

Life at low water activity

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Two major types of environment provide habitats for the most xerophilic organisms known: foods preserved by some form of dehydration or enhanced sugar levels, and hypersaline sites where water availability is limited by a high concentration of salts (usually NaCl). These environments are essentially microbial habitats, with high-sugar foods being dominated by xerophilic (sometimes called osmophilic) filamentous fungi and yeasts, some of which are capable of growth at a water activity (a_w) of 0.61, the lowest a_w value for growth recorded to date. By contrast, high-salt environments are almost exclusively populated by prokaryotes, notably the haloarchaea, capable of growing in saturated NaCl (a_w 0.75). Different strategies are employed for combating the osmotic stress imposed by high levels of solutes in the environment. Eukaryotes and most prokaryotes synthesize or accumulate organic so-called 'compatible solutes' (osmolytes) that have counterbalancing osmotic potential. A restricted range of bacteria and the haloarchaea counterbalance osmotic stress imposed by NaCl by accumulating equivalent amounts of KCl. Haloarchaea become entrapped and survive for long periods inside halite (NaCl) crystals. They are also found in ancient subterranean halite (NaCl) deposits, leading to speculation about survival over geological time periods.

Keywords: xerophiles; halophiles; haloarchaea; hypersaline lakes; osmoadaptation; microbial longevity

1. INTRODUCTION

There are two major types of environment in which water availability can become limiting for an organism. One is a solution in which water availability is determined by the concentration of solutes in that solution, whereas in the other case, the availability is determined mainly by capillary and surface-binding effects, as, for example, in a complex physically heterogeneous environment such as soil. There is no *a priori* reason to suppose that an organism would react differently to water stress imposed by either set of conditions, but in practice, in the heterogeneous environment there are other surface-associated effects that complicate and compromise any quantitative analysis. For that reason, the vast majority of laboratory studies relate to environments where water availability is imposed by the presence of solutes. A widely adopted way of defining the availability of water in a particular environment is the use of the term a_w (water activity), originally developed by the food and pharmaceutical industry, where it is used to determine shelf life and quality of product. The simple water content of a material (percentage water) takes no account of water that is thermodynamically available and has little application to the majority of situations. Water activity (a_w) is based on Raoult's Law for ideal solutions and does not take into consideration solute interactions with components other than water; accordingly the accuracy of the calculation is greater for dilute solutions. Put simply:

$$a_w = P/P_0 = n_1/n_1 + n_2,$$

where n_1 is moles of solvent (water); n_2 is moles of solute; P is vapour pressure of solution and P_0 is vapour pressure of pure water at the same temperature.

Water activity is simply the effective water content expressed as its mole fraction, which is also reflected in the relative humidity that is reached at equilibrium in a sealed container where a hygroscopic product or solution has been placed.

It follows that pure water has a water activity of 1 and all other solutions have values of a_w less than 1. The advantages of using a_w include its ready application to solutions and the ease with which it can be measured. It is less useful in complex particulate systems like soil, where a different term ψ is often used, which addresses the question of capillarity. However, capillarity is negligible for organisms in essentially liquid environments and can be ignored under these conditions. The underlying theory of ψ and the consideration of the capillary component for environments such as soil is described in Griffin (1981) and Griffin & Luard (1979) and is not considered further in this review, which is largely concerned with homogeneous liquid environments.

Organisms capable of growing under conditions of low water activity are commonly referred to under the blanket terms xerotolerant or xerophilic (although see later in this section). The suffix 'tolerant' indicates that the organism is capable of growth conditions of low a_w , but does not necessarily require low a_w for growth. The suffix 'philic' on the other hand indicates that the organisms actually require low a_w conditions for growth. Generally, xerophilic organisms are capable of growing at a_w values lower than

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xerotolerant organisms, but there are exceptions to this general rule, particularly among the prokaryotes, where there are organisms in both categories capable of growing in saturated salt (Vreeland 1987; Brown 1990). Two major types of environment provide habitats for the most xerophilic organisms, namely foods preserved by some form of dehydration or organic solute-promoted lowering of a_w , and saline lakes, where low a_w values are a consequence of inorganic ions. These environments are essentially exclusively populated by micro-organisms and contain the most xerophilic organisms described to date. Saline soils, often adjacent to saline lakes, share micro-organisms in common with saline lakes (Ventosa *et al.* 1998a).

The stresses imposed by ions and organic solutes are not necessarily the same. Many xerophilic micro-organisms from high-sugar foods are tolerant of low a_w levels imposed by ions. *Zygodaccharomyces rouxii*, a xerophilic yeast food-spoilage organism, will grow in media containing 20% (w/v) NaCl (Eriksen & McKenna 1999) and in media supplemented with glucose or glycerol at similar a_w levels (although it will grow at much lower a_w levels in these media). The limiting a_w for food-poisoning strains of *Staphylococcus aureus* isolated from food is the same whether the solute is salt or sugars (Scott 1957). However, the converse is generally not true—micro-organisms growing in saturated salt lakes (a_w 0.75) for example, as a rule, cannot grow in media of similar a_w solely imposed by organic solutes (Kushner 1978). In particular, micro-organisms inhabiting low a_w , high-salt environments have features specifically adapted to higher levels of ions, in addition to an overarching adaptation to low a_w values. Such organisms are generally described as halophilic or halotolerant rather than xerophilic or xerotolerant. Hence, the terms xerophilic and xerotolerant are often now restricted to describing those organisms growing at a_w values imposed by other than inorganic ions, and this usage is followed here. There are a number of subcategories of terms for organisms that have intermediate properties of halotolerance and halophily, not all of which are taken to mean the same thing by different authors (see Grant *et al.* 1998a). This review is concerned with organisms, the majority of which are capable of growth at a_w values of 0.80 or less, whether they be xerophilic/halophilic or xerotolerant/halotolerant.

2. HYPERSALINE ENVIRONMENTS

Hypersaline waters are defined as those with total salt concentrations greater than that of seawater. *Thalassohaline* waters are derived from seawater, and initially at least, have a proportional composition of ions similar to that of seawater. Solar salterns, where seawater is evaporated to produce sea salt, are typical examples of thalassohaline environments. Calcite (CaCO_3), gypsum ($\text{CaSO}_4 \cdot 2\text{H}_2\text{O}$), halite (NaCl), sylvite (KCl) and finally carnallite ($\text{KCl} \cdot \text{MgCl}_2 \cdot 6\text{H}_2\text{O}$), precipitate out sequentially as evaporation occurs. It therefore follows that the final proportional composition of a hypersaline brine will be different from that of seawater. Brines that have undergone halite precipitations are dominated by Mg^{2+} and Cl^- and are more acidic than seawater (table 1). By contrast, athalassohaline waters are markedly influenced by the

geology of the area where they develop, for example by the resolution of salt deposits from a previous evaporative event, or significant leaching of ions from the surrounding geology. The Dead Sea is an example of a hypersaline environment profoundly influenced by an earlier Mg^{2+} -rich brine, somewhat depleted in Na^+ . Eugster & Hardie (1978) have attempted to define the key geological and chemical features that influence how a hypersaline brine develops.

Apart from total salinity and ionic composition, pH is important in determining the composition of any microbial population in any hypersaline brine. The amount of Ca^{2+} (and to a lesser extent Mg^{2+}) is critical in determining the final pH of a brine. The equilibrium between CO_3^{2-} , HCO_3^- and CO_2 is one of the principal buffer systems in the aquatic environment, being, for example, one of the buffer systems that maintains the pH of seawater. The presence of Ca^{2+} , which removes alkaline CO_3^{2-} through the precipitation of insoluble calcite (CaCO_3), obviously influences this equilibrium. Brines derived from seawater have relatively high concentrations of Ca^{2+} and remain around neutrality even after extensive concentration because the molarity of Ca^{2+} always exceeds that of CO_3^{2-} . Profoundly alkaline lakes develop in areas where the surrounding geology is deficient in Ca^{2+} , for example in the East African Rift Valley. Here, surrounding high Na^+ trachyte lavas are deficient in both Ca^{2+} and Mg^{2+} , allowing the development of lakes with pH values in excess of 11 (Grant & Tindall 1986; Grant *et al.* 1990; Jones *et al.* 1998). Levels of Mg^{2+} also influence the systems by removing CO_3^{2-} as dolomite ($\text{CaMg}(\text{CO}_3)_2$), and in the case of the Dead Sea, whose composition is markedly influenced by a previous Mg-rich evaporite, cause slightly acidic conditions through the generation of Mg^{2+} minerals such as sepiolite, which generates H^+ during the precipitative process. Figure 1 shows a schematic representation of the genesis of major neutral and alkaline hypersaline lake types. High levels of other ions in the surrounding topography will also influence the final composition, and there are exceptional hypersaline lakes dominated by Ca^{2+} . Javor (1989) should be consulted for a list of brine types and the chemical and physical parameters that influence their development.

Surface hypersaline lakes and solar salterns are the major habitats for halophilic and halotolerant micro-organisms, but there are other, less well-studied high-salt habitats such as hypersaline soils, salt marshes, desert plants, wall paintings, sea floor brines (such as the Atlantis Deep and the Discovery Deep in the Mediterranean), oil field brines and ancient evaporite deposits, where isolations of halophilic and halotolerant micro-organisms have been recorded. Accounts of these unusual environments and their microbial ecology are to be found in Oren (2002).

