viable cells, supporting the idea that enough necromass is required to maintain an intraterrestrial microbial community [66].

Cellulose found inside evaporites could provide carbon and energy [68]. The cells also store energy in the form of polyhydroxyalkanoates [69]. Permian archaea *Hcc. salifodinae*, *Hcc. dombrowskii*, and *Hbt. noricense* produce intracellular polyhydroxyalkanoate granules, whereas the modern *Hbt. salinarum* R1 or NRC-1 do not [69]. Molecular hydrogen has also been suggested to be an important energy source for subsurface microbes [70]. Many bacteria and archaea oxidize hydrogen for energy, although haloarchaea are not able to do so (see review by Schwartz *et al.* [71] and references therein). Leakage-resistant haloarchaeal membranes might help in maintaining membrane potential by inhibiting ion flow through the membrane, thus conserving energy [72]. The anaerobic conditions present in the liquid inclusions also allow saving energy in biosynthesis. It has been calculated that aerobic biosynthesis in microbes requires approximately 17 kJ $(g\ cells)^{-1}$ more energy than anaerobic to synthesize the same biomass [73].

DNA damage is often caused by radiation, reactive metabolites, or hydrolysis. Microbes employ a system called the SOS response to identify and repair damaged DNA. The damage can vary from a single nucleotide alteration to single- and double-strand breaks, and there are specific repair proteins for each situation. Halophiles are often exposed to great amounts of UV radiation in their living environment. Because adjacent thymines in DNA are prone to dimerization in a photochemical reaction caused by UV irradiation, halophile genomes commonly have a high GC content. Halophiles also have efficient photoreactive DNA repair systems [74].

Most extreme halophiles use intracellular potassium to obtain osmotic balance. Genomes of halite-buried microbes are protected from radiation by the sediments and high levels of intracellular potassium chloride [75]. The potassium uptake system KdpFABC has been shown to enhance *Hbt. salinarum* survival in salt crystals [57]. However, no *kdp* genes were found in the genome of Early Cretaceous *Hbt. hubeiense* [10]. Haloarchaea are usually polyploid [23,75], including archaea from 40 Mya salt [23]. Polyploidy reduces mutation rate and helps repair double-strand breakage [76,77]. The extra DNA also serves as phosphate storage, allowing polyploid cells to grow even in the absence of phosphate, which is often a growth-limiting nutrient [78].

Halophiles are extremely well adapted for survival in harsh environments. The strategies used for thriving in a salt lake, of which some are also acidic, alkaline, or very hot or cold, are also useful for prolonged survival inside halite. The way halophilic microbes are able to overcome radiation, desiccation, starvation, and high osmotic stress makes them one of the best candidates for surviving for millions of years, and possibly even space travel.

Relationship between Buried and Surface Microbes

Microbes inside ancient halite are surprisingly closely related to contemporary halophiles. The most thoroughly analyzed example of this is the genome of *Hbt. hubeiense*, with most of its genes being homologues of known haloarchaeal genes [10]. The same seems to be true with the genome of Permo-Triassic *Hcc. salifodinae* (GenBank: NZ_AOME00000000.1). It is around 4.2 Mb in size and has over 4000 genes, of which ~61% have a predicted function, meaning that they have counterparts in sequenced genomes.

The Permian *Hcc. salifodinae* and *Hcc. dombrowskii* have been compared to five *Halococcus* type strains from the contemporary hypersaline environments [79]. *Halococcus* species divide into three separate phylogenetic groups [79]. Because the Permian strains reside in different groups, these evolutionary divisions must have taken place before or during the Permian period. The gene content and physiological attributes of halite-buried microbes can also reveal details of haloarchaeal evolution. Because halophilic archaea are strongly affected by lateral gene transfer, their evolutionary relationships are unclear. Halite-buried microbes can help in the analysis of gene transfer between species and the development of metabolic pathways. For example, the ancient *Hbt. hubeiense* has no genes for bacteriorhodopsin [10], unlike *Hbt. salinarum* [80], suggesting that the genes have been obtained more recently, or have been lost in *Hbt. hubeiense*. In contrast, the DNA repair genes in *Hbt. salinarum* and *Hbt. hubeiense* are similar, showing that there has been little change in this system [10].

