Extremophiles (1998) 2:279–287

REVIEW

William D. Grant · Renia T. Gemmell
Terry J. McGenity

Halobacteria: the evidence for longevity

Received: January 22, 1998 / Accepted: February 16, 1998

Abstract Subterranean salt deposits are the remains of ancient hypersaline waters that presumably supported dense populations of halophilic microorganisms including representatives of the haloarchaea (halobacteria). Ancient subterranean salt deposits (evaporites) are common throughout the world, and the majority sampled to date appear to support diverse populations of halobacteria. The inaccessibility of deep subsurface deposits, and the special requirements of these organisms for survival, make contamination by halobacteria from surface sites unlikely. It is conceivable that these subterranean halobacteria are autochthonous, presumably relict populations derived from ancient hypersaline seas that have been revived from a state of dormancy. One would predict that halobacteria that have been insulated and isolated inside ancient evaporites would be different from comparable bacteria from surface environments, and that it might be possible to use a molecular chronometer to establish if the evolutionary position of the subsurface isolates correlated with the geological age of the evaporite. Extensive comparisons have been made between the 16S rRNA genes of surface and subsurface halobacteria without showing any conclusive differences between the two groups. A further phylogenetic comparison exploits an unusual feature of one particular group of halobacteria that possess at least two heterogeneous copies of the 16S rRNA gene, the sequences of which may have been converging or diverging over geological time. However, results to date have yet to show any gene sequence differences between surface and evaporite-derived halobacteria that might arguably be an indication of long-term dormancy.

Key words Halobacteria · Haloarchaea · Evaporites · Dormancy · 16S rRNA genes · rRNA gene heterogeneity · Salt mines · Longevity

Communicated by K. Horikoshi

W. D. Grant (✉)
Department of Microbiology and Immunology, University of Leicester, P.O. Box 138, Leicester, LE1 9HN, UK
Tel. +44-116-252-2948; Fax +44-116-252-5030
e-mail: WDG1@le.ac.uk

R. T. Gemmell
Oxford Centre for Environmental Biotechnology, Institute of Virology and Environmental Microbiology, Mansfield Road, Oxford, OX1 3SR, U.K.

T. J. McGenity
Postgraduate Institute for Sedimentology, University of Reading, Whiteknights, Reading, RG6 6AH, U.K.

Taxonomy and phylogeny of halobacteria

The term "halobacteria" is the trivial name used to describe the members of the family *Halobacteriaceae*, the only family in the order *Halobacteriales* (Grant and Larsen 1989) within the euryarchaeotal kingdom of the Archaea. An alternative trivial name for the group is "haloarchaea," and the organisms can be readily differentiated from halophilic bacteria on the basis of their archaeal characteristics, notably the possession of ether-linked isopranoid lipids (Ross et al. 1981).

Halobacteria are the most halophilic organisms known, and form the dominant microbial population when hypersaline waters approach saturation, frequently importing a red coloration to the brines because of C_{50} carotenoids. Indeed, as long ago as 2500 B.C., the Chinese noted the red color of saturated salterns (Baas-Becking 1931). It is known that the carotenoid pigments of halobacteria trap solar radiation, increasing the ambient temperature and evaporation rates in salterns, thus promoting rapid precipitation of sea salt.

In recent times, the application of chemotaxonomic and gene sequence methodologies has shown that the halobacteria, previously considered to be a relatively homogeneous group, are in fact quite diverse with at least ten major taxa that all diverged from a common ancestor at about the same time (Fig. 1). Representatives of the majority of genera are characteristic of neutral saline environments (*Halobacterium*, *Halorubrum*, *Haloarcula*, *Haloferax*, *Halococcus*, *Halobaculum*, and *Natrialba* spp.) (Grant and Larsen 1989; McGenity and Grant 1995;

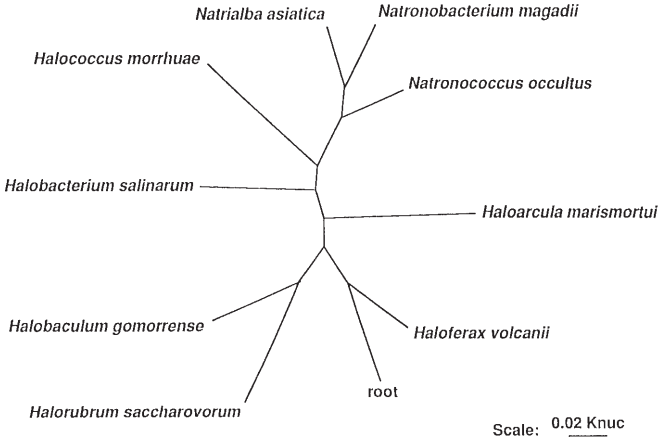

Fig. 1. A maximum likelihood tree of 16S rRNA gene sequences of halobacteria. The root was imposed by the outgroup *Methanospirillum hungatei*. A preliminary sequence alignment was obtained using CLUSTAL V, which was manually optimized. A phylogenetic tree was drawn using the DNAML Maximum Likelihood program in PHYLIP 3.4