Several terms describe the salt tolerance/requirement of organisms. The term halophilic is generally restricted to those organisms that have a specific requirement for salt (almost always assumed to be NaCl). Such organisms will not grow in the absence of relatively high concentrations of salt, usually greater than 1.0–1.5 M. Halotolerance is generally taken to mean that the organism has no specific requirement for salt, but will continue to grow in the presence of high concentrations. There are examples of

Table 1. Concentration of ions in thalassohaline and athalassohaline brines. (Modified from Grant *et al.* 1998a.)

ion	concentration (g l ⁻¹)						
	seawater	seawater at onset of NaCl precipitation	seawater at onset of KCl precipitation	Great Salt Lake (North America)	Dead Sea	Lake Magadi	Wadi Natrun Lake Zugm
Na ⁺	10.8	98.4	61.4	105.0	39.7	161.0	142.0
Mg ²⁺	1.3	14.5	39.3	11.1	42.4	0	0
Ca ²⁺	0.4	0.4	0.2	0.3	17.2	0	0
K ⁺	0.4	4.9	12.8	6.7	7.6	2.3	2.3
Cl ⁻	19.4	187.0	189.0	181.0	219.0	111.8	154.6
SO ₄ ²⁻	2.7	19.3	51.2	27.0	0.4	16.8	22.6
CO ₃ ²⁻ /HCO ₃ ⁻	0.3	0.1	0.1	0.7	0.2	23.4	67.2
pH	8.2	7.3	6.8	7.7	6.3	11.0	11.0

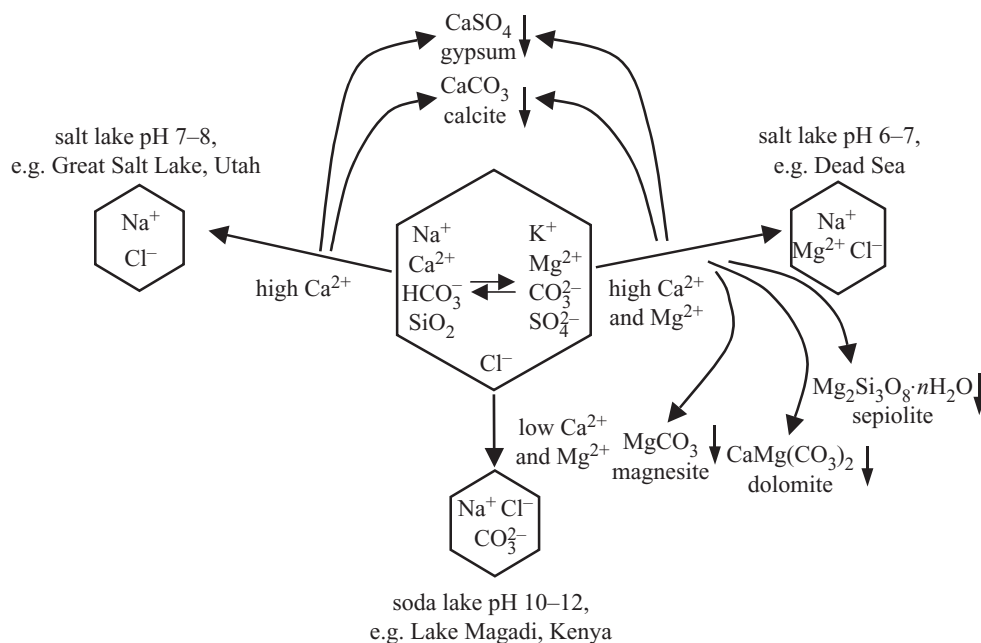


Figure 1. Schematic representation of the genesis of hypersaline brines. The centre box indicates the leaching of minerals by CO₂-charged waters. Alkaline lake development is dependent on low levels of Ca²⁺ and Mg²⁺. Neutral lakes develop where Ca²⁺ and Mg²⁺ levels are high. High Mg²⁺ lakes are more acidic due to reactions involving sepiolite precipitation. (Modified from Grant (2004).)

organisms that are capable of growth over the range of salt concentrations from zero to saturation (sometimes called haloversatile), and, indeed, other terms for organisms that grow within a particular window of salt concentration. Kushner (1978) and Vreeland (1987) have extensively discussed the terms in use. This review is mainly concerned with organisms growing at salinities exceeding 1.0 M and does not attempt such semantic descriptors for the organisms.

There is no doubt that the majority of hypersaline sites harbour significant populations of micro-organisms. Values of a_w do not generally fall much below 0.75, the limiting value obtainable at the saturation point of NaCl (5.2 M). One of the exceptions is Don Juan Pond, a small unfrozen Antarctic lake dominated by very large concentrations of CaCl₂. Total dissolved salts may exceed 47% (w/v) and the a_w value is recorded at 0.45 (Siegel *et al.* 1979). There has been some dispute over the evidence for

microbial colonization of this site (Horowitz *et al.* 1972) and the prevailing opinion is that life is unlikely to exist at this a_w value, which is substantially below the lowest recorded a_w for growth recorded to date, that in high-sugar food (0.61). This particular site is long overdue for a re-examination using direct molecular technologies.

Primary productivity in many hypersaline lakes, mainly by halophilic and halotolerant cyanobacteria, anoxygenic phototrophic bacteria, and also eukaryotic algae of the genus *Dumaliella* may be the source of the significant levels of organic compounds often present (Oren 1994; Jones *et al.* 1998). Phototrophic productivity is probably greatest during periods of dilution, since most of the recorded phototrophs, with the possible exception of *Dumaliella* spp., are unable to grow significantly at saturation point for NaCl. The organic compounds generally support significant populations of aerobic heterotrophs that may form dense blooms that impart coloration to the brines.

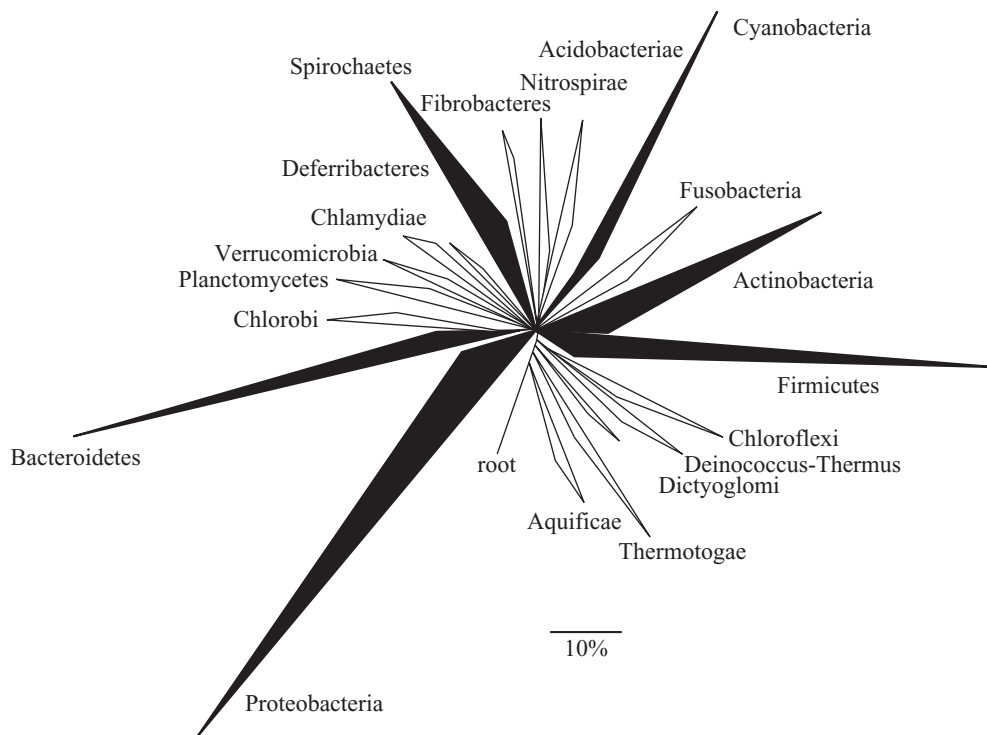


Figure 2. 16S rRNA gene sequence tree showing the major bacterial lines of descent. Lines of descent with halophilic or halotolerant representatives are shown shaded. (Modified from Ludwig & Klenk (2001).)

Hypersaline environments are relatively low in oxygen due to reduced oxygen solubility (2 p.p.m. in saturated NaCl, compared with 7 p.p.m. in seawater) and the brines also harbour substantial populations of anaerobic heterotrophs.

3. LIFE IN LOW a_w HYPERSALINE ENVIRONMENTS

The upper limit of NaCl concentration for vertebrates and invertebrates is *ca.* 1.5 M, although the brine shrimp (*Artemia salina*) is an exception, often present in extremely hypersaline brines but not in extremely alkaline types. Eukaryotes in general are scarce above this salt concentration, with the exception of phototrophic flagellates of the genus *Dunaliella* that frequently impart pigmentation to brines. Between concentrations of 1.5 M and 3.0 M, prokaryotes become predominant, with the haloarchaea and a few rare bacterial types such as *Salinibacter ruber* forming the climax population at the point of halite precipitation (Oren 1994; Antón *et al.* 2002; Grant *et al.* 2001). A few unusual fungi and protozoa are also present, but these are probably active at lower salt concentrations (Post *et al.* 1983; Gunde-Cimerman *et al.* 2000, 2004). Figure 2 indicates that the halophilic or halotolerant character has appeared in most of the main evolutionary lines of bacteria. Within the domain Archaea, halophilic prokaryotes occur in three families: the Halobacteriaceae (also known as the haloarchaea or halobacteria) and a few examples of halophilic methanogens in the Methanospirillaceae and the Methanosarcinaceae. Unlike the Halobacteriaceae, where members are all extremely halophilic, the Methanospirillaceae and the Methanosarcinaceae have representatives that are not halophilic. Among the bacteria, most halophiles described to date fall into

established genera with non-halophilic representatives, but the family Haloanaerobiaceae exclusively contains halophiles and the Halomonadaceae predominantly contains halophiles. Table 2 lists examples of taxonomic groups containing prokaryote representatives isolated from hypersaline environments.