In addition to determining when speciation or gene transfers have occurred, halite-buried microbes might help re-evaluate the concept of a 'molecular clock', referring to the amount of changes occurring within a certain time frame in a certain sequence. Evaluating realistic molecular clock rates is difficult because most microbes inhabit oligotrophic niches, where growth rates are considerably slower than in laboratory conditions. Genes of halite-buried microbes have been suggested to be used for estimating the mutation speed in natural low-nutrient conditions [79]. Comparing the sequences of *recA* and *splB* (coding for the DNA repair protein and the serine protease, respectively) as well as the ribosomal 16S gene of *Virgibacillus* sp. 2-9-3 to their contemporary counterparts in closely related species led to the estimation of 850 years generation time for halite-buried microbes [81]. The authors state also that microbe replication would be unlikely under the ion concentrations present in the liquid inclusions [81].

The sequence similarities between halite-buried and surface microbes have sometimes been claimed to result from the isolates being modern contaminants [82]. However, obtaining isolates from evaporites on four different continents using modern surface sterilization and isolation methods speak against this explanation. The evaporites can function as microbe reserves, constantly releasing buried cells back to surface environments [10]. This would mean that microbes from inside evaporites contribute to the surface microbial flora, in which case it would be difficult to define any species as contemporary [10].

It has been suggested that certain microbes could function as indicator organisms for evaporites of the same age [79]. For example, similar strains of *Hcc. salifodinae* have been isolated from Permo-Triassic salt-mine environments at several locations in Austria, England, and Germany [49,79]. *Hbt. noricense* could also be indicative of this geological era [3]. However, it should be noted that similar microbes could be encountered in surface environments as well, as seen with *Hbt. noricense* [5]. Since halite-buried microbes can be released and revived, identifying the origin of a microbe could prove to be problematic.

Concluding Remarks

Most of the biosphere is still largely unknown to us. The finding that microbes commonly inhabit evaporite formations has only recently been accepted. Halite is optimal for studying ancient microbes since the authenticity of the findings can be backed up by signs of primary crystallization, efficient surface sterilization, microbe observations *in situ*, and reproducibility of the results. The numerous adaptations allowing halophiles to survive in demanding environments make them optimal candidates for prolonged survival. Finding out what species colonize evaporite formations helps us to understand halophile evolution, paleoenvironments, and factors affecting microbial survival in this environment (see Outstanding Questions). Miniaturized cells observed in high salinity could be counterparts for bacterial spores, suggesting a novel survival strategy for archaea. The mechanism might be specific for halophilic archaea, although

Outstanding Questions

What are the requirements for prolonged survival in halite?

What is the maximum age for a microbe in optimal conditions?

Are some genes more frequent in halite-buried microbes?

Are the bacteria in ancient halite polyploid?

Do all of the ribosomal 16S sequences in ancient halite originate from viable cells?

What can diversity and sequences of halite-buried microbes reveal about halophile evolution?

How do microbes released from halite reservoirs influence the contemporary halophilic flora?

Could halophiles be detected on other celestial bodies?

miniaturized cells have been observed in ancient ice as well [62]. As sequence data collection is becoming faster and cheaper, it is to be expected that more complete genomes of halite-buried microbes will become available. With more data, we can analyze which genes are essential for survival in halite, and whether some microbes are specialized for living underground. It could also be revealed how much of the genetic similarity between surface and underground species is explained by slow evolution, and how strongly the microbes released from halite affect the surface environments.

Acknowledgments

We thank the Academy of Finland for funding (Academy Professor funding grants 283072 and 255342, D.H.B.).