Oren et al. 1995; Kamekura and Dyall-Smith 1995), whereas alkaline saline environments harbor haloalkaliphilic halobacteria, representatives of the genera *Natronobacterium* and *Natronococcus* in particular, although there has been a recent reappraisal of the phylogeny of the haloalkaliphiles resulting in the transfer of one representative to a new genus, *Natronononas*, and indicating that genera such as *Natrialba* and *Halorubrum* may have haloalkaliphilic members (Kamekura et al. 1997).

Isolates from neutral hypersaline environments can be readily assigned to genera on the basis of polar lipid signatures (Grant and Larsen 1989), although this is less helpful for haloalkaliphilic isolates. However, 16S rRNA gene sequence alignments have been used to determine unique oligonucleotides that can be used as signature sequences for the rapid identification of new isolates (McGenity et al., in press), so that populations in natural environments can be analyzed with precision. It is now clear, for example, that the climax population of halobacteria in neutral environments like the Great Salt Lake and seawater solar salterns is dominated by members of the genera *Haloarcula*, *Halorubrum*, *Halobacterium*, and, occasionally, *Halococcus* (Oren 1994, unpublished results).

Halite precipitation and the genesis of evaporite deposits

Salt deposits or evaporites result from the evaporitic concentration of dissolved salt water of marine, lacustrine, or mixed origin and their consequential precipitation and crystallization as chemical sediments. Climatic factors such as temperature (air and water difference), wind speed, and relative humidity of the atmosphere determine the rate of evaporation. These factors, together with the origins of the salt water, determine the nature of the salts deposited and their relative proportions. Typically, the evaporitive concentration of seawater precipitates salts in the following order, with the least soluble being deposited first: $CaCO_3$ and other carbonates, anhydrite ($CaSO_4$) or gypsum ($CaSO_4 \cdot 2H_2O$), halite (NaCl), magnesium and potassium chlorides, and sulfates (bittern salts). The highly soluble bittern salts are the last to crystallize out after prolonged desiccation. Theoretically, the complete evaporation of a column of sea water 300 m deep would precipitate about 4.8 m of salts, of which 0.0192 m would be carbonates, 0.173 m gypsum, 3.7 m halite, and 0.85 m potassium and magnesium salts (Handford 1991).

Hypersaline waters usually contain relatively large amounts of carbon because of concentration processes and are thus capable of supporting significant populations of microorganisms. With increasing salinity, the oxygen concentration drops, pH decreases, and temperature increases. The populations of eukaryotic and prokaryotic halophilic microorganisms change with salt concentration, with a decrease in diversity as salt concentration increases (Rodriguez-Valera, et al. 1985). Throughout the world, halobacteria become dominant as these environments approach the point of halite precipitation, numbers of more than $10^8 ml^{-1}$ being recorded at various sites (Oren 1994). Following halite precipitation, halobacteria are seldom recovered from magnesium- and potassium-dominated bitterns brines and are recorded as being unable to grow in bitterns brines (Javor 1984). Failure to recover halobacteria from bitterns is probably not a consequence of loss of viability, however, but rather their absence from the brines in question results from the entrapment of the halobacteria within the freshly crystallised halite (Fig. 2). As halite crystallizes, representatives of the climax population of halobacteria become incorporated in a viable state within tiny pockets of brine called fluid inclusions that become a permanent feature of the crystal structure (Norton and Grant 1988). Laboratory experiments indicate that these entrapped halobacteria remain viable for a considerable number of years, providing an explanation for the ready cultivation of these organisms from solar salts that have been stored for very long periods of time.

Ancient evaporite deposits of various ages are common throughout the world (Table 1). These deposits are the remains of ancient hypersaline sites that presumably once supported dense populations of halobacteria (or halobacterial ancestors). Many are directly accessible because they are mined for rock salt, or alternatively, material is indirectly accessible though solution mining of the subterranean deposits, where brines pumped to the surface may be sampled. Primary fluid inclusions of ancient salt deposits should contain ancient brines, gases, and organic material representing the environment at the point of halite precipitation. Fluid inclusions containing material can be readily seen in ancient halite (Fig. 3). Although it is not possible to identify the precise nature of the entrapped material, fluorescence under ultraviolet light often is consistent with organic origins. Any microorganisms found within these fluid inclusions would be autochthonous with the salt deposit at the time of precipitation. If these microorganisms

