Chapter 5
Microorganisms in Evaporites: Review of Modern Geomicrobiology

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Introduction

The "geomicrobiology" of evaporites—microorganisms and associated biomaterials preserved in saline minerals—has seen great progress over the past decade. There are many new reports of culturing archaea and bacteria (Stan-Lotter et al. 1999, 2002; Vreeland et al. 2000, 2007; Mormile et al. 2003; Gruber et al. 2004; Schubert et al. 2009b, 2010a; Gramain et al. 2011), sequencing prokaryote DNA (Radax et al. 2001; Fish et al. 2002; Park et al. 2009; Panieri et al. 2010; Gramain et al. 2011), and identifying organic compounds such as beta carotene and cellulose (Griffith et al. 2008; Schubert et al. 2010b; Lowenstein et al. 2011) from ancient samples of halite (NaCl) and gypsum ($CaSO_4 \cdot 2H_2O$). Tiny droplets of brine trapped within evaporite minerals, called fluid or brine inclusions, seem to be an important, but not exclusive, haven for microbes and biomaterials in buried evaporites. Given the expanded interest in microbial life in evaporites, and the potential implications regarding the search for life in the solar system, it seemed worthwhile to summarize the most important findings in the geomicrobiology of evaporites. The last such summary of advances in the geomicrobiology of ancient evaporites was by Vreeland and Powers (1999), so the focus here is on the last 10 years.

Five important aspects for geomicrobiologists studying ancient evaporites form the core of this review.

1. The timing of formation of the samples studied, whether "syndepositional" and formed at the time of deposition, or soon after deposition, by processes controlled by the contemporary surface environment, or "burial" and formed by later processes that existed in the subsurface burial environment (Hardie et al. 1985).

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R. H. Vreeland (ed.), *Advances in Understanding the Biology of Halophilic Microorganisms,* 117
DOI 10.1007/978-94-007-5539-0_5,

The syndepositional versus burial origin of an evaporite deposit in its present state should be known before any studies of biological materials are undertaken because without definitive information on the timing of formation of the samples under consideration, little can be conclusively said about the age of any microorganisms and other biomaterials discovered.

2. Most evaporite deposits formed from the evaporation of ancient seawater. Analysis of the chemical composition of fluid inclusions in ancient marine halites over the past 10 years has shown that there have been secular changes in the major ion chemistry of seawater during the Phanerozoic Eon, the past 542 million years (Lowenstein et al. 2001; Horita et al. 2002). These changes occurred slowly over periods of millions of years and most notably involved the ions Ca^{2+}, Mg^{2+}, and SO_4^{2-}, which in turn, impacted the development and evolution of $CaCO_3$ shell building organisms (Stanley and Hardie 1998). It is not known how such variations in the major ion chemistry of seawater influenced halophilic microorganisms living in concentrated marine brines.
3. Microthermometric techniques used on primary fluid inclusions in halite can document the water temperatures at which the halite originally crystallized (Roberts and Spencer 1995; Lowenstein et al. 1998, 1999; Benison and Goldstein 1999; Satterfield et al. 2005a, b). Such information is a quantitative record of the surface water temperatures at which microorganisms were trapped in fluid inclusions, and has potential significance for paleoenvironmental interpretations and for designing cultivation experiments.
4. Geomicrobiological studies of ancient evaporites have seen important advances using *in situ* light microscopy (Benison et al. 2008; Panieri et al. 2008; Schubert et al. 2009a, b, 2010b), *in situ* Raman spectroscopy (Fendrihan et al. 2009), scanning electron microscopy, and transmission electron microscopy (Griffith et al. 2008). Such studies, in particular *in situ* microscopy, help establish authenticity of microbial materials trapped in evaporite minerals and fluid inclusions.
5. Culturing studies have become more sophisticated, using new methods, improved surface sterilization techniques, and reproduction of laboratory results. Beginning in 2001, ancient DNA from halite and gypsum has been extracted, purified, amplified, and sequenced (Radax et al. 2001; Fish et al. 2002; Park et al. 2009; Panieri et al. 2010; Gramain et al. 2011).