References

1. Whitman, W.B. *et al.* (1998) Prokaryotes: the unseen majority. *Proc. Natl. Acad. Sci. U.S.A.* 95, 6578–6583
2. Vreeland, R.H. *et al.* (2000) Isolation of a 250 million-year-old halotolerant bacterium from a primary salt crystal. *Nature* 407, 897–900
3. Gruber, C. *et al.* (2004) *Halobacterium noricense* sp. nov., an archaeal isolate from a bore core of an alpine Permian salt deposit, classification of *Halobacterium* sp. NRC-1 as a strain of *H. salinarum* and emended description of *H. salinarum*. *Extremophiles* 8, 431–439
4. McGenity, T.J. *et al.* (2000) Origins of halophilic microorganisms in ancient salt deposits. *Environ. Microbiol.* 2, 243–250
5. Gramain, A. *et al.* (2011) Archaeal diversity along a subterranean salt core from the Salar Grande (Chile). *Environ. Microbiol.* 13, 2105–2121
6. Radax, C. *et al.* (2001) Novel haloarchaeal 16S rRNA gene sequences from Alpine Permo-Triassic rock salt. *Extremophiles* 5, 221–228
7. Park, J. *et al.* (2009) Haloarchaeal diversity in 23, 121 and 419 MYA salts. *Geobiology* 7, 515–523
8. Fish, S.A. *et al.* (2002) Recovery of 16S ribosomal RNA gene fragments from ancient halite. *Nature* 417, 432–436
9. Sankaranarayanan, K. *et al.* (2014) Characterization of ancient DNA supports long-term survival of Haloarchaea. *Astrobiology* 14, 553–560
10. Jaakkola, S.T. *et al.* (2015) The complete genome of a viable archaeum isolated from 123 million years old rock salt. *Environ. Microbiol.* Published online December 2, 2015. http://dx.doi.org/10.1111/1462-2920.13130
11. Vreeland, R.H. *et al.* (2002) *Halosimplex carlsbadense* gen. nov., sp. nov., a unique halophilic archaeon, with three 16S rRNA genes, that grows only in defined medium with glycerol and acetate or pyruvate. *Extremophiles* 6, 445–452
12. Gillow, J. *et al.* (2000) The potential of subterranean microbes in facilitating actinide migration at the Grimsel Test Site and Waste Isolation Pilot Plant. *Radiochim. Acta* 88, 769
13. Swanson, J.S. *et al.* (2013) Degradation of organic complexing agents by halophilic microorganisms in brines. *Geomicrobiol. J.* 30, 189–198
14. Vreeland, R.H. *et al.* (1998) Distribution and diversity of halophilic bacteria in a subsurface salt formation. *Extremophiles* 2, 321–331
15. Oren, A. (2014) Halophilic archaea on Earth and in space: growth and survival under extreme conditions. *Philos. Trans. R. Soc. Lond. A: Math. Phys. Eng. Sci.* 372, 20140194
16. Osterloo, M. *et al.* (2008) Chloride-bearing materials in the southern highlands of Mars. *Science* 319, 1651–1654
17. Vítek, P. *et al.* (2012) The miniaturized Raman system and detection of traces of life in halite from the Atacama Desert: some considerations for the search for life signatures on Mars. *Astrobiology* 12, 1095–1099
18. Kempe, S. and Kazmierczak, J. (2002) Biogenesis and early life on Earth and Europa: favored by an alkaline ocean? *Astrobiology* 2, 123–130
19. Konstantinidis, K. *et al.* (2015) A lander mission to probe subglacial water on Saturn's moon Enceladus for life. *Acta Astronaut.* 106, 63–89