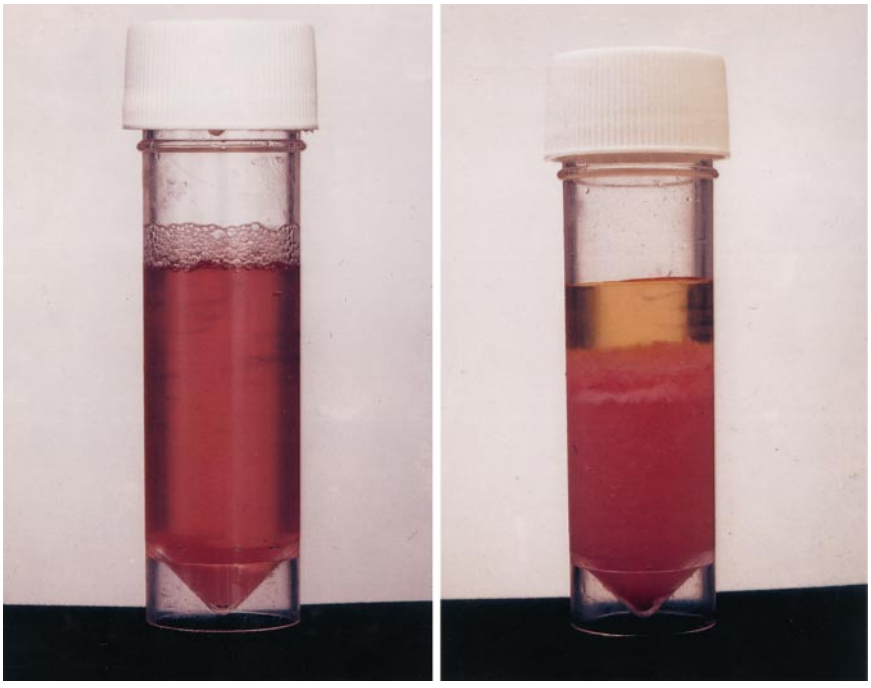

Fig. 2. *Left*: saltern liquor before the onset of halite precipitation. *Right*: after halite precipitation, halobacteria become entrapped within the halite

Table 1. Evaporite deposits and their geological ages

Era	Period/Series	Evaporite deposit	
Cenozoic	Holocene		Present-day sabkhas, salinas, salt lakes, etc.
	Pleistocene	−2 Ma	California; Nevada; Russia; Mexico; Israel
	Pliocene	5 Ma	Virgin Valley, Nevada; Utah; Italy; Jordan
	Miocene	23 Ma	Mediterranean; Red Sea; Trucial Coast (Arabian Gulf); Dominican Republic; Romania; Wieliczka, Poland
	Oligocene	36 Ma	France; Germany; Spain; Turkey; Iran; Iraq
	Eocene	53 Ma	Green River, Wyoming; Potwar, Pakistan
	Palaeocene	65 Ma	None known
Mesozoic	Cretaceous	135 Ma	Gabon, Congo and Angola; Brazil; Colombia; Russia; Florida; Khorat Basin, Thailand; Morocco
	Jurassic	205 Ma	Montana; Bulgaria; Black Sea; Cuba; Chile; Idaho; Gulf Coast
	Triassic	250 Ma	Cheshire Basin; Portugal; Spain; N. and S.W. France; Netherlands; Germany; Switzerland; N. Africa; Peru; Persian Gulf
Palaeozoic	Permian	290 Ma	Zechstein Basin, N.W. Europe; Permian Basin, Texas; Salado Formation, Mexico; Emba, Caspian Sea
	Carboniferous	355 Ma	Canadian Arctic Islands; Paradox Basin, S.W. USA
	Devonian	410 Ma	Elk Point Basin, Canada; Mongolia; Moscow Basin
	Silurian	438 Ma	Salina Basin (New York, Ohio, W. Virginia, Pennsylvania, Michigan; Ontario)
	Ordovician	510 Ma	Williston Basin, USA/Canada
	Cambrian	570 Ma	Mackenzie, NW Territories; Siberia; Iran (Persian Gulf); India; Australia
Proterozoic	Precambrian		Amadeus Basin, C. Australia; McArthur Group, Queensland, Australia; Ontario, Canada; Iran

Geological age given in Ma Bp (mega annons, 10^6 years, before present.
Source: Halbouty (1967); Lefond (1969); Cowie and Bassett (1989).

have somehow survived any physicochemical changes that would have taken place since the primary crystallization event, then demonstration of viability (by culture) under conditions that would exclude contamination would be evidence for very long-term survival.