It is possible to make some predictions as to the roles played by different organisms in the utilization and recycling of organics. Despite apparently inhospitable conditions, salt and soda lakes are extremely productive environments (particularly soda lakes, presumably because of unlimited access to CO₂ for photosystems via the HCO₃⁻/CO₃²⁻/CO₂ equilibrium). Cyanobacteria and, in neutral salt lakes, species of the eukaryotic alga *Dunaliella* are the key primary producers, although anoxygenic phototrophic bacteria of the genus *Halorhodospira* may be significant from time to time (Grant & Tindall 1986; Oren 1994). The primary productivity supports large numbers of aerobic heterotrophic Gram-negative bacteria. These are mainly Proteobacteria, in particular members of the Halomonadaceae (the halomonads), probably the most important group of bacterial heterotrophs in both alkaline and neutral hypersaline environments, although other Proteobacteria related to pseudomonads and vibrios are also present (Duckworth *et al.* 1996; Ventosa *et al.* 1998a; Arahal *et al.* 2002). Heterotrophic Gram-positive bacteria of both the high G+C (Firmicutes) and low G+C (Actinobacteria) lineages are also readily isolated from hypersaline brines. Especially abundant are members of the low G+C lineage associated with the diverse *Bacillus* spectrum (Ventosa *et al.* 1998a,b). There are also high G+C relatives of streptomycetes. Isolates from soda lakes have proven to have commercial potential in that they secrete many extracellular hydrolytic enzymes, including

Table 2. Halophilic and halotolerant prokaryotes.

groups	species	habitat
Bacteria		
Cyanobacteria	<i>Arthrospira platensis</i>	soda lake
	<i>Dactylococcopsis salina</i>	Dead Sea sabka
	<i>Aphanothece halophytica</i>	salt lakes
	<i>Microcoleus chthonoplastes</i>	salterns
	<i>Halospirulina tapeticola</i>	salterns (Mexico)
Proteobacteria		
halomonads	<i>Halomonas elongata</i>	salterns (Spain)
	<i>Halomonas subglaciescola</i>	salt lake (Antarctica)
	<i>Halomonas halodurans</i>	river estuary
	<i>Halomonas halmophila</i>	salt lake (Dead Sea)
	<i>Halomonas eurihalina</i>	salterns (Spain)
	<i>Halomonas halophila</i>	saline soil (Spain)
	<i>Halomonas salina</i>	salterns (Spain)
	<i>Halomonas halodentrificans</i>	salterns, curing brines
	<i>Halomonas variabilis</i>	salt lake (Great Salt Lake)
	<i>Halomonas pantelleriensis</i>	alkaline saline soil (Italy)
	<i>Halomonas magadiensis</i>	soda lake (Kenya)
	<i>Halomonas desiredata</i>	sewage plant
	<i>Halomonas meridiana</i>	salt lake (Antarctica)
	<i>Halomonas campisalis</i>	soda lake (Washington)
	<i>Halomonas maura</i>	salterns (Morocco)
	<i>Halomonas alimentaria</i>	salterns, fermented sea food
	<i>Chromohalobacter marismortui</i>	salt lakes (Dead Sea)
	<i>Chromohalobacter canadiensis</i>	culture contaminant
	<i>Chromohalobacter israeliensis</i>	salt lake (Dead Sea)
	<i>Chromohalobacter salexigens</i>	salterns (Spain)
anoxygenic phototrophs	<i>Rhodothalassium salexigens</i>	brackish seawater (Oregon)
	<i>Rhodovibrio sodomensis</i>	salt lake (Dead Sea)
	<i>Rhodovibrio salinarum</i>	saltern (Portugal)
	<i>Halochromatium salexigens</i>	saltern (France)
	<i>Halochromatium glycolyticum</i>	salt lake (Egypt)
	<i>Thiohalocapsa halophila</i>	saltern (France)
	<i>Ectothiorhodospira mobilis</i>	salt lakes
	<i>Ectothiorhodospira marismortui</i>	salt lake (Dead Sea)
	<i>Ectothiorhodospira haloalkaliphila</i>	soda lakes
	<i>Halorhodospira halophila</i>	salt and soda lakes
	<i>Halorhodospira halochloris</i>	soda lake (Egypt)
	<i>Halorhodospira abdelmalekii</i>	soda lake (Egypt)
	<i>Alcalilimnicola halodurans</i>	soda lake (Tanzania)
pseudomonads/vibrios/alteromonads	<i>Pseudomonas halophila</i>	salt lake (Great Salt Lake)
	<i>Pseudomonas beijerinckii</i>	salterns?, salted beans
	<i>Marinobacter hydrocarbonoclasticus</i>	seawater
	<i>Marinobacter aquacolar</i>	oil well (Vietnam)
	<i>Salinivibrio costicola</i>	saltern? Cured meats
sulphate reducers	<i>Desulfovibrio halophilus</i>	salt lake (Egypt)
	<i>Desulfovibrio senezii</i>	saltern (California)
	<i>Desulfovibrio oxyclimae</i>	salt lake (Egypt)
	<i>Desulfohalobium retbaense</i>	salt lake (Senegal)
	<i>Desulfobacter halotolerans</i>	salt lake (Great Salt Lake)
	<i>Desulfonatronovibrio hydrogenovorans</i>	soda lake (Kenya)
	<i>Desulfonatronum lacustre</i>	soda lake (Kenya)
	<i>Desulfosalsa halophila</i>	salt lake (Great Salt Lake)
sulphur oxidizers	<i>Halothiobacillus halophilus</i>	salt lake (Australia)
	<i>Halothiobacillus kellyi</i>	hydrothermal vent (Aegean Sea)
hyphomicrobia	<i>Dichotomicrobium thermohalophilus</i>	salt lake (Egypt)
Firmicutes (low G+C% Gram-positives)		
unknown affiliation	<i>Thermohalobacter berrensensis</i>	saltern (France)
haloanaerobes	<i>Haloanaerobium praevalens</i>	salt lake
	<i>Haloanaerobium alcaliphilum</i>	salt lake (Great Salt Lake)

(Continued.)

Table 2. (Continued.)

groups	species	habitat
	<i>Haloanaerobium acetyethylicum</i>	oil well (Mexico)
	<i>Haloanaerobium salsuginis</i>	oil well (Oklahoma)
	<i>Haloanaerobium saccharolyticum</i>	salt lake (Crimea)
	<i>Haloanaerobium congolense</i>	oil well (Congo)
	<i>Haloanaerobium lacusrosei</i>	salt lake (Senegal)
	<i>Haloanaerobium kushneri</i>	oil well (Oklahoma)
	<i>Haloanaerobium fermentans</i>	salterns, salted fish
	<i>Halocella cellulositytica</i>	salt lake (Crimea)
	<i>Halothermothrix orenii</i>	salt lake (Tunisia)
	<i>Natronella acetigenica</i>	soda lake (Egypt)
	<i>Halobacteroides halobius</i>	salt lake (Dead Sea)
	<i>Halobacteroides elegans</i>	salt lake (Crimea)
	<i>Acetohalobium arabatania</i>	salt lake (Crimea)
	<i>Haloanaerobacter chitinivorens</i>	saltern (California)
	<i>Haloanaerobacter lacunarum</i>	salt lake (Kerech)
	<i>Haloanaerobacter salinarius</i>	saltern (France)
	<i>Orenia marismortui</i>	salt lake (Dead Sea)
	<i>Orenia salinaria</i>	saltern (France)
	<i>Orenia sivashensis</i>	salt lake (Crimea)
	<i>Halonatronum saccharophilum</i>	soda lake (Kenya)
	<i>Natronella acetigena</i>	soda lake (Kenya)
<i>Bacillus/Clostridium</i>		
	<i>Sporhalobacter lortetii</i>	salt lake (Dead Sea)
	<i>Bacillus halophilus</i>	seawater
	<i>Bacillus haloalkaliphilus</i>	soda lake (Egypt)
	<i>Gracibacillus halotolerans</i>	soda lake (Great Salt Lake)
	<i>Gracibacillus dipsosauri</i>	salt glands of iguanas
	<i>Halobacillus halophilus</i>	salt marsh
	<i>Halobacillus literalis</i>	salt lake (Great Salt Lake)
	<i>Halobacillus thailandensis</i>	salterns, fish sauce
	<i>Salibacillus salexigens</i>	salterns
	<i>Salibacillus marismortui</i>	salt lake (Dead Sea)
	<i>Oceanobacillus iheyensis</i>	deep sea ridge
	<i>Tindallia magadiensis</i>	soda lake (Kenya)
	<i>Clostridium halophilus</i>	salt lakes
	<i>Desulfotomaculum halophilus</i>	oil field brine (France) sulphate reducer
cocci		
	<i>Salinicoccus roseus</i>	salterns (Spain)
	<i>Salinicoccus hispanicus</i>	salterns (Spain)
	<i>Marinococcus halophilus</i>	salterns
	<i>Marinococcus albus</i>	salterns
	<i>Tetragenococcus halophilus</i>	salterns/salted food
	<i>Tetragenococcus muraticus</i>	salterns? fermented squid
Actinobacteria (high %G+C Gram-positives)		
	<i>Nesterenkonia halobia</i>	salterns
	<i>Actinopolyspora halophila</i>	culture contaminant
	<i>Actinopolyspora mortivallis</i>	saline soil (Death Valley)
	<i>Actinopolyspora iraqiensis</i>	saline soil (Iraq)
	<i>Nocardiopsis lucentensis</i>	saline soil (Spain)
	<i>Nocardiopsis halophila</i>	saline soil
	<i>Nocardiopsis kunsanensis</i>	saltern (Korea)
Bacteroidetes		
	<i>Flavobacterium salegens</i>	salt lake (Antarctica)
	<i>Salinibacter ruber</i>	salterns (Spain)
Archaea		
haloarchaea		
	<i>Halobacterium salinarum</i>	salterns/salted hides
	<i>Haloarcula vallismortis</i>	salt ponds (Death Valley)
	<i>Haloarcula marismortui</i>	salt lake (Dead Sea)
	<i>Haloarcula hispanica</i>	salterns (Spain)
	<i>Haloarcula japonica</i>	salterns (Japan)
	<i>Haloarcula argentinensis</i>	salt flats (Argentina)

(Continued.)