Sedimentology and Microscopy of Evaporites and Fluid Inclusions: Syndepositional (Primary) Versus Burial (Secondary) Origin and Interpretation of Paleoenvironments

Evaporites are salt deposits that form from the evaporation of water at the Earth's surface in marine and inland lake settings with arid climates and no drainage out of the basin. Modern environments of evaporite deposition include coastal lagoons,

such as Santa Pola, Spain; inland saline lakes such as the Dead Sea, Israel and Jordan; and desiccated saline pans such as Death Valley, California. There is a spectrum of environments, from permanent density-stratified deep lakes (i.e., the Dead Sea), to shallower perennial lakes (i.e., Great Salt Lake), to ephemeral lakes (i.e., Death Valley), that may form standing bodies of water for years to days. Some evaporite environments, almost always dry, contain thick surface salt crusts and shallow groundwaters normally less than one meter below the surface.

Samples of halite used to study microorganisms and ancient DNA have so far come from borehole cores and from underground mine outcrops. Gypsum is much less soluble than halite and therefore samples for geomicrobiological studies have come from surface outcrops (Panieri et al. 2008, 2010). The ages of these halites and gypsums vary from Pleistocene, tens of thousands of years old, to Silurian, greater than 400 million years in age. The depths from which core and mine samples were obtained range from meters to hundreds of meters. For all these samples, before beginning microbiological studies, it is important to distinguish the minerals, textures, structures, and fluid inclusions of sedimentary syndepositional origin from those formed from burial alteration processes. These features are easily observed in large (5 × 7.5 cm) thin sections, which may be prepared without heating or dissolving samples, preferably using a diamond wire saw (Lowenstein and Brennan 2001).

Discussion of the syndepositional versus burial origin will be limited to halite and gypsum because they are the most common evaporite minerals and the only ones that have been used for geomicrobiological studies to date. Syndepositional evaporites that formed at or soon after the time of deposition, should be the focus for geomicrobiological studies because they are expected to be the richest source of living prokaryotes and associated microorganisms and biomaterials. Such syndepositional evaporites, now buried, contain biomass that was originally trapped at or near the Earth's surface. Fortunately, the syndepositional versus burial origin of evaporites, and specific surface environments of deposition can be evaluated and interpreted through sedimentologic and microscopic studies. How are syndepositional features recognized? Detailed information on the analysis of syndepositional sedimentary features in gypsum and halite is described in Hardie et al. (1985), Smoot and Lowenstein (1991), and Lowenstein and Brennan (2001). Diagnostic sedimentary structures common in gypsum and halite include layering on the millimeter to meter scale defined by textural and mineralogical variations (Fig. 5.1). Repetitious interlayering of clay or carbonate mud with gypsum or halite is common. Evaporite layers may form cross lamination and cross stratification structures which are the grains making up ripples and dunes formed by movement of grains by water currents, waves, and air (Fig. 5.2). Detrital framework textures, the settle out layers of halite crystal hoppers, cubes, and rafts, and gypsum plates, that all precipitated at the air-water interface, are widely recognized in modern and ancient evaporites (Figs. 5.3 and 5.4). Such detrital accumulations of halite and gypsum form well sorted layers of loosely packed crystals, which can later be reworked into ripples.

Fig. 5.1 Slab sample of halite from the F-salt, Silurian Salina Salt, Michigan Basin (408.5–411 million years old), showing halite beds (light) and dark millimeter-thick laminae of anhydrite (*arrows*). Such layering is common in evaporites with well-preserved syndepositional features. Scale at bottom is in centimeters. (Modified from Satterfield et al. (2005b))

Crystalline framework crusts of halite and gypsum, formed at the brine bottom by in place growth into the water column, are common in modern and ancient evaporites. These crusts are made of vertically oriented, upward widening and elongated crystals that grew competitively off a common substrate at the brine bottom (Fig. 5.4 and 5.7). Such frameworks are of great significance because the crystals in these layers are typically large, centimeters in size, with relatively abundant and large fluid inclusions. These samples have therefore been the focus of recent geomicrobiological studies because it is relatively easy to visualize fluid inclusions and microorganisms in the crystalline frameworks using *in situ* microscopy. The large crystals also simplify procedures for surface sterilization and for drilling and extracting brine from individual fluid inclusions (Mormile et al. 2003; Vreeland et al. 2007; Schubert et al. 2009a, b, 2010a, b; Panieri et al. 2008, 2010).