Isolation of ancient microorganisms

Kennedy et al. (1994) have compiled a database of reports of more than 5000 preserved or revived microorganisms

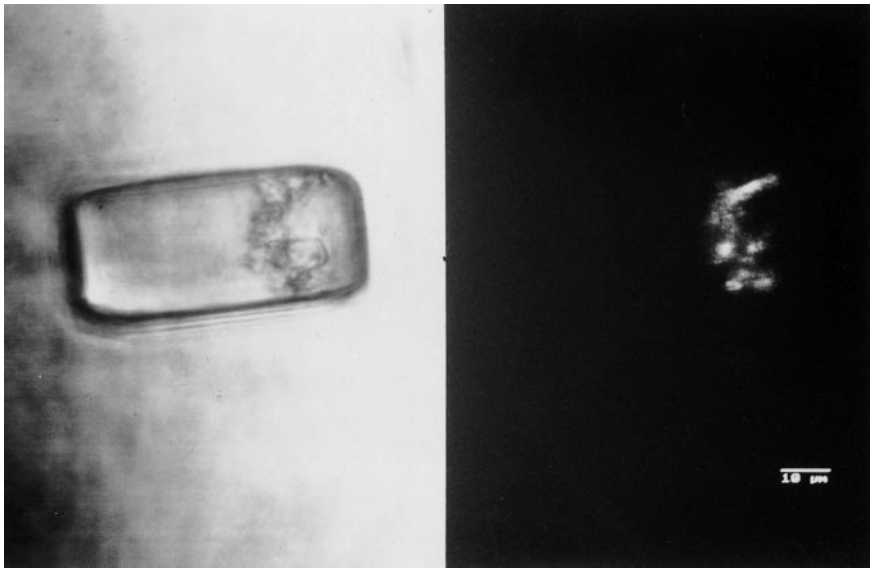

Fig. 3. Visible light (*left*) and UV light (*right*) photomicrographs of a fluid inclusion in a primary halite crystal from Wieliczka, Poland (about 20×10^6 years, or mega annons, before present). The inclusion contains fluorescent material that is probably organic in origin

older than 50 years. These ancient bacteria are often spore-formers, such as *Bacillus* spp. Sources of these ancient microorganisms include 166-year-old Porter beer, mastodon intestines, soils, coal, deep sediment cores, and rock salt.

Possibly the earliest claim that living microorganisms can be isolated from ancient sites was made by Lipman in 1928, who followed this with a claim of having isolated bacteria from coal, suggesting that they were survivors since the coal was formed, remaining in a state of suspended animation as a spore or some similarly resistant resting stage until conditions became favorable (Lipman 1931). Despite rigorous precautions against contamination, Lipman's claims that viable bacteria could be recovered after millions of years were largely disbelieved. In *The Quest for Life in Amber*, Poinar and Poinar (1994) have, for more than two decades, examined many life-forms preserved in amber, including bacterial cells inside nematodes, as well as various insects, some with intact tissues and cells complete with organelles. In 225-million-year-old amber, the Poinars identified intact bacteria, algae, fungi, and protozoa, in a "flashphoto of life in a drop of Triassic water, preserved in three-dimensional form." In 1984 they succeeded in isolating *Bacillus* sp., presumably from spores, from a stingless bee embedded in 25- to 40-million-year-old Dominican amber, but were unable to prove longevity conclusively. Cano continued this particular study, and in 1994 (Cano and Borucki 1995) announced the revival and culture of 25- to 40-million-year-old bacillus spores from this amber under stringent, aseptic, and controlled conditions.

There is a long history of reported successful isolations from ancient evaporites, although as late as 1981 Larsen (Larsen 1981) described mined rock salts as bacteria free. This perception of salt mines as sterile environments is widespread, and may have been one of the criteria for choosing salt mines as repositories for nuclear and chemical waste. Brine pools and efflorescences of recrystallized salt within salt mines are not sterile; in fact, they harbor dense populations of halophilic microorganisms (Namyslowski 1913; Nehrhorn and Schwartz 1961; Norton et al. 1993). However, the evidence for the presence of viable microorganisms in primary rock salt is more controversial. Dombrowski made several reports, from the late 1950s, on the isolation of bacteria from salt deposits. Following the isolation of *Pseudomonas halocrenaea* (Dombrowski 1960), from thermal saline (Zechstein) carbon dioxide springs at Bad Nauheim and also from salts mined from the Zechstein deposit, Dombrowski made further bacteriological investigations of Zechstein salt (Dombrowski 1966). Using elaborate modes of sterilization of equipment and surroundings, he claimed to have isolated bacteria from surface-sterilized pieces of primary, undisturbed Zechstein salt. Microscopic observation of thin sections of salt showed the bacteria to be embedded in the crystalline matrix. Additionally, bacteria were reported to have been isolated from Middle Devonian, Silurian, and Precambrian salt. Dombrowski suggested that a combination of dehydration and salification conserved these bacteria for millions of years. Schwartz was involved in several investigations in the early 1960s into the halophilic bacterial inhabitants of various saline environments, and although he was able to isolate halophiles (e.g., "*Sarcina litoralis*' and *Halobacterium halobium*") from salt lakes and salt works brine (Nehrkorn and Schwartz 1961), was unable to isolate living bacteria from rock salt samples (Bien and Schwartz 1965), although variable amounts of diverse forms of conserved dead bacteria were observed in rock salt. Reiser and Tasch (1960) looked at Permian salt from Kansas and observed diplococcus-like bacteria inside salt crystals. Taking extreme care to prevent contamination, they claimed to have cultured these halotolerant bacteria from a small proportion of the salt samples. Dombrowski's experiments