Table 2. (Continued.)

groups	species	habitat
	<i>Haloarcula mukohataei</i>	salt flats (Argentina)
	<i>Haloarcula quadrata</i>	sabka (Egypt)
	<i>Halobaculum gomorrense</i>	salt lake (Dead Sea)
	<i>Halococcus morrhuae</i>	salterns? salted cod
	<i>Halococcus saccharolyticus</i>	salterns (Spain)
	<i>Halococcus salifodinae</i>	salt mine (Austria)
	<i>Haloferax volcanii</i>	salt lake (Dead Sea)
	<i>Haloferax gibbonsii</i>	saltern (Spain)
	<i>Haloferax dentrificans</i>	saltern (California)
	<i>Haloferax mediterranei</i>	salt lake (Spain)
	<i>Halogeometricum borinquense</i>	saltern (Puerto Rico)
	<i>Halorhabdus utahensis</i>	salt lake (Great Salt Lake)
	<i>Halorubrum saccharovororum</i>	saltern (California)
	<i>Halorubrum sodomense</i>	salt lake (Dead Sea)
	<i>Halorubrum lacusprofundi</i>	salt lake (Antarctica)
	<i>Halorubrum coriense</i>	saltern (Australia)
	<i>Halorubrum distributans</i>	saline soil (USSR)
	<i>Halorubrum vacuolatum</i>	soda lake (Kenya)
	<i>Halorubrum trapanicum</i>	saltern (Sicily)
	<i>Halorubrum tebequense</i>	salt lake (Chile)
	<i>Haloterrigina turkmenica</i>	alkaline soil (USSR)
	<i>Haloterrigina thermotolerans</i>	saltern (Puerto Rico)
	<i>Natrialba asiatica</i>	beach sand (Japan)
	<i>Natrialba taiwanensis</i>	saltern (Taiwan)
	<i>Natrialba magadii</i>	soda lake (Kenya)
	<i>Natrialba hulunbeirensis</i>	soda lake (China)
	<i>Natrialba chahannoensis</i>	soda lake (China)
	<i>Natrinema pellirubrum</i>	saltern? salted hide
	<i>Natrinema pallidum</i>	saltern? salted cod
	<i>Natrinema versiforme</i>	salt lake? (China)
	<i>Natronobacterium gregoryi</i>	soda lake (Kenya)
	<i>Natronobacterium nitratireducens</i>	soda lake (China)
	<i>Natronococcus occultus</i>	soda lake (Kenya)
	<i>Natronococcus amylolyticus</i>	soda lake (Kenya)
	<i>Natronococcus pharaonis</i>	soda lake (Kenya)
	<i>Natronorubrum bangense</i>	soda lake (Tibet)
	<i>Natronorubrum tibetense</i>	soda lake (Tibet)
methanogens	<i>Methanocalculus halotolerans</i>	oil well (France)
	<i>Methanohalobium evestigatum</i>	saline lagoon (Crimea)
	<i>Methanohalophilus mahii</i>	salt lake (Great Salt Lake)
	<i>Methanohalophilus halophilus</i>	salt lake (Australia)
	<i>Methanohalophilus portucalensis</i>	saltern (Portugal)
	<i>Methanosalsum zhilinae</i>	soda lake (Egypt)
	<i>Methanococcus doii</i>	saltern (California)

proteinases, cellulases and lipases (Rees *et al.* 2003). Genencor BV (Leiden) currently markets two different soda lake cellulases derived from Gram-positive isolates for use in laundry and textile processes.

Hydrolysis products of complex polymers are also substrates for anaerobes, especially members of the Haloanaerobiales and organisms related to other clostridial groups in the low G+C division of the Gram-positive bacteria. These bacteria ferment organic compounds to acetic acid, hydrogen and CO₂ (Grant *et al.* 1998a; Zavarzin *et al.* 1999), which in turn may be used by methanogens, although most of the methanogens isolated to date from hypersaline environments are methylotrophic, using compounds such as methanol and methylamine (Oren 1999). Such C1 compounds are likely to be abundant in hypersaline brines, probably derived from the anaerobic decomposition of cyanobacterial mats.

Sulphidogenesis is usually pronounced in hypersaline sediments. Isolation of halophilic sulphate-reducing bacteria has proven difficult, but there are now several of these in cultures from both neutral and alkaline hypersaline environments including *Desulfovibrio*, *Desulfonatronovibrio* and *Desulfobacter* species (Caumette *et al.* 1991; Zhilina *et al.* 1997; Tsu *et al.* 1998). Oxidation of sulphide is brought about by anoxygenic phototrophic bacteria such as *Halorhodospira* spp. and under aerobic conditions in alkaline hypersaline lakes by *Thioalkalivibrio* spp. (Sorokin *et al.* 2001). Halophilic thiobacilli are also found in neutral salt lakes (Kelly & Wood 2000). Ammonia oxidation is known to take place in soda lakes (Khmelenina *et al.* 2000), but nitrifying bacteria have not yet been recorded in any neutral lakes.

The climax population in sodium-dominated hypersaline lakes at the point of halite (NaCl) precipitation

almost always comprises haloarchaea, with only a few bacterial types such as *S. ruber* able to compete. These saturated brines provide among the most extreme a_w values possible via inorganic solutes. Soda lakes impose the additional stress of very alkaline pH values, up to pH 12 in some cases. Haloarchaea now comprise 15 genera (table 2) on the basis of phylogenetic analysis, although phenotypically they are all rather similar. Dense blooms of these organisms colour neutral and alkaline saturated hypersaline lakes bright red. These organisms are the most halophilic known, most requiring at least 2 M NaCl for growth and many capable of growing in saturated NaCl (5.2 M). As might be expected, the isolates from alkaline soda lakes have an additional requirement for high pH in growth media, usually growing between pH 8.5 and 11.0 with an optimum at pH 9.5–10.0, whereas those haloarchaea from neutral lakes generally have pH optima for growth between pH 6 and pH 8. Alkaliphilic haloarchaea are currently classified in six genera (table 2); four of these, *Natronococcus*, *Natronobacterium*, *Natronorubrum* and *Natronomonas*, harbour only alkaliphiles. Haloarchaea from neutral sites comprise the remaining 11 genera, two of which contain both alkaliphilic and neutrophilic types. It is not usually clear whether blooms of these haloarchaea comprise predominantly one species or a mixture of several species. Often a majority of isolates from neutral brines are *Halorubrum* or *Haloarcula* spp. (W. D. Grant, unpublished results), but an analysis of lipids in Dead Sea biomass suggested mainly *Haloferax* and *Halobaculum* spp. (Oren & Gurevich 1993). Early work relied on phenotypic characterization, and most rod-shaped isolates were assigned to the genus *Halobacterium*. However, *Halobacterium salinarum*, the sole representative of this genus, is seldom isolated from hypersaline brines and is typically isolated from salted hides and salted fish, as a consequence of its proteolytic capacity. Alkaline brines have yielded a considerable number of isolates, but it is not clear as yet, which types, if any, are dominant.

Direct molecular analysis of both alkaline and neutral hypersaline brines by 16S rDNA amplification of environmental DNA, preparation of gene libraries, followed by sequence determinations of individual 16S rRNA genes, has revealed novel lineages that have yet to be brought into culture (Grant *et al.* 1999; Benlloch *et al.* 2001), notably enabling the phylogenetic identification of the square flat cells first described by Walsby (1980) that are frequently observed in neutral hypersaline brines from a variety of geographical locations (Antón *et al.* 1999). There have also been reports of the retrieval of haloarchaeal sequences from environments that are not particularly saline, suggesting that less halophilic haloarchaea may exist (Munson *et al.* 1997).

Haloarchaeal blooms in solar salterns are known to promote crystallization of halite. It is possible that the cells may serve as templates in the nucleation of halite crystals and their subsequent development (López-Cortés & Ochoa 1998). There is no doubt that salt yields are diminished in the absence of haloarchaeal blooms, and laboratory experiments support this view (Javor 2002). Haloarchaeal blooms, by virtue of their red carotenoid pigments, also increase light absorption of the brines and promote evaporation by increasing the temperature. Observations of halite crystallization show that halorchaea

Table 3. Approximate water activity (a_w) values of selected foods.

(Adapted from Brewer (1999).)

a_w value	foods
1.00–0.95	fresh meat, fresh and canned fruit and vegetables, sausages, eggs, margarine, butter, low-salt bacon
0.95–0.90	processed cheese, bakery goods, raw ham, dry sausage, high-salt bacon, orange juice concentrate
0.90–0.80	hard cheese, sweetened condensed milk, jams, margarine, cured ham, white bread
0.80–0.70	molasses, maple syrup, heavily salted fish
0.70–0.60	Parmesan cheese, dried fruit, corn syrup, rolled oats, jam
0.60–0.50	chocolate, confectionary, honey
0.40	dried egg, cocoa
0.30	dried potato flakes, potato crisps, crackers, cake mix
0.20	dried milk, dried vegetables

become entrapped within the crystals, leaving behind a so-called bitter brine dominated by $MgCl_2$ and KCl, which does not support significant growth of haloarchaea, although it is not actually toxic (Norton & Grant 1988). The failure to recover haloarchaea from these bitter brines is because these have been physically removed by entrapment within halite. Crude solar salt contains many viable haloarchaea, typically 10^6 viable cells per gram. Since crude solar salt is generally used in the salting of hides and fish, this is the explanation for the recovery of haloarchaea from these products, notably *Halobacterium salinarum*.

4. LIFE IN LOW a_w FOODS

Low a_w foods fall broadly into two types: cured foods whose water activity is lowered by the presence or addition of a solute, either salt or sugar, and those foods that are dehydrated by the removal of water by freeze-drying or simple evaporation. Freezing food owes part of its effectiveness to the removal of water. Clearly, other factors such as temperature, pH, oxygen and the presence of antimicrobial inhibitors also markedly affect any microbial population (Houtsma *et al.* 1996). Table 3 lists the a_w values for particular types of food.