20. Rosenzweig, W.D. *et al.* (2000) Development of a protocol to retrieve microorganisms from ancient salt crystals. *Geomicrobiol. J.* 17, 185–192
21. Sankaranarayanan, K. *et al.* (2011) Ancient microbes from halite fluid inclusions: optimized surface sterilization and DNA extraction. *PLoS ONE* 6, e20683
22. Mormile, M.R. *et al.* (2003) Isolation of *Halobacterium salinarum* retrieved directly from halite brine inclusions. *Environ. Microbiol.* 5, 1094–1102
23. Jaakkola, S.T. *et al.* (2014) Halophilic archaea cultivated from surface sterilized middle-late Eocene rock salt are polyploid. *PLoS ONE* 9, e110533
24. Norton, C.F. *et al.* (1993) Archaeal halophiles (halobacteria) from two British salt mines. *J. Gen. Microbiol.* 139, 1077–1081
25. Feng, C. *et al.* (2011) SHRIMP zircon U–Pb and molybdenite Re–Os isotopic dating of the tungsten deposits in the Tianmenshan–Hongtaoling W–Sn orefield, southern Jiangxi Province, China, and geological implications. *Ore Geol. Rev.* 43, 8–25
26. Belmaker, R. *et al.* (2013) 10 Be dating of Neogene halite. *Geochim. Cosmochim. Acta* 122, 418–429
27. Leitner, C. *et al.* (2014) 40Ar/39Ar ages of crystallization and recrystallization of rock-forming polyhalite in Alpine rocksalt deposits. *Geol. Soc. Lond. Spec. Publ.* 378, 207–224
28. Renne, P.R. *et al.* (2001) 40 Ar/39 Ar dating of Late Permian evaporites, southeastern New Mexico, USA. *Earth Planet. Sci. Lett.* 193, 539–547
29. Zhang, J. *et al.* (2005) Feasibility of optical dating using halite. *J. Luminesc.* 114, 234–240
30. Roedder, E. (1984) Fluids in salt. *Am. Miner.* 69, 5–6
31. Lowenstein, T.K. (2012) Microorganisms in evaporites: review of modern geomicrobiology. In *Advances in Understanding the Biology of Halophilic Microorganisms* (Vreeland, R.H., ed.), pp. 117–139, Springer
32. Meng, F. *et al.* (2011) Ediacaran seawater temperature: Evidence from inclusions of Sinian halite. *Precambrian Res.* 184, 63–69
33. Meng, F-W. *et al.* (2015) A newly isolated haloalkaliphilic bacterium from middle–late Eocene halite formed in salt lakes in China. *Carbonate Evaporite* 30, 321–330
34. Satterfield, C.L. *et al.* (2005) New evidence for 250 Ma age of halotolerant bacterium from a Permian salt crystal. *Geology* 33, 265–268
35. Schubert, B.A. *et al.* (2010) Halophilic archaea cultured from ancient halite, Death Valley, California. *Environ. Microbiol.* 12, 440–454
36. Schubert, B.A. *et al.* (2009) Microscopic identification of prokaryotes in modern and ancient halite, Saline Valley and Death Valley, California. *Astrobiology* 9, 467–482
37. Fendrihan, S. *et al.* (2009) Raman spectroscopy as a potential-method for the detection of extremely halophilic archaea embedded in halite in terrestrial and possibly extraterrestrial samples. *J. Raman Spectrosc.* 40, 1996
38. Winters, Y.D. *et al.* (2013) Identification of carotenoids in ancient salt from Death Valley, Saline Valley, and Searles Lake, California, using laser Raman spectroscopy. *Astrobiology* 13, 1065–1080
39. Wang, R. (1998) Acyclic isoprenoids–molecular indicators of archaeal activity in contemporary and ancient Chinese saline/hypersaline environments. *Hydrobiologia* 381, 59–76