were reproduced with success by Bibo et al. (1983), who used careful precautions against contamination, with surface sterilization of the salt samples, and stringently conducted control experiments to show that viable microorganisms could be isolated from salt samples from primary salt deposits and cultivated.

More recent reports have been made of halobacteria isolated from Triassic and Permian salt deposits (Norton et al. 1993; Stan-Lotter et al. 1993; Denner et al. 1994; McGenity 1994). In one of these deposits, the presence of well-preserved pollen suggests that they either did not experience or were able to tolerate metamorphic events; this may hold true for embedded microorganisms (Stan-Lotter et al. 1993). Norton et al. (1993) isolated approximately one halobacterium per 500 g of freshly blasted, surface-sterilized rock salt and suggested that viable halobacteria are found, although with rarity, within such deposits. Vreeland and Huval (1991) also recovered halobacteria and moderately halophilic bacteria from brines derived from Permian salt strata that had been dissolved by meteoric water and from a flooded salt dome.

Many other subterranean environments are being explored for microbial life (Ghiorse and Wobber 1989). A variety of microorganisms have been cultured from subsurface environments, such as 500 m below the ocean floor (Parkes et al. 1990). The biosphere is no longer considered to be a thin crust on the earth's surface, but has the added dimension of depth. An interesting analogy to halobacteria in salt mines was the discovery of hyperthermophilic Archaea in hot oil reservoirs beneath the ocean floor (Stetter et al. 1993). The isolation of prokaryotes from other deep subsurface environments has received much attention in recent years, particularly in relation to repositories for petroleum (Bock et al. 1994) or radioactive waste (Pedersen and Ekendahl 1990). Viable bacteria have been isolated from a rock matrix at 450-m depth where, without nutrient flux, they may have existed in a state of starvation for several million years (Amy et al. 1993).

The scientific community is rightly skeptical about these claims of microbial longevity, because so little is known about the movement of microorganisms through halite, rocks, and groundwater and because sterilization techniques are always open to question.

Molecular evidence for possible long-term dormancy of halobacteria

We have isolated more than 100 halobacteria from various habitats in geologically and geographically different evaporites (Norton et al. 1993; Grant et al. 1995). Most notably, we have occasionally, but consistently, isolated halobacteria from surface-sterilized crystals obtained from freshly blasted rock salt (Norton et al. 1993; Denner et al. 1994). Ancient evaporites almost always harbor representatives of the genera *Haloarcula*, *Halorubrum*, *Halobacterium*, and occasionally *Halococcus*, but strains belonging to previously undescribed genera are also common (Norton et al. 1993; Grant et al. 1995). We have previously established that halobacteria become entrapped in a viable state for many years inside brine inclusions formed within halite crystals grown in the laboratory (Norton and Grant 1988), providing a conceptual basis with which to understand our finding that halobacteria can be isolated from ancient halite. There are two possible hypotheses regarding the origins of halobacteria: either they are recent contaminants, introduced from surface sites in recent times, or they are halobacteria (or halobacterial ancestors) that once flourished in ancient saline seas which gave rise to these halite deposits, so that we are seeing representatives of a relict population entrapped since the seas evaporated to form salt deposits. Examination of many evaporite sites, including solution mined sites, leads us to the conclusion that the second hypothesis should be not be dismissed out of hand. Entrapped halobacteria might be in a state of suspended animation or exhibiting periods of growth over geological time as resources become available.

The case for long-term suspended animation and revival of specimens is almost always based on circumstantial evidence, namely:

1. Inaccessibility of the environments from which the specimen(s) originated
2. Conditions that mitigate against growth in situ
3. Stringent precautions against contamination by present-day material
4. The recovered organisms differing from those in the surrounding environment.