Preventing pathogen growth and retarding spoilage are crucial in the preservation of foods. Most fresh foods have a_w values of 0.95–0.99, allowing the growth of many types of micro-organism. The minimum a_w for most bacteria is ca. 0.90 (table 4) and foods with a_w values below this provide environments largely for xerophilic and xerotolerant fungi and a few highly resistant prokaryotes (Brewer 1999). The vast majority of human pathogens are suppressed by a_w values below 0.90 (Houtsma *et al.* 1996). One exception is *Staphylococcus aureus*, which is extremely xerotolerant and will survive in salted foods at a_w values of ca. 0.82 (15% w/v NaCl) (Scott 1957; Kushner 1978).

Curing foods in baths of brine extracts nutrients that promote the growth of halophilic and halotolerant micro-organisms. Often, specific concentrations of salt are

Table 4. Minimum inhibitory a_w values for growth of micro-organisms in food. (Adapted from Brewer (1999).)

a_w value	prokaryotes	yeasts	moulds
0.97–0.95	<i>Clostridium</i> spp. <i>Pseudomonas</i> spp. <i>Escherichia</i> spp. <i>Bacillus</i> spp. <i>Pediococcus</i> spp. <i>Citrobacter</i> spp. <i>Vibrio</i> spp. <i>Lactobacillus</i> spp.		
0.95–0.90	<i>Streptococcus</i> spp. <i>Corynebacterium</i> spp. <i>Micrococcus</i> spp.	<i>Rhodotorula</i> spp. <i>Pichia</i> spp. <i>Candida</i> spp. <i>Trichosporon</i> spp.	<i>Rhizopus</i> spp. <i>Mucor</i> spp.
0.90–0.85	<i>Staphylococcus</i> spp.	<i>Saccharomyces</i> spp. <i>Hansenula</i> spp. <i>Torulopsis</i> spp.	<i>Cladosporium</i> spp.
0.85–0.80		<i>Zygosaccharomyces bailii</i>	<i>Aspergillus patulum</i>
0.80–0.75	haloarchaea		<i>Aspergillus glaucus</i> <i>Aspergillus conicus</i> <i>Aspergillus flavus</i>
< 0.70		<i>Zygosaccharomyces rouxii</i>	<i>Xeromyces bisporus</i>

necessary to favour a succession of organisms such as lactic acid bacteria during fermentation, for example in pickles and sausages (Brewer 1999). At very high salt concentrations (a_w values below 0.8) the only prokaryotes to flourish are the haloarchaea, originally introduced into the food via solar salt. These may cause the spoilage of heavily salted proteinaceous products, being responsible for the 'pink' in salted fish and 'red heat' of salted hides (Grant & Larsen 1989). Here, one particular species, the proteolytic *H. salinarum* seems to be dominant.

The minimum a_w that can be achieved by the addition of NaCl is 0.75 (saturation point for NaCl). Values below this in food can occur only in the presence of organic solutes such as glucose and fructose, and sometimes there may be additional significant surface effects in heterogeneous foods through drying. Foods, which include honey, fruit syrups, jams, marmalades and dried fruits, are dominated by xerophilic fungi and yeasts. The vast majority of fungi and yeasts are inhibited at a_w values between 0.8 and 0.75, but the spoilage mould *Xeromyces bisporus* is exceptional in being able to grow at a lower water activity (a_w 0.61) than any other organism described to date and can thus cause spoilage of foods that are normally considered safe from microbial attack (Hocking & Pitt 1999; table 4). The organism will not grow at a_w values greater than 0.97, is thus an obligate xerophile, and is also capable of completing a sexual life cycle, forming ascospores, at a_w values below 0.7. Originally isolated from spoiled liquorice, *X. bisporus* also causes problems with dried fruit, tobacco and honey, although its natural habitat is as yet unknown (Hocking & Pitt 1999). *Zygosaccharomyces rouxii* is one of three *Zygosaccharomyces* spp. that are troublesome spoilage organisms at very low a_w values, particularly under acidic conditions in products such as fruit concentrates and brined vegetables (Eriksen & McKenna 1999). Under extreme conditions, internal CO₂ pressure due to sugar fermentation can cause explosions of glass bottles to occur (Thomas & Davenport

1985). Growth of *Z. rouxii* has been recorded at a_w 0.62 and the organism also, unusually, is capable of growing in relatively high NaCl concentrations (20% (w/v); a_w 0.85; Tokuoka (1993)), sharing with *X. bisporus* the capacity to produce ascospores at a_w values below 0.7.

5. OSMOADAPTATION

Micro-organisms exposed to a low a_w environment must possess mechanisms to avoid water loss by osmosis. At least some level of turgor pressure has to be maintained to allow cell survival and growth. There are two basic strategies:

- (i) a minority of halophiles use counterbalancing levels of inorganic ions (usually KCl) to achieve osmotic stability;
- (ii) the majority of halophiles (and all the xerophiles) produce or accumulate low-molecular-mass organic compounds that have osmotic potential.

These osmolytes, also known as compatible solutes because of the need for them to be compatible with cell machinery (Brown 1978, 1990), must also protect against inactivation, inhibition and denaturation of both enzymes and macromolecular structures under conditions of low water activity. Compatible solutes are polar, normally uncharged or zwitterionic compounds under the conditions experienced inside cells. Compatible solutes belong to several classes of compounds and there are some common structural motifs, particularly among amino acid derivatives. Figure 3 shows the most important compatible solutes of micro-organisms. They include the following:

- (i) polyols such as glycerol, arabitol, mannitol, sugars or sugar derivatives such as trehalose, sucrose, sulphotrehalose and glucosylglycerol;

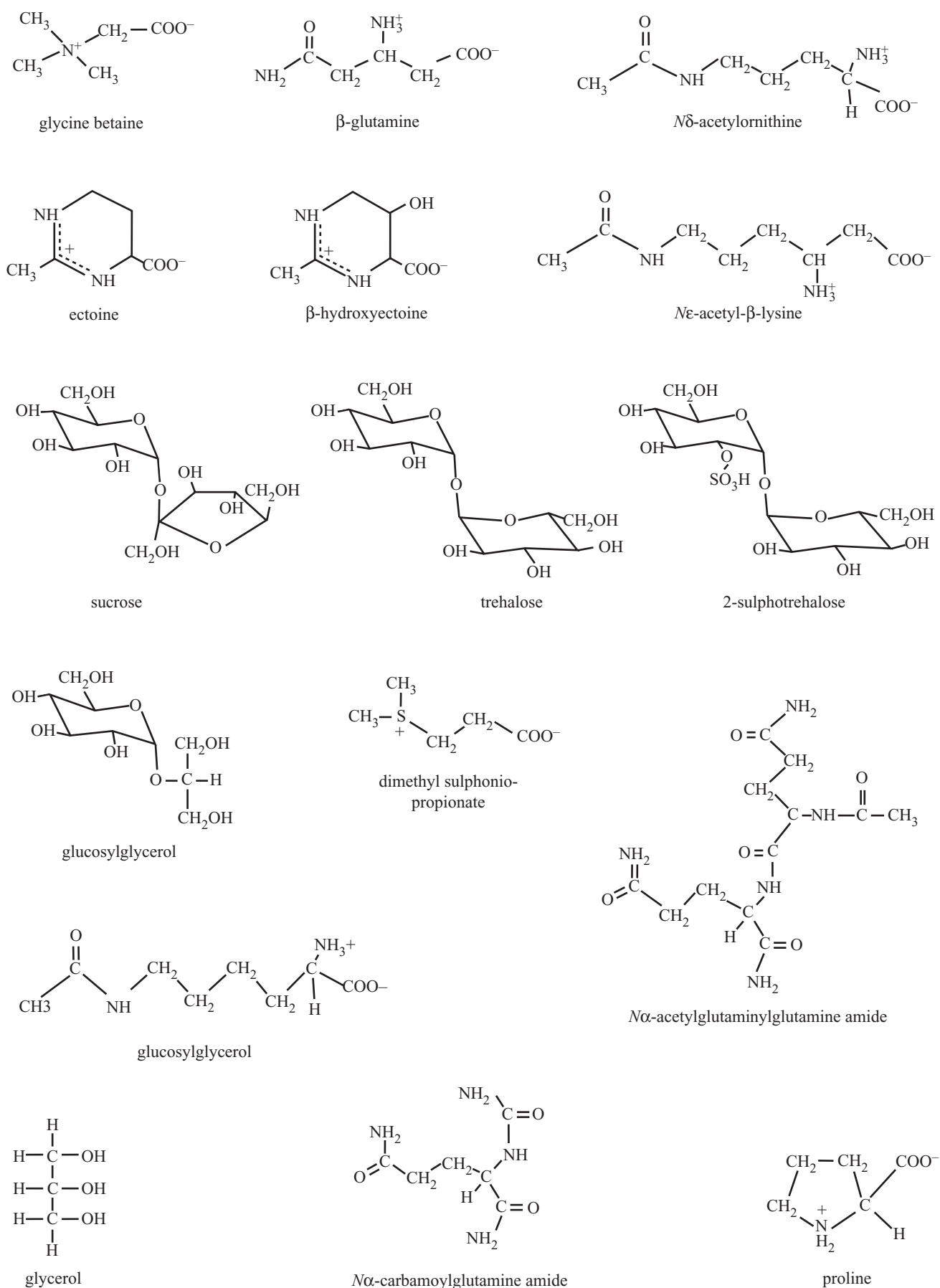
Figure 3. Structure of compatible solutes. (Modified from Grant *et al.* (1998a) and Oren (2002).)