Fig. 5.2 Ripple marks preserved in modern halite crust, Dabusun Lake, Qaidam Basin, China; Swiss army knife for scale. The ripple marks record reworking of halite crystals by waves along a lake shoreline. *Arrow* points to ripple crest and shows direction of wave approach. Inset shows rippled bedding surface in anhydrite-polyhalite rock from the Permian Salado Formation, New Mexico, (~250 million years old) with pencil for scale

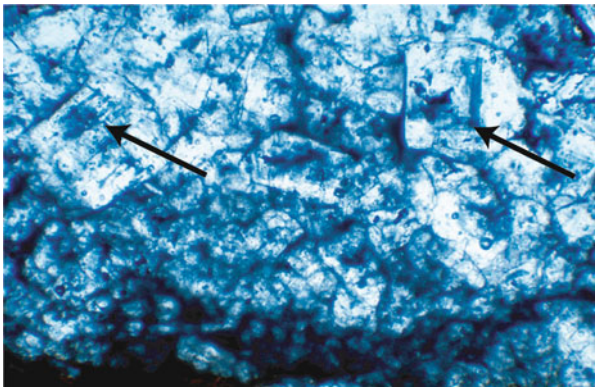

Fig. 5.3 Thin section photograph of detrital halite cubes (viewed perpendicular to layering), which precipitated at the air-water interface and settled to the brine bottom. Dark patches and bands in the cores of halite cubes are arrays of fluid inclusions (*arrows*). Dark material between halite crystals is polyhalite. Sample from Permian Salado Formation, New Mexico. Horizontal field of view is 7 mm

Layered gypsum and halite deposits may have syndepositional dissolution textures from contact with undersaturated waters. These features may be preserved as rounded dissolution cavities, truncated crystal surfaces, and vertical dissolution pipes (Figs. 5.5 and 5.6). They are important because they indicate contact with undersaturated waters, which is most likely to occur in a shallow lake, lagoon, or salt pan setting, and not in a deep brine pool. Deep saline lakes and marine saline basins are stratified and contain dense brine bodies that separate dilute undersaturated

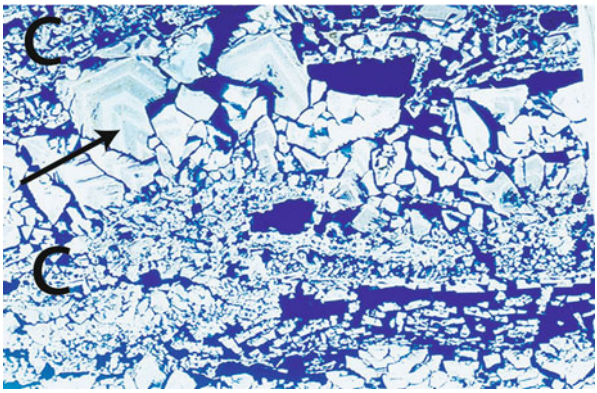

Fig. 5.4 Thin section photograph of modern halite crust from Salina Omotepec, Baja California, Mexico (viewed perpendicular to layering). Note layer of vertically-oriented, fluid inclusion banded "chevrons" in upper half (*arrow*). Smaller crystals below and above are layers made of sunken detrital rafts and cubes of halite (C). Open pore spaces are dark blue. Horizontal field of view is 5.5 cm. (Modified from Lowenstein and Hardie (1985))