All four criteria are seldom met, although the salt mine sites meet these criteria better than most. We have previously applied an indirect approach to attempting to prove that halobacteria recovered from ancient halite might have been revived after very long periods of dormancy. If the dormancy hypothesis is correct, then revived organisms would be expected to be more primitive at the molecular level relative to their contemporary descendants. We had originally taken a straightforward approach to address this question, comparing the 16S rRNA genes of isolates of halobacteria from deposits of a range of geological ages. It would be expected, in such a phylogenetic analysis, that modern isolates (i.e., those from surface sites) would appear at the ends of branches on any phylogenetic tree, whereas ancient isolates of the same lineage would appear as short branches or points close to the root of the tree, their position perhaps dependent on whether the isolates had experienced periods of growth or had been dormant over the entire period. However, we were unable to find any clear correlation between phylogenetic position and geological age of the sample site, calibrating the tree with geological age using the data of Moran et al. (1993) (Fig. 4). Analysis of a large number of surface isolates has now indicated that the range of sequence variation in any halobacterial line probably is as great as the difference in sequence we would expect to see between, say, a 200-million-year-old isolate and a present-day isolate.

An alternative approach (Gemmell et al. 1998) exploits an unusual feature of *Haloarcula* isolates. Members of this

Code	Site	Geological Age (Ma)
GSL	Great Salt Lake, USA	Recent
S	Spanish salterns	Recent
PW	Wieliczka, Poland	*c.* 20
T	Khorat Basin, Thailand	60-70
W	Winsford, Cheshire, UK	235-240
Nh	Hengelo, Netherlands	250-260
PIG	Gora, Inowroclaw, Poland	250-260
B	Boulby, Cleveland, UK	250-260

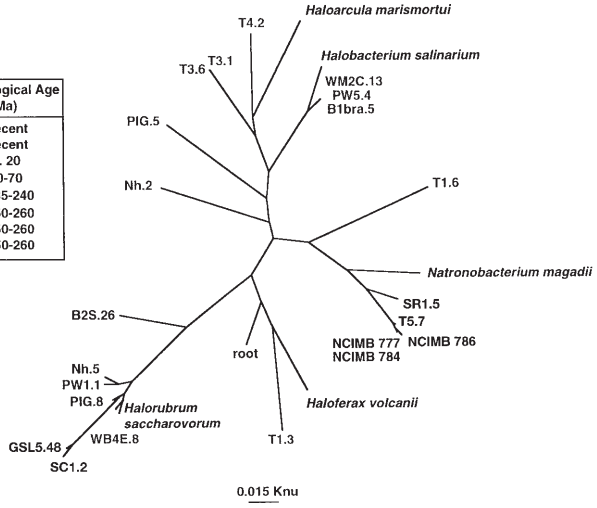

Fig. 4. A 16S rRNA gene tree of extremely halophilic Archaea from ancient salt deposits, showing their phylogenetic relatedness to isolates from recent deposits and culture collection strains. The outgroup *Methanospirillum hungatei* was included to infer a root. 16S rRNA gene sequences were aligned in CLUSTAL V and manually adjusted. The phylogenetic tree was derived using the maximum likelihood method DNAML (version 3.4c) in PHYLIP 3.4, the phylogenetic inference package (colpywrite 1986–1993) of Joseph Felsenstein and the University of Washington, and drawn in DRAWTREE (PHYLIP 3.54c). The tree was calibrated, with a phylogenetic distance of 0.015 Knu being equivalent to 50 million years (Moran et al. 1993). NCIMB 777, 786, and 784 are misclassified surface isolates formerly considered to be *Halobacterium* spp (Grant and Larsen 1989)

Table 2. Number of base changes between different 16s rRNA genes of haloarculas

Isolate	Number of genes	Base differences between genes			Age of site where isolated
		A/B	B/C	A/C	
Haloarcula marismortui	2 (A, B)	52			Present-day salt lake
Haloarcula sinaiiensis	2 (A, B)	26			Present-day salt lake
T 3.2	3 (A, B, C)	52 (+3)	53 (+4)	6 (+1)	Halite deposit, 65 million years
T. 208.9	2 (A, B)	52 (+4)			Halite deposit, 65 million years
WB4 E.4	2 (A, B)	53 (+1)			Halite deposit, 240 million years
CFN E4	3 (A, B, C)	5 (+27)	1 (+14)	7 (+21)	Halite deposit, 240 million years
Br 6	3 (A, B, C)	56 (+7)	57 (+3)	2 (+6)	Halite deposit, 240 million years
BI bra 10	2 (A, B)	52			Halite deposit, 260 million years

Based on analysis of sequence between positions 415 and 1032, which cover the two variable regions where most of the sequence differences are known to reside. (+), ambiguous bases.

group possess at least two dissimilar 16S rRNA genes. If this gene multiplicity is the result of duplication, then fewer differences would be expected between the genes of a haloarcula revived from dormancy; the reverse would be expected if the extra gene(s) have been obtained by lateral gene transfer because of crossover mechanisms that would reduce differences between gene sets with time. Sixty genes from 21 isolates have been separated and analyzed. Eight of these isolates had 2 genes, 9 had 3 genes, and 4 had 4 genes. Isolates with more than 2 genes generally had 2 or 3 virtually identical genes and 1 gene that was significantly different in sequence. Comparisons of the genes of some of these isolates are shown in Table 2. Ambiguities in gene sequence were observed in a considerable number of cases. Nevertheless, when ambiguities are taken into account two broad groups can be defined, isolates with more than 51 base changes in the region we have sequenced and isolates with 23–37 base changes. However, these sequence groups are represented by isolates from both ancient evaporites and present-day salt lakes. Therefore, we are unable to detect any sequence pattern that correlates with geological age of the sample site.