40. Wang, H. *et al.* (2013) Assessing the ratio of archaeol to caldarchaeol as a salinity proxy in highland lakes on the northeastern Qinghai–Tibetan Plateau. *Org. Geochem.* 54, 69–77
41. Birgel, D. *et al.* (2014) Hypersaline conditions during deposition of the Calcare di Base revealed from archaeal di-and tetraether inventories. *Org. Geochem.* 77, 11–21
42. Ten Haven, H. *et al.* (1988) Application of biological markers in the recognition of palaeohypersaline environments. *Geol. Soc. Lond. Spec. Publ.* 40, 123–130
43. Damsté, J.S.S. *et al.* (1995) Evidence for gammacerane as an indicator of water column stratification. *Geochim. Cosmochim. Acta* 59, 1895–1900
44. Kim, J.S. *et al.* (2012) Diversity of bacteria and archaea in hypersaline sediment from Death Valley National Park, California. *Microbiol. Open* 1, 135–148
45. Schinteie, R. and Brocks, J.J. (2014) Evidence for ancient halophiles? Testing biomarker syngeneity of evaporites from Neoproterozoic and Cambrian strata. *Org. Geochem.* 72, 46–58
46. Brocks, J.J. (2011) Millimeter-scale concentration gradients of hydrocarbons in Archean shales: Live-oil escape or fingerprint of contamination? *Geochim. Cosmochim. Acta* 75, 3196–3213
47. Kovalevych, V. *et al.* (2008) Geochemical aureoles around oil and gas accumulations in the Zechstein (Upper Permian) of Poland: Analysis of fluid inclusions in halite and bitumens in rock salt. *J. Petrol. Geol.* 31, 245–262
48. Han, D. and Cui, H-L. (2014) *Halosimplex pelagicum* sp. nov. and *Halosimplex rubrum* sp. nov., isolated from salted brown alga *Laminaria*, and emended description of the genus *Halosimplex*. *Int. J. Syst. Evol. Microbiol.* 64, 169–173
49. Stan-Lotter, H. *et al.* (1999) Very similar strains of *Halococcus salifodinae* are found in geographically separated Permo-Triassic salt deposits. *Microbiology* 145, 3565–3574
50. Stan-Lotter, H. *et al.* (2002) *Halococcus dombrowskii* sp. nov., an archaeal isolate from a Permian alpine salt deposit. *Int. J. Syst. Evol. Microbiol.* 52, 1807–1814
51. Schubert, B.A. *et al.* (2010) *Dunaliella* cells in fluid inclusions in halite: significance for long-term survival of prokaryotes. *Geomicrobiol. J.* 27, 61–75
52. Engelhardt, T. *et al.* (2011) Induction of prophages from deep-subseafloor bacteria. *Environ. Microbiol. Rep.* 3, 459–465
53. Conner, A.J. and Benison, K.C. (2013) Acidophilic halophilic microorganisms in fluid inclusions in halite from Lake Magic, Western Australia. *Astrobiology* 13, 850–860
54. Benison, K. (2013) Acid saline fluid inclusions: examples from modern and Permian extreme lake systems. *Geofluids* 13, 579–593
55. Benison, K.C. *et al.* (2008) Hairy blobs: microbial suspects preserved in modern and ancient extremely acid lake evaporites. *Astrobiology* 8, 807–821
56. Oren, A. *et al.* (1995) A bloom of *Dunaliella parva* in the Dead Sea in 1992: biological and biogeochemical aspects. *Hydrobiologia* 297, 173–185
57. Kixmüller, D. and Greie, J.C. (2012) An ATP-driven potassium pump promotes long-term survival of *Halobacterium salinarum* within salt crystals. *Environ. Microbiol. Rep.* 4, 234–241
58. Fendrihan, S. *et al.* (2004) Effects of embedding *Halobacterium* sp. NRC-1 in salt crystals and potential implications for long term preservation. In *Proceedings of the Third European Workshop on Exo-Astrobiology*, pp. 203–204
59. Kminek, G. *et al.* (2003) Radiation-dependent limit for the viability of bacterial spores in halite fluid inclusions and on Mars. *Radiat. Res.* 159, 722–729
60. Nicholson, W.L. (2003) Using thermal inactivation kinetics to calculate the probability of extreme spore longevity: implications for paleomicrobiology and lithopanspermia. *Orig. Life Evol. Biosph.* 33, 621–631
61. Fendrihan, S. *et al.* (2012) Spherical particles of halophilic archaea correlate with exposure to low water activity–implications for microbial survival in fluid inclusions of ancient halite. *Geobiology* 10, 424–433
62. Miteva, V.I. *et al.* (2004) Phylogenetic and physiological diversity of microorganisms isolated from a deep Greenland glacier ice core. *Appl. Environ. Microbiol.* 70, 202–213
63. Rothschild, L.J. *et al.* (1994) Metabolic activity of microorganisms in evaporites. *J. Phycol.* 30, 431–438
64. Johnson, S.S. *et al.* (2007) Ancient bacteria show evidence of DNA repair. *Proc. Natl. Acad. Sci. U.S.A.* 104, 14401–14405