Table 5. Organic compatible solutes in different xerophiles/halophiles.
(Adapted from Oren (2002).)

group	genera	solutes
Archaea		
haloarchaea	<i>Natronobacterium</i> <i>Natronococcus</i>	2-sulphotrehalose glycine betaine
methanogens	<i>Methanohalophilus</i>	glycine betaine glutamine Nε-acetyl-β-lysine
Bacteria		
Proteobacteria	<i>Halorhodospira</i> <i>Salinivibrio</i> <i>Halochromatium</i> <i>Halomonas</i>	glycine betaine ectoine ectoine glycine betaine ectoine hydroxyectoine
Firmicutes	<i>Marinococcus</i> <i>Halobacillus</i> <i>Salinibacillus</i>	ectoine hydroxyectoine proline Nε-acetyl lysine ectoine hydroxyectoine
Cyanobacteria	<i>Anthrospira</i>	glycine betaine glycine betaine glucosyl glycerol
Eukaryota		
phototrophs	<i>Dunaliella</i>	glycerol
marine algae	<i>Porphyra</i>	dimethylsulphoniopropionate
yeasts/fungi	<i>Zygosaccharomyces</i> <i>Xeromyces</i>	glycerol arabitol

- (ii) betaines (trimethylammonium compounds) and thetines (dimethyl sulphonium compounds);
- (iii) amino acids, including proline, glutamate and glutamine;
- (iv) N-acetylated amino acids such as Nδ-acetylornithine;
- (v) glutamine amide derivatives such as Nα-carbamoylglutamine amide;
- (vi) ectoines, notably ectoine and β-hydroxyectoine.

Arguably, dimethylsulphoniopropionate is the major compatible solute on Earth, since the majority of marine algae synthesize this compound, whose eventual breakdown leads to a major flux of organic sulphur to the atmosphere, mostly as dimethylsulphide (Archer *et al.* 2001). However, it is not generally considered as a microbial compatible solute.

With the exception of the haloarchaeon *H. salinarum*, which accumulates KCl as an osmoprotectant, food spoilage prokaryotes generally accumulate mainly proline, glutamate, trehalose and glycine betaine as compatible solutes, whereas the fungi and yeasts tend to accumulate polyhydric alcohols such as glycerol and arabitol (Brewer 1999). As a general rule, polyols such as glycerol and arabitol are also significant compatible solutes in xerophilic and xerotolerant plants and halophilic eukaryotic micro-organisms such as the microscopic alga *Dunaliella viridans*, but are not significantly present in prokaryotic halophiles (Brown 1990; Eriksen & McKenna 1999). Disaccharides and amino acids have only a limited potential as compatible solutes and are never accumulated to high concentrations (Oren 2002), whereas glycine betaine

and the ectoines are the compatible solutes accumulated by halophilic micro-organisms capable of growing at NaCl concentrations close to saturation (5.2 M) (Galinski 1993, 1995). Galinski & Trüper (1994) have reviewed the biosynthetic pathways for a number of these compatible solutes and apart from glycine betaine, all nitrogen-containing compatible solutes are derived from glutamate or aspartate biosynthetic routes. Table 5 lists compatible solutes in different groups of xerotolerant/xerophilic and halotolerant/halophilic micro-organisms.

The use of inorganic ions (usually KCl) to provide osmotic balance is exclusively confined to halophilic prokaryotes (Kushner 1978), mainly haloarchaea and anaerobic bacteria of the order Haloanaerobiales. The discovery of *S. ruber*, a red-pigmented bacterium that competes with haloarchaea in saturated salterns, has recently added another example of this kind of osmoadaptation (Antón *et al.* 2002).

We now have a reasonably good understanding of the mechanisms used by haloarchaea and other halophiles for the removal of Na⁺ from the cell and the accumulation of K⁺. The membranes of all halophiles investigated possess high Na⁺/H⁺ antiporter activity, driven by the proton electrochemical gradient, thus constantly extruding Na⁺ from the cells (Lanyi & MacDonald 1976). Accumulation of K⁺ is, in part, by passive transport (Lanyi 1979), K⁺ entering the cells as Na⁺ is ejected, thus maintaining electroneutrality, but active transport is also involved which is ATP dependent (Meury & Kohiyama 1989). Chloride is also important in haloadaptation in this group, but not necessarily in osmoregulation *per se* (Oren 2002; Müller &

Oren 2003). Two systems have been identified in haloarchaea, one being a light-independent transport system coupled to the efflux of Na^+ (Duschl & Wagner 1986), whereas the second is the light-dependent, inwardly directed Cl^- pump, halorhodopsin. There is a vast literature on halorhodopsin and related retinal-based ion pumps that can be accessed in reviews by Lanyi (1998, 1999) and Oesterhelt (1995, 1998). Chloride cannot be replaced in growth media by other ions, but there is little information on the specific requirement for cellular functions (Müller & Oren 2003). However, *Halobacillus halophilus*, which osmoregulates primarily with organic compatible solutes, requires chloride for endospore formation, motility and the expression of a large number of proteins, is chloride regulated (Oren 2002; Roessler *et al.* 2000).

It is worth noting that almost all other halophiles accumulate some K^+ and Na^+ to varying amounts, K^+ usually being the dominant cation and the only one at levels above that in the medium. However, the intracellular concentrations that have been measured are insufficient to compensate for the osmotic potential of the growth medium, and the majority of the osmoregulation for these organisms is provided by organic compatible solutes (Ventosa *et al.* 1998a).

Ectoine and its derivative hydroxyectoine are considered to be the most abundant compatible solutes synthesized by prokaryotes (Galinski 1995; Galinski & Trüper 1994; Ventosa *et al.* 1998a). Glycine betaine, another common solute, is often accumulated from the environment where levels are relatively high through rapid secretion from organisms that have experienced a reduction in osmotic stress, although phototrophic bacteria synthesize this *de novo*. Polyols are also generally synthesized *de novo* (Galinski & Trüper 1994).

Compatible solutes must, by definition, have no significant interaction with cell machinery, and are to a large extent excluded from the hydration shell of proteins. It has been suggested that water in protein hydration shells (and closely associated with molecular surfaces in general) is thermodynamically different from that in the bulk phase (Wiggins 1990). Compatible solutes may preferentially interact with water in the bulk phase due to their own capacity to form strong water structures (Galinski 1995). It is this bulk-phase water that responds to osmotic changes of the environment. Exclusion of compatible solutes from the hydration shell of proteins may cause a decrease in entropy and subsequent stabilization of proteins, a feature exploited by the biotechnology industry in their use as protectants for otherwise labile enzymes, increasing shelf life and activity (Galinski 1993; Ventosa & Nieto 1995). Ectoine and derivatives are commercially produced by Bitop (Witten, Germany), have also been used in the cosmetic industry as moisturizers in cosmetics and also show beneficial effects in reducing UV damage to skin cells (Beyer *et al.* 2000).

Cells that accumulate or synthesize compatible solutes must clearly have rapid response systems that react to a sudden dilution of the environment. Some compatible solutes are rapidly catabolized or polymerized to an osmotically inert state. Others, such as glycine betaine and ectoine, are rapidly excreted, explaining their ready availability in the environment for organisms that have to

acquire them (Trüper & Galinski 1990). Osmotic shift-up and shift-down regimes are used as a means to produce such compounds for the biotechnology industry (Galinski & Tindall 1992; Galinski & Sauer 1998).

Organisms that osmoadapt by accumulating KCl must have cellular components that are specifically adapted to very high levels of KCl. Normally, high salt concentrations would withdraw water from proteins, making hydrophobic bonds strong and inducing polypeptides to denature. Detailed physical studies of proteins from halophiles are mainly confined to members of the haloarchaea. Most enzymes and other proteins of the haloarchaea denature when suspended in solutions of low ionic strength (1–2 M) (Kushner 1978). Some of these enzymes are also markedly stimulated by KCl rather than NaCl. Most proteins of haloarchaea contain a large excess of acidic amino acids (glutamate and aspartate) and a low content of basic amino acids (lysine and arginine) (Lanyi 1974; Mevarech *et al.* 2000; Oren 2002). Recent analysis of protein sequences deduced from the genome analysis of *H. salinarum* NRC1 confirms this general trend, showing particular abundance of aspartate and relative scarcity of lysine (Ng *et al.* 2000). Haloarchaeal proteins are also relatively low in hydrophobic amino acid residues. It has been suggested that high levels of cations are necessary to shield negative charges on the protein surface, which would otherwise make the structure unstable and prone to denaturing, although there is some doubt that this is the only mechanism preventing protein instability (Mevarech *et al.* 2000). High salt may also help to maintain necessary weak hydrophobic interactions important in structural configuration. Less halophilic bacteria that osmoadapt mainly with organic compatible solutes show less excess of acidic amino acids and decrease in basic amino acids than the haloarchaea, but still show some of the trend when compared with non-halophilic types, as a consequence of somewhat elevated levels of K^+ (Oren 2002). Curiously, representatives of one group of alkaliphilic haloarchaea, although accumulating KCl to high levels, also appear to have an organic compatible solute, 2-sulphotrehalose (Desmarais *et al.* 1997). Much less is known about protein–nucleic-acid interactions in the presence of a high concentration of salt, although there is evidence for differential transcription dependent on salt concentration (Ferrer *et al.* 1993; Juez 2003).

Many haloarchaea (the exceptions being the coccoid types in the genera *Halococcus* and *Natronococcus*) also require high concentrations of ions to maintain cell integrity. These prokaryotes, in common with many others, have a single layer of hexagonally arranged subunits outside the cytoplasmic membrane known as the S-layer (Oren 2002). The cell wall is optimally stabilized by a combination of Na^+ and Mg^{2+} . As with the cytoplasmic proteins, the glycoprotein that comprises most of the S layer is negatively charged, and maintenance of structure also depends on stabilization of hydrophobic interactions and charge shielding effects (Kushner 1978; Oren 2002).

6. HALOARCHAEA, ANCIENT HALITE AND MICROBIAL LONGEVITY

It has been shown that haloarchaea probably become entrapped within fluid inclusions in halite crystals rather

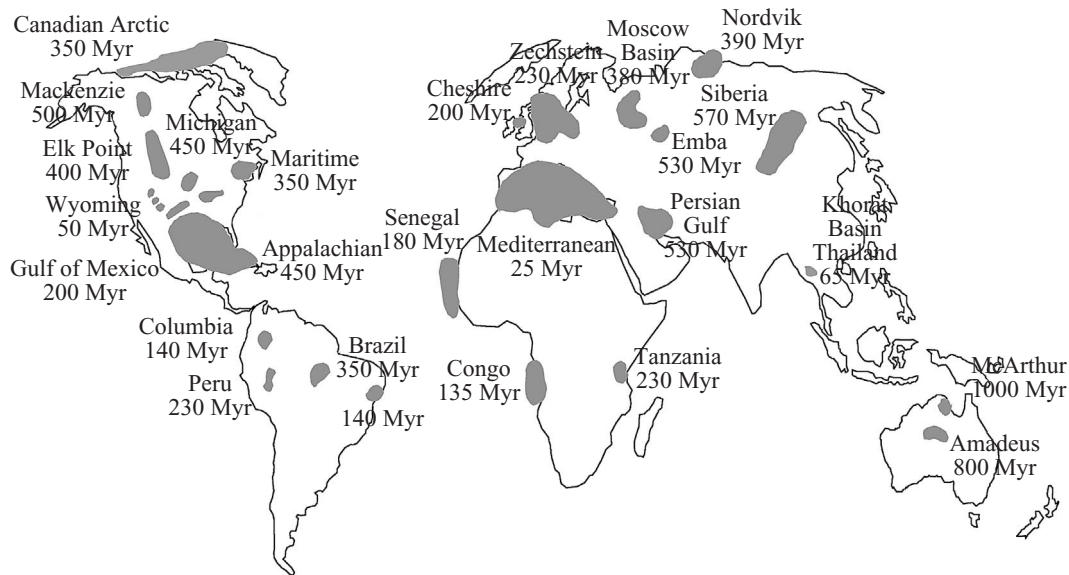


Figure 4. Location of ancient evaporite deposits on Earth. (Modified from Grant (2004).)

Table 6. Evaporite deposits and their geological ages. (Taken from Grant *et al.* (1998b).)

era	period/series	Myr ^a	evaporite deposit
Cenozoic	Holocene		present-day sabkhas, salinas, salt lakes, etc.
	Pleistocene	2	California; Nevada; Russia; Mexico; Israel
	Pliocene	5	Virgin Valley, Nevada; Utah; Italy; Jordan
	Miocene	23	Mediterranean; Red Sea; Trucial Coast (Arabian Gulf); Dominican Republic; Romania; Wieliczka, Poland
	Oligocene	36	France; Germany; Spain; Turkey; Iran; Iraq
	Eocene	53	Green River, Wyoming; Potwar, Pakistan
Mesozoic	Palaeocene	65	Khorat basin, Thailand
	Cretaceous	135	Gabon, Congo and Angola; Brazil; Colombia; Russia; Florida; Khorat Basin, Thailand; Morocco
	Jurassic	205	Montana; Bulgaria; Black Sea; Cuba; Chile; Idaho; Gulf Coast
Palaeozoic	Triassic	250	Cheshire Basin; Portugal; Spain; north and southwest France; The Netherlands; Germany; Switzerland; North Africa; Peru; Persian Gulf
	Permian	290	Zechstein Basin, northwest Europe; Permian Basin, Texas; Salado Formation, Mexico; Emba, Caspian Sea
	Carboniferous	355	Canadian Arctic Islands; Paradox Basin, southwest USA
	Devonian	410	Elk Point Basin, Canada; Mongolia; Moscow Basin
	Silurian	438	Salina Basin (New York, Ohio, West Virginia, Pennsylvania, Michigan; Ontario)
	Ordovician	510	Williston Basin, USA/Canada
	Cambrian	570	Mackenzie, Northwest Territories; Siberia; Iran (Persian Gulf); India; Australia
Proterozoic	Precambrian		Amadeus Basin, central Australia; McArthur Group, Queensland, Australia; Ontario, Canada; Iran

^a Geological age given in Myr BP (10^6 yr BP).

than within the halite crystal structure (Norton & Grant 1988). Fluid inclusions contain small pockets of brine (of similar concentration to bittern brine) and range in size from structures too small to incorporate cells, to large structures visible to the naked eye. Haloarchaea remain viable within halite crystals for considerable periods of time—one of the old ways of preserving haloarchaeal cultures was to dry these down in salt, which was then kept desiccated at room temperature. However, it is not clear how long the cells might remain viable under these

conditions, although cultures kept in this way have been recovered after more than 10 years. Could haloarchaea remain viable under these conditions for very long periods of time?

One way to examine possible long-term viability is to examine old halite deposits. Ancient halite deposits are widely distributed on Earth (figure 4), ranging in age from the Recent (some of the sediments from present-day salt lakes may be 10 000 years old) to Precambrian (table 6). These are the remains of ancient surface hypersaline sites

that presumably once supported dense populations of haloarchaea (or their ancestors). Halite, and occasionally thermonatrite ($\text{Na}_2\text{CO}_3 \cdot \text{H}_2\text{O}$) derived from soda lakes as in the Green River deposits in Wyoming, has dried out and undergone burial to depths of up to 1500 m over geological time periods. Many such sites are directly accessible because they are mined for rock salt. Alternatively, they may be indirectly accessible through solution mining, where water is pumped into the deposit and is then recovered at another borehole, where brines may be sampled for any microbial population. There is a long history of reports of viable micro-organisms, usually prokaryotes, being recovered from within supposedly ancient halite crystals (Grant *et al.* 1998b; McGenity *et al.* 2000), suggesting that the organisms themselves might be the same age, originally entrapped when the ancient brine dried down. Fluid inclusions containing material can often be seen in ancient halite. Although it is not possible to identify what the material might be, fluorescence under UV is consistent with organic origins. Some of the early work on ancient halite must be called into question since the understanding of halophily and growth conditions was much less well developed than today. Isolations were, for example, often made using media without salt, readily opening the way for the recovery of contaminants. More recent isolations have used media developed for halophilic micro-organisms, notably the haloarchaea, and in view of their obligate requirement for high salt for survival, these are unlikely candidates as present-day contaminants.

There is no doubt that ancient halite deposits support substantial populations of halophilic bacteria and, particularly, haloarchaea, although as late as 1981, Larsen described mined rock salts as microbe-free. This perception of salt mines as sterile environments is widespread and may have been one of the criteria for choosing such sites as repositories for nuclear and chemical waste. Salt mines, which access ancient halite beds, may contain primary rock salt (original chevron crystals), brine pools, brine lakes, plus efflorescences of freshly recrystallized salt as well as relatively dry layered halite. The brine pools and efflorescences harbour dense populations of haloarchaea and other halophilic prokaryotes (Norton *et al.* 1993). However, the evidence for the presence of viable organisms in primary rock salt is more controversial. Dombrowski (1963) and Reiser & Tasch (1960) were among the first to isolate organisms from rock salt. These authors used rigorous techniques to avoid contamination, and careful reading of these papers suggests that at least some of the isolates might have been haloarchaea. Bibo *et al.* (1983) were able to repeat this work, again using extensive precautions against surface contaminants. More recent isolations have tended to concentrate on haloarchaea. Unfortunately little is known about the transport of haloarchaea outside hypersaline environments. Wind-blown salt crystals might be a potential source of mine contamination, and Saharan sand that frequently blows over and deposits throughout mainland Britain is known to contain halite crystals (Wheeler 1985). These, however, have not been rigorously examined for the presence of halophiles.

More recent reports have recorded the presence of haloarchaea in Triassic and Permian deposits (summarized in McGenity *et al.* 2000; Stan-Lotter *et al.*

2004). These organisms are consistently isolated from surface-sterilized crystals obtained from freshly blasted rock salt, suggesting that these were survivors from the original depositional event. The scientific community is rightly sceptical about these claims of microbial longevity. The case for long-term suspended animation is almost always based on circumstantial evidence, including the following:

- (i) inaccessibility of the environments from which the isolate(s) originated;
- (ii) conditions that instigate against growth *in situ*, and any isolates differing significantly from those in the surrounding environments;
- (iii) appropriate precautions against contamination by present-day micro-organisms.

All three criteria are seldom met!

There are many arguments against the possibility of very long-term survival. Hydrolysis and oxidation of DNA occur in cells and if energy is not available for repair, then DNA will degrade over several thousand years, largely through depurination (Lindahl 1993). However, high salt concentration markedly reduces the rate of depurination (Lindahl 1993) and significantly protects RNA from heat inactivation (Tehei *et al.* 2002). Haloarchaea have an intracellular KCl concentration of 4–5 M and would be particularly at risk from ionizing radiation from ^{40}K , although calculations based on Sneath (1962) suggest that a lethal dose would not be generated even over at least 10^9 years (Grant *et al.* 1998b). Haloarchaea do not form resting stages like endospores, which are known to survive for some thousands of years in a profoundly dehydrated state, largely excluding oxygen (Nicholson *et al.* 2000). Haloarchaea may nevertheless be good candidates for long-term suspended animation, in that high levels of ions in the environment may generate conditions for macromolecules akin to dehydration, plus producing low oxygen concentration.

There may of course be actively growing populations in large bodies of original entrapped brine, comparable with those seen in recently developed brines in salt mines. If these organisms have been growing over geological time, then they must somehow be supplied with growth substrates. Slow movement of fluid inclusions through halite driven by thermal gradients can be demonstrated in laboratory experiments, and those could, in theory, replenish entrapped sites with nutrients from elsewhere, but there is no good evidence for movement of inclusions in salt deposits over hundreds of millions of years (Roedder 1984).

There are few good negative controls in studies of halite deposits. It would be expected that a deposit that had undergone extensive heating after burial would be sterile, and, indeed, brines from a highly deformed salt diapir that had been heated to at least 80 °C were found to be sterile (McGenity *et al.* 2000). However, a detailed study of highly transformed and heated salt deposits, in comparison with bedded and relatively unheated deposits has, to my knowledge, never been carried out.

One possible approach to proving that an isolate has been in suspended animation is to compare gene sequences between such isolates and contemporary descendants. Straightforward comparisons of 16S rRNA

genes of supposedly ancient isolates would be expected to position these closer to the root of a phylogenetic tree as compared with present-day isolates, which should be at the end of branches. Furthermore, the phylogenetic distance from the present-day relatives should correspond to the supposed geological age of the ancient isolates (assuming that any evolutionary clock calibration is correct). There have been a considerable number of haloarchaeal 16S rRNA gene sequence comparisons of this type and none of these has found consistent differences between ancient and modern types. McGenity *et al.* (2000) have summarized these results. In a recent paper Vreeland *et al.* (2000) isolated a halophilic *Bacillus* sp. by culturing brine from a fluid inclusion that was believed to be 250 Myr old, but again, the 16S rRNA gene sequence was virtually identical to a present-day organism from the Dead Sea. An alternative approach (Gemmell *et al.* 1998) exploits an unusual feature of one group of the haloarchaea, members of the genus *Haloarcula*, which possess at least two dissimilar 16S rRNA genes. Regardless of whether the gene multiplicity is a consequence of gene duplication or lateral transfer, one would expect differences in sequence between the dissimilar genes when comparing 'ancient' with contemporary isolates, because of sequence changes brought about by various genetic mechanisms as a consequence of growth. However, again, Gemmell *et al.* (1998) were unable to find any significant differences between 'ancient' and contemporary isolates.

Salt deposits are particularly prone to ingress of water, or of brines of different age from those around at the original precipitative event. Crystals identified as primary, may, in fact, be much younger, having been formed by a much more recent solubilization and recrystallization. Contemporary micro-organisms could then be incorporated into some areas of apparently ancient primary halite crystals. Fish *et al.* (2002) used laser ablation microprobe inductively coupled plasma mass spectrometry to analyse fluid inclusion brines and establish whether their composition was consistent with an original primary precipitative event. Small pieces of verified primary halite were then excised and, after surface cleaning and sterilization, subjected to DNA extraction. Fragments of 16S rRNA genes were detected by polymerase chain reaction amplification. In particular, a haloarchaeal sequence was detected in 11–16-Myr-old salt that matched that of haloarchaea that had repeatedly been isolated from Permo-Triassic salt. However, other samples, including a 425-Myr-old sample, failed to yield haloarchaeal amplicons and instead, bacterial amplicons related to Proteobacteria were detected. Terminal restriction fragment polymorphism analyses of 16S rRNA amplicons revealed quite complex patterns of prokaryotes in ancient halites of different ages. However, again, there were no significant sequence differences between supposedly ancient amplicons and those derived from contemporary relatives.

There have been repeated claims that the failure to find significant gene sequence differences between 'ancient' and 'modern' micro-organisms must be proof of contamination (Graur & Pupko 2001; Nickle *et al.* 2002). Assertions of contamination rely on the calibration of the molecular clock in prokaryotes based on endosymbionts in insects, which suggest that 16S rRNA gene sequence changes by ca. 1% for every 50 Myr (Ochman *et al.* 1999).

However, it is not clear whether the clock necessarily runs at the same rate in different groups. Generation times of environmental isolates are often months or even years (Parkes *et al.* 2000) and if the clock were to run 5–10 times more slowly than in endosymbionts, differences between 'ancient' and 'modern' organisms would fall in the range that has been recorded for the halite isolates (Nickle *et al.* 2002). What is not in doubt is that ancient halite does harbour substantial populations of halophiles, particularly haloarchaea, and salt does have significant protective properties for biological molecules, but the evidence for longevity still remains circumstantial.

Finally, there has been speculation that halite or other evaporites might be appropriate places for the long-term preservation of life on other planets (Litchfield 1998; Grant 2004). Evaporites have been detected in meteorites including Martian meteorites (Zolensky *et al.* 1999; Whitby *et al.* 2000; Stan-Lotter *et al.* 2004) and are probably present on Mars (Sims *et al.* 1997), and haloarchaea are known to survive a space environment (Mancinelli *et al.* 1998). If these halophilic prokaryotes really do remain viable for very long periods on Earth, it is not unreasonable to assume that they may exist in similar subterranean evaporites elsewhere in the solar system. Haloarchaea (or haloarchaeal remains) are arguably plausible targets for the new generation of spacecraft charged with the remote sensing of past or present life on other planets.

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Discussion

M. J. Danson (*Centre for Extremophile Research, Department of Biology and Biochemistry, University of Bath, Bath BA2 7AY, UK*). A major physiological difference between bacterial and archaeal halophiles is that bacteria solve the osmotic problem by synthesizing intracellular compatible solutes, whereas Archaea accumulate high levels of KCl. Are there any exceptions to this division?

W. D. Grant. The vast majority of bacteria osmoadapt by synthesizing or accumulating organic compatible solutes. However, there are a few that seem to have adopted the same ion-accumulating strategy as the haloarchaea, notably the extremely halophilic *Salinibacter ruber*, which coexists with haloarchaea in salterns. Anaerobic halophilic bacteria belonging to the *Haloanaerobiales* also seem to accumulate ions, and organic compatible solutes have not been detected in this group to date. As far as haloarchaea are concerned, the organic compatible solute 2-sulphotrehalose has been detected in a small number of haloalkaliphiles, but it is not clear if this is the main mechanism for osmoadaptation in this group since they also accumulate ions (KCl) like the rest of this group.

D. J. Scott (*National Centre for Macromolecular Hydrodynamics, University of Nottingham, Sutton Bonington, Leicestershire LE12 5RD, UK*). Given Watson Fuller's beautiful talk yesterday about DNA structure at low humidities, what are your thoughts on DNA structure in halophiles?

W. D. Grant. Interactions between haloarchaeal proteins and high levels of inorganic ions such as KCl have been extensively studied, whereas the interactions between the intracellular concentrations of KCl (4 M) and DNA remain completely unknown, particularly the structural configuration adopted. Analysis of the *Halobacterium salinarum* genome shows that its systems for DNA replication, transcription and translation resemble those of eukaryotes rather than prokaryotes and codon usage is what would be expected from a high G+C content organism. Exposure to different salt concentrations does result in differential transcription.

P. J. Halling (*Department of Chemistry, University of Strathclyde, Glasgow GL1 1XL, UK*). When water activity is too low, these organisms can no longer grow. Is anything known about what goes wrong with the physiology at the limit? What is the most sensitive part of the cell machinery?

W. D. Grant. It is not known if some systems are more affected by water activity than others. In the case of the haloarchaea, some enzymes require very high levels of inorganic ions, others much less so—here the effects are primarily a consequence of ion interaction rather than water activity *per se*. I would hazard a guess that membranes and membrane-associated systems must be particularly at risk from changes in water regime.

J. B. F. N. Engberts (*Physical Organic Chemistry Unit, University of Groningen, 9747 AG Groningen, The Netherlands*). At the end of your lecture, you have characterized the high-salt environments in terms of water activity (a_w ca. 0.7). Now, it is our experience that enzymic and organic reactions in cytosol-mimetic media correlate much better with water concentration than with water activity. What is in your opinion the best parameter for characterizing such thermodynamically non-ideal aqueous solutions?

W. D. Grant. The majority view would be that water content does not adequately describe the availability of water at an organismal level in the majority of environments, due to binding effects promoted by solutes and/or surfaces. However, at the subcellular level, it is possible that some measured biochemical parameters might correlate better with other ways of assessing water regimes. There are, however, additional complications if the system under study derives from an organism that osmoadapt using high levels of ions—here there may be specific ion effects as well as osmotic effects.

M. W. Ho (*Institute of Science in Society, PO Box 32097, London NW1 0XR, UK*). Is anything known concerning how these compatible solutes interact with proteins?

W. D. Grant. It is generally held that compatible solutes do not interact significantly with proteins by being largely excluded from protein hydration shells. While it is true that compatible solutes are strong water structure formers, it is known that the water molecules in the hydration shell of proteins are ordered differently to those in the bulk phase, and it is assumed that the compatible solutes interact preferentially with the bulk-phase water structures. Compatible solutes thus alter the osmotic potential of the free water within the cell and are 'compatible' with cell machinery by failure to interact strongly with water molecules bound to the surface of macromolecules.

H. J. C. Berendsen (*Laboratory of Biophysical Chemistry, University of Groningen, 9747 AG Groningen, The Netherlands*). In the early 1970s, we worked with Professor Ben-Zion and Dr Margareth Ginzburg of the University of Jerusalem on *Haloarcula marismortui*, a halophile that accumulates K^+ . It has been reported that this organism, under starved conditions, can take up K^+ in preference to Na^+ by a factor of at least 1000, without the expenditure of any metabolic energy. We studied intracellular water and D_2O by proton and deuteron magnetic resonance and found very broad resonances indicating greatly reduced rotational mobility of water. This work has only been

published in a thesis (H. T. Edzes) and broadening by paramagnetic ions could not be rigidly excluded. The possibility exists that at low water activity, the hydrated protein matrix in the cell preferentially binds K^+ over Na^+ . My question is: what is known about the mechanism of K^+ accumulation in halophiles?

W. D. Grant. Haloarchaea eject Na^+ via an electrogenic Na^+/H^+ antiporter, and the majority of these organisms

also pump Cl^- into the cell using the light-driven halorhodopsin ion pump. Accumulation of K^+ is thus in part passive driven by membrane potential and charge difference. However, there is also evidence for ATP-dependent accumulation in at least one haloarchaeon, and analysis of the *Halobacterium salinarum* genome shows the presence of multiple K^+ transport systems including an ATP-driven type.