The results do not necessarily disprove the possibility of long-term suspended animation because it is possible that these techniques do not have sufficient resolving power, given the relatively limited geological time span that has been examined; unfortunately, to date older evaporite deposits (e.g., the Australian Precambrian sites) have not been subjected to any microbiological survey. The remote possibility that salt deposit halobacteria could have seeded surface sites should also be considered.

The case for longevity

There are several plausible hypotheses relating to the origins of microorganisms in salt deposits:

1. Microorganisms in salt mines originate from present-day populations inhabiting surface saline sites (recent microorganisms hypothesis).
2. Mine populations represent ancient, autochthonous populations entrapped when the salts were deposited by

evaporative concentration (ancient microorganisms hypothesis).

The recent microorganism hypothesis relies on a method of dispersal for the microorganisms, such as wind-blown dust or salt crystals, animal carriers (man, birds, rodents), or percolation from the surface through fissures in the rock. Halobacteria can survive for several years within salt crystals (Dombrowski 1966; Tew 1980; Norton and Grant 1988), which may keep them viable while being dispersed by wind (cyclic salt) (Hatch et al. 1971) or by animals. Both rats and humans are renowned for their ability to travel and colonize new areas, inadvertently acting as vectors of microorganisms.

If the microbial populations are indigenous, then we must explain how they have survived over geological time. There may be actively growing populations in large bodies of brine that are found in the rock strata. Microorganisms may have survived within fluid inclusions in salt crystals, either growing slowly or in a state of dormancy. Alternatively, they could be embedded in the crystal matrix of the salt, or in interstitial brine between crystalline salt. It should also be considered that the presence of microorganisms in salt deposits may result from a combination of both hypotheses; i.e., microorganisms were not laid down with the salt, but somehow subsequently entered the evaporite, and since then have survived over geological time within the salt deposit by one of the processes described.

Crucial to the discussion in whether microorganisms found in salt deposits are separated and insulated from other halophiles, effectively forming an island population, or whether there are means by which halophiles from surface saline sites can reach deep evaporites. Potential transfer depends on the proximity of the salt deposit to surface saline sites, the dispersal strategies of halophiles, and the ability of halophiles to survive outside concentrated brines. Halotolerant bacteria survive and even flourish in a relatively low salt environment such as sea water. Halobacteria usually lyse in such a milieu, although *Halococcus* spp. are an exception, surviving in seawater and being resistant to lysis in distilled water (Rodriguez-Valera et al. 1979, 1982). The sporelike structures (halocysts) observed by Kostrikana et al. (1991) in halobacteria from soil may be common to all halobacteria. Some species of *Haloarcula* (Cline and Doolittle 1992) and other halobacteria (Wais 1985; Kostrikana et al. 1991) form an amorphous mass of cellular material (thallus) often more than 20 μm in diameter with a common capsule. Wais (1985) demonstrated that this thallus did not lyse when dialyzed with distilled water. Both these strategies may enable *Haloarcula* species and other halobacteria to survive in a low-salt environment, allowing them to disperse more easily.

The question can be posed as to how heterotrophic microorganisms could survive, if they have been in evaporites since they were laid down, many millions of years ago. If these organisms have been growing over geological time, then they must have a constant and sufficient supply of organic matter that can be used for energy. As brines evaporate, organic matter is concentrated and deposited with the salt, together with extremely halophilic microorganisms. It is reasonable to suppose that extreme halophiles are adapted to using this organic matter (Tasch 1970). Therefore, for a limited time, heterotrophic halophiles would have sufficient carbon and energy sources to sustain biological systems. These food sources would quickly become depleted in fluid inclusions, but in large bodies of brine would last much longer. There are no studies that model such an enclosed system, so it is impossible to estimate the length of time before nutrient depletion. Slow movement of large bodies of brine or fluid inclusions through rock salt would replenish the brines with organic matter. It has been demonstrated that fluid inclusions migrate up a thermal gradient, but even though there is a slight thermal gradient (greater than $15°C\,km^{-1}$) in salt strata, there is no evidence for downward movement of inclusions over hundreds of millions of years (Roedder 1984). If nutrients were limiting, microorganisms in salt could then enter a state of starvation-survival or dormancy. If available energy is low, microorganisms commonly enter a condition of "starvation-survival" in which growth and reproduction stop and only maintenance energy is expended (Morita 1988). Typical starvation-survival patterns were observed by Amy et al. (1993) in microorganisms isolated from subsurface pore waters that allegedly had not been replenished for 1 to 10 million years. This pattern starts with an increase in cell numbers, caused by fragmentation, followed by a decrease in numbers and than a leveling off. Starved cells are typically miniaturized and show improved chemotaxis; their lipids change and some proteins are lost while new ones appear (Morita 1985). Vegetative cells have been shown to survive for 5 years in a starved state with constant cell numbers (Morita 1988). Maintenance energy is used for maintaining a functional membrane, which is essential for starved cells, so that when nutrients are available these can be transported into the cell (Dawes 1985). Production and repair of essential cell components must continue in the starved state. Many starved cells use storage compounds or degrade protein and RNA as sources of maintenance energy (Dawes 1985). It is still uncertain whether microorganisms become completely dormant in a state of suspended animation or if energy of maintenance, however small, is always required.