65. Tuorto, S.J. *et al.* (2014) Bacterial genome replication at subzero temperatures in permafrost. *ISME J.* 8, 139–149
66. Lomstein, B.A. *et al.* (2012) Endospore abundance, microbial growth and necromass turnover in deep sub-seafloor sediment. *Nature* 484, 101–104
67. Lowenstein, T.K. *et al.* (2011) Microbial communities in fluid inclusions and long-term survival in halite. *GSA Today* 21, 4–9
68. Griffith, J.D. *et al.* (2008) Discovery of abundant cellulose microfibers encased in 250 Ma Permian halite: a macromolecular target in the search for life on other planets. *Astrobiology* 8, 215–228
69. Legat, A. *et al.* (2010) Identification of polyhydroxyalkanoates in *Halococcus* and other haloarchaeal species. *Appl. Microbiol. Biotechnol.* 87, 1119–1127
70. Morita, R. (1999) Is H2 the universal energy source for long-term survival? *Microb. Ecol.* 38, 307–320
71. Schwartz, E. *et al.* (2013) H_2-metabolizing prokaryotes. In *The Prokaryotes* (Rosenberg, E. *et al.*, eds), pp. 119–199, Springer
72. Tenchov, B. *et al.* (2006) Salt tolerance of archaeal extremely halophilic lipid membranes. *J. Biol. Chem.* 281, 10016–10023
73. McCollom, T. and Amend, J. (2005) A thermodynamic assessment of energy requirements for biomass synthesis by chemolithoautotrophic micro-organisms in oxic and anoxic environments. *Geobiology* 3, 135–144
74. Moeller, R. *et al.* (2010) UV photoreactions of the extremely haloalkaliphilic euryarchaeon Natronomonas pharaonis. *FEMS Microbiol. Ecol.* 73, 271–277
75. Shahmohammadi, H.R. *et al.* (1998) Protective roles of bacterioruberin and intracellular KCl in the resistance of *Halobacterium salinarium* against DNA-damaging agents. *J. Radiat. Res.* 39, 251–262
76. Soppa, J. (2011) Ploidy and gene conversion in Archaea. *Biochem. Soc. Trans.* 39, 150
77. Soppa, J. (2013) Evolutionary advantages of polyploidy in halophilic archaea. *Biochem. Soc. Trans.* 41, 339–343
78. Zerulla, K. *et al.* (2014) DNA as a phosphate storage polymer and the alternative advantages of polyploidy for growth or survival. *PLoS ONE* 9, e94819
79. Legat, A. *et al.* (2013) Properties of *Halococcus salifodinae*, an isolate from Permian rock salt deposits, compared with halococci from surface waters. *Life* 3, 244–259
80. Pfeiffer, F. *et al.* (2008) Evolution in the laboratory: the genome of *Halobacterium salinarum* strain R1 compared to that of strain NRC-1. *Genomics* 91, 335–346
81. Maughan, H. *et al.* (2002) The paradox of the "ancient" bacterium which contains "modern" protein-coding genes. *Mol. Biol. Evol.* 19, 1637–1639
82. Graur, D. and Pupko, T. (2001) The Permian bacterium that isn't. *Mol. Biol. Evol.* 18, 1143–1146
83. Ma, Y. *et al.* (2010) Halophiles 2010: life in saline environments. *Appl. Environ. Microbiol.* 76, 6971–6981
84. Paul, S. *et al.* (2008) Molecular signature of hypersaline adaptation: insights from genome and proteome composition of halophilic prokaryotes. *Genome Biol.* 9, R70
85. DasSarma, S. and DasSarma, P. (2012) *Halophiles,* John Wiley
86. Culka, A. *et al.* (2014) Detection of pigments of halophilic endoliths from gypsum: Raman portable instrument and European Space Agency's prototype analysis. *Philos. Trans. R. Soc. Lond. A: Math. Phys. Eng. Sci.* 372, 20140203
87. Kozary, M.T. *et al.* (1968) Incidence of saline deposits in geologic time. *Geol. Soc. Spec.* 88, 43–58
88. Stan-Lotter, H. *et al.* (1993) Comparison of membrane ATPases from extreme halophiles isolated from ancient salt deposits. *Orig. Life Evol. Biosph.* 23, 53–64
89. Denner, E.B. *et al.* (1994) *Halococcus salifodinae* sp. nov., an archaeal isolate from an Austrian salt mine. *Int. J. Syst. Bacteriol.* 44, 774–780

90. Vreeland, R. *et al.* (2007) Isolation of live Cretaceous (121-112 million years old) halophilic Archaea from primary salt crystals. *Geomicrobiol. J.* 24, 275–282

91. Cosmidis, J. *et al.* (2013) Nanometer-scale characterization of exceptionally preserved bacterial fossils in Paleocene phosphorites from Ouled Abdoun (Morocco). *Geobiology* 11, 139–153

92. Peters, K. *et al.* (2005) In *The Biomarker Guide, Biomarkers and Isotopes in Petroleum Exploration and Earth History* (vols 1–2), Cambridge University Press

93. Tamura, K. *et al.* (2013) MEGA6: molecular evolutionary genetics analysis version 6.0. *Mol. Biol. Evol.* 30, 2725–2729