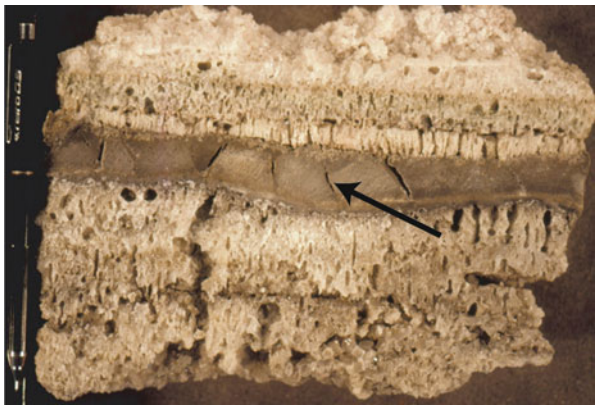

Fig. 5.5 Slabbed hand sample of modern saline pan halite from Saline Valley, California. Note the large number of vertical voids formed by dissolution of the halite crust when the saline pan is flooded. Dark mud layer in middle (*arrow*) was deposited during a flood. Sample is 10 cm thick. (Modified from Casas and Lowenstein (1989))

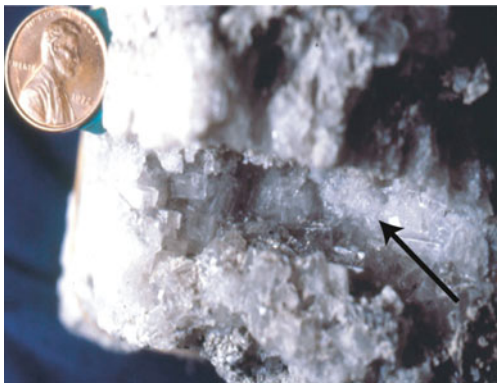

Fig. 5.6 Hand sample of modern halite crust from Salina Omotepec, Baja California, Mexico, from just below the surface. Large dissolution cavity (*arrow*) is lined with halite cement crystals that have grown in the cavity. Coin is 20 mm in diameter. (Modified from Lowenstein and Hardie (1985))

inflow waters from the evaporites accumulated at the brine bottom. Therefore the preservation potential of evaporite deposits formed in deep perennial settings is greater than in shallow and ephemeral systems. Dissolution features in gypsum and halite are diagnostic of very shallow water and ephemeral environments of deposition and contrast with the "pristine" unaltered deposits that commonly form in deeper water, density stratified settings. Syndepositional dissolution features found in shallow water and ephemeral deposits preserve important paleoenvironmental information.

Gypsum and halite may form "diagenetically" in the subsurface by crystallization from saline groundwaters as displacive crystals, commonly millimeter- to centimeter-sized single crystals or as nodular aggregates composed of sub-millimeter sized crystals. They may also form mineral cements that occur as cavity fillings or crystal overgrowths (Fig. 5.6). Such diagenetic cements and displacive crystals are difficult to interpret in terms of their timing of formation because they can form in either syndepositional or burial environments (Hardie et al. 1985). For example, the large halite cement crystal studied by Vreeland et al. (2000) from which a Permian bacterium was cultured, is difficult to interpret, in terms of the timing of its formation, from its texture alone. It took study of fluid inclusions from these halite cements to prove that they formed syndepositionally, early in the diagenetic history, from evaporated Permian seawater (Satterfield et al. 2005a).

Finally, because of the ease with which evaporites may be altered, one should always be on the lookout for burial diagenetic alteration features that deform, disrupt or destroy the original sedimentary features. If evaporites have sutured interpenetrating crystalline textures, recrystallized polygonal mosaics, and deformation features (folds, faults, etc.), described more fully in Hardie et al. (1985), they have been modified during burial. Such samples should not be used in geomicrobiological studies.

Fluid Inclusions in Halite and Gypsum

Fluid inclusions are cavities within crystals filled with fluid, normally water. They may also contain other liquid (i.e., hydrocarbon), vapor (i.e., CO_2 and H_2S), and a variety of solids including minerals, organic material and of course, microbes (Schubert et al. 2009a). Fluid inclusions trapped during crystal growth are called primary inclusions. Crystal imperfections and irregularities that form during crystal growth may be enclosed by the growing crystal to become fluid inclusions. It is important to note that primary fluid inclusions can form during crystal growth in either surface or burial environments. Therefore, sedimentologic and microscopic examination, outlined above, should first be conducted to determine the syndepositional versus burial origin of the deposit under consideration. Details on fluid inclusion microscopy are found in Roedder (1984), Goldstein and Reynolds (1994), Lowenstein and Brennan (2001), Schubert et al. (2009a, 2010b), and Lowenstein et al. (2011).

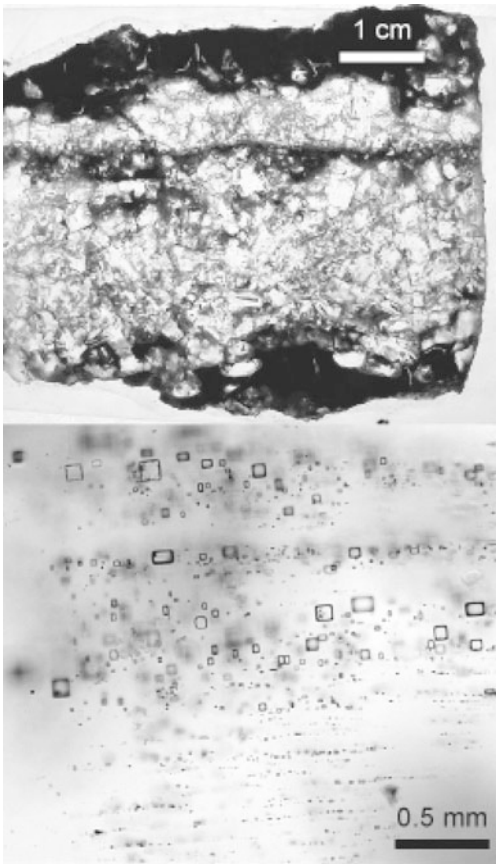

Fig. 5.7 Photomicrograph of halite in thin section from the Death Valley core, depth of 14.1 m (age of 25,000 years). *Top:* Interlayered halite (crusts of vertically oriented halite crystals grown on the bottom of an ancient lake) and dark mud (viewed perpendicular to layering). *Bottom:* Close-up of primary fluid inclusions in halite crystal, showing bands rich and poor in fluid inclusions. This sample yielded a positive culture in the genus *Natronomonas*. (Modified from Schubert et al. (2010a))

Secondary fluid inclusions form later, by healing of fluid-filled microfractures (Roedder 1984; Goldstein and Reynolds 1994) and are to be avoided in most geomicrobiological studies. First, the ages of the fluids trapped in secondary fluid inclusions are not known with certainty except that they are younger than the host mineral. In addition, secondary fluid inclusions may be related to fluids associated with burial and deformation processes, not the concern of most geomicrobiological studies if the primary aim is the isolation and study of surface microbial communities. But secondary fluid inclusions may be of interest in studies seeking to understand the activities and identification of subsurface microbes.

Fluid inclusions in halite are quite common and have been studied by geologists for decades (Roedder 1984; Hardie et al. 1985; Lowenstein and Hardie 1985; Lowenstein and Spencer 1990; Goldstein and Reynolds 1994; Roberts and Spencer 1995; Kovalevych et al. 1998; Benison and Goldstein 1999; Lowenstein and Brennan 2001; Schubert et al. 2009a, b, 2010b; Lowenstein et al. 2011). Primary fluid inclusions, composed of halite saturated brine, occur in halite crusts in which crystals grew at the bottom of the brine body as "chevrons" and vertically oriented crystals (Lowenstein and Hardie 1985) (Figs. 5.4, 5.7 and 5.8). They also occur in halite

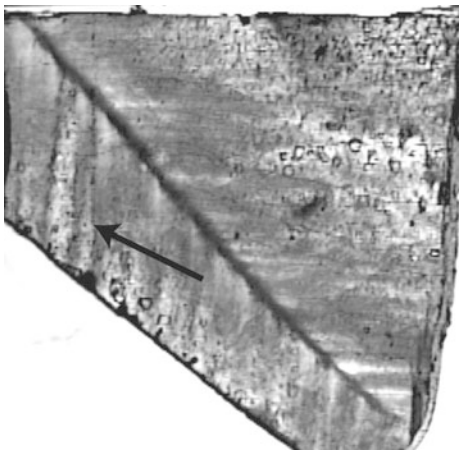

Fig. 5.8 Photograph of Cretaceous (112–121 million years old) chevron halite crystal with well defined primary fluid inclusion bands (dark, *arrow*) that formed parallel to crystal growth faces. Crystals like this yielded live halophilic *Archaea* (Vreeland et al. 2007). Crystal is approximately 5 mm in size

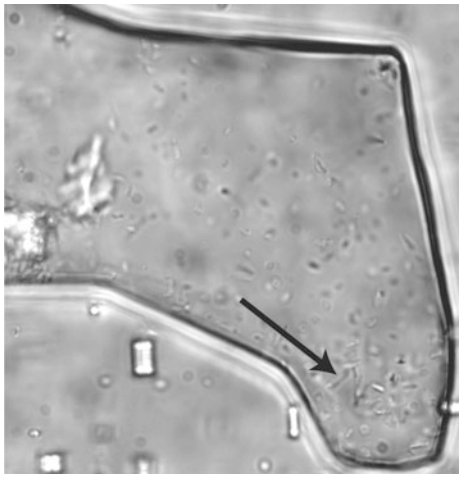

Fig. 5.9 Photomicrograph of a large, irregularly-shaped fluid inclusion in halite crystallized in Saline Valley, California, in March, 2004. Note the large number of prokaryote cells (rod and coccoid shapes, *arrow*) within the brine inclusion. Width of inclusion is ~100 μm

crystal plates, rafts and cubes that grew at the air water interface and sank down to the brine bottom to form "cumulate" crystal layers (5.3 and 5.4). Fluid inclusions in halite can be quite abundant, with as many as 10^{10} cm^{-3} (Roedder 1984). Fluid inclusions commonly occur in zones parallel to crystal growth faces (Benison and Goldstein 1999; Lowenstein and Brennan 2001) (Figs. 5.4, 5.7 and 5.8). Such fluid inclusion zonation results from variations in the rate of crystal growth, which in turn, controls the amount of ambient fluid trapped. Faster growing crystals trap more fluid inclusions, resulting in inclusion rich zones, whereas halite crystals that grow slowly have fewer fluid inclusions.

Primary fluid inclusions in halite are aqueous, negative cubes, rectangular prisms, and irregular shapes, including tubes, from <1 μm to several millimeters in size (Figs. 5.7 and 5.9). Fluid inclusions in halite are normally single phase brines because that is the medium in which they grew, but they may also contain solids and vapor.

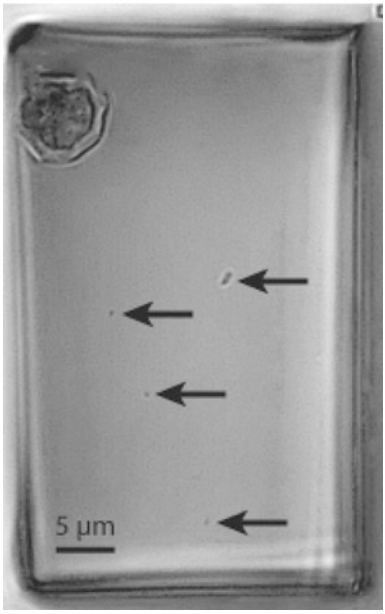

Fig. 5.10 Photomicrograph of probable *Dunaliella* cell and prokaryote cells (*arrows*) in a fluid inclusion from the Death Valley core, depth of 8.7 m (age 12,000 years). (Modified from Schubert et al. (2010b))

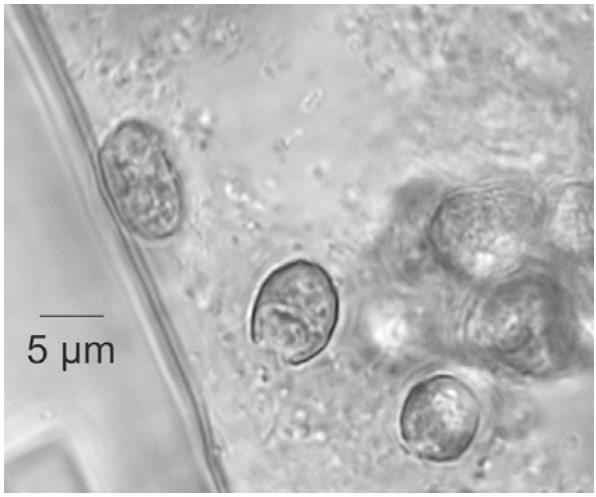

Fig. 5.11 Photomicrograph of a portion of a large fluid inclusion in halite, crystallized in Saline Valley, California in 2004, with numerous, small prokaryote cells and larger spherical and ellipsoidal cells of *Dunaliella*. (Modified from Lowenstein et al. (2011))

Minerals, organic materials, and microorganisms, including prokaryotes and algae, have all been observed within fluid inclusions in halite (Schubert et al. 2009a, 2009b, 2010b; Lowenstein et al. 2011) (Figs. 5.9, 5.10, and 5.11). It has been assumed that microorganisms living in the water column are passively trapped inside fluid inclusions during halite crystallization, but experiments documenting the modes and mechanisms by which microorganisms are trapped in fluid inclusions have not yet been done.

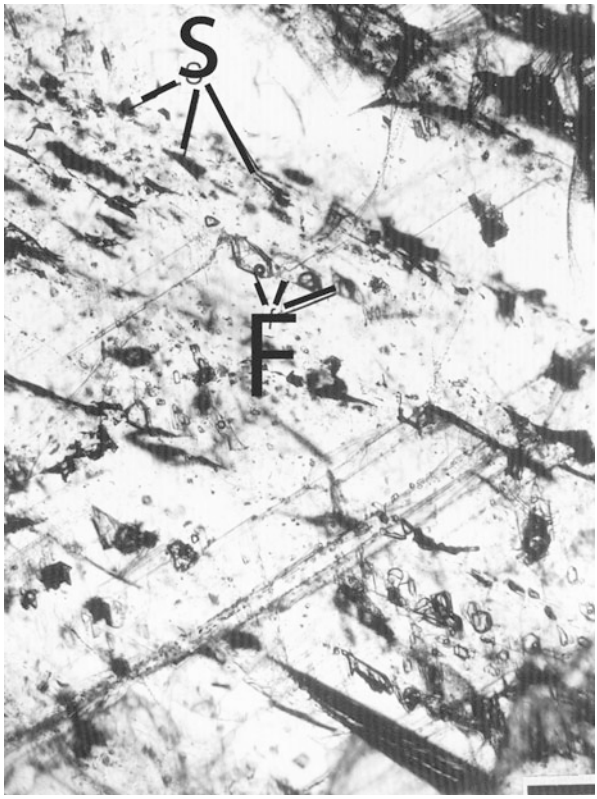

Fig. 5.12 Photomicrograph of fluid inclusions in gypsum crystal, Middle Miocene (~11–16 million years old), Gulf of Suez, Egypt. Solid (S) and fluid inclusions (F, with liquid water and vapor bubbles) occur in planes parallel to the growth direction of the gypsum crystal. Vapor bubbles, not present in original samples, were produced in the laboratory after freezing and melting experiments. Scale bar is 60 μm. (Modified from Attia et al. (1995