Many microorganisms have evolved specialized structures such as spores or cysts, which allow prolonged survival in a dormant state. Sporulation is not a known feature of halobacteria, and ultramicro-forms and viable but noncultivable (VBNC) cells are not known, but other forms of cellular morphogenesis, which may be a response to suboptimal environmental conditions, are known. The unidentified halophilic archaeon studied by Wais (1985) exhibited cellular morphogenesis, one stage of which was existence within a thallus that, unlike the individual unicells, was resistant to lysis in distilled water. This thallus may well be a structure enabling long-term survival of these halobacteria in an environment reported to be subject to extreme variations in salinity. Similarly, Kostrikina et al. (1991) described pleomorphic halobacteria ("*Halobacterium distributum*," "*Halococcus turkmenicus*,"

Natronobacterium pharaonis, and *Natronococcus occultus*) from saline soils, also subject to fluctuations in salinity. "*H. distributum*" had four morphological forms; in one form cells were packaged within a common capsule and in another, single cells were rounded with a thick, multilayered cell wall and thought to be cystlike resting cells (*halocysts*).

To understand whether it is possible for microorganisms to live for thousands, millions, or even billions of years, it is important to know how they die. If cell proteins and nucleic acids are degraded beyond repair, then that cell will die. Sneath (1962) discussed the lethal effect of an accumulation of ionizing radiation in dormant microorganisms over geological time, both from outside the cell and the potassium-40 within the cell. This generation of free radicals, together with free radicals that are produced as intermediates of metabolism (if the cells are metabolizing), damage proteins and nucleic acids (Dean et al. 1993). Ambler and Daniel (1991) discussed the ways in which the covalent structure of fossil protein could be altered, such as hydrolysis of peptide bonds, cross-linking of peptide chains by modifications of amino acids, and racemization. Presumably the same processes will occur within a cell over geological time. Hydrolysis, oxidation, and nonenzymatic methylation of DNA occur in cells and are countered by DNA repair processes (Lindahl 1993). If energy is not available for repair, then DNA will degrade over several thousand years at moderate temperatures, and the cell will die (Lindahl 1993).

The environment must be very important for maintaining the integrity of DNA, but mechanisms are poorly understood. Environmental factors may explain why DNA remains intact when entombed in amber for millions of years (Cano et al. 1993; Poinar et al. 1993). Low temperatures also decrease the rate of chemical breakdown. Spores exclude oxygen and are partially dehydrated, conditions that would stabilize DNA and additionally, specifically synthesize chaperonin-like proteins which stabilize nucleic acids (Lindahl 1993); there is a commitment to dormancy rather than a gradual nonplanned progression. It has also been observed that at very high ionic strength there is a five- to tenfold reduction in the rate of DNA depurination (Lindahl 1993). Depurination is believed to be the main process of DNA decay and so halobacteria, which have an intracellular K^+ concentration of 5 M, would be at an advantage. However, they would be more likely to be at risk from ionizing radiation from ^{40}K, although calculations based on Sneath (1962) suggest that a lethal dose would not be generated by internal ^{40}K over at least 10^9 years.

Halobacteria may therefore constitute ideal candidates for long-term suspended animation in that they have an internal ionic environment that minimizes chemical deterioration of key polymers such as DNA, where all the internal machinery is on the edge of dehydration produced by high levels of solutes. Whether a commitment to dormancy is dependent on specific synthesis of polymers analogous to those seen in endospores remains to be established. In the final analysis, supportive evidence for a truly ancient origin of a microorganism from an ancient site must include inaccessibility of the source environment, strict avoidance of contamination during sampling and culture, demonstration of unique characteristics of the organism, its repeated culture from related samples, and its phylogenetic distinctness. To date, all of these criteria have not been met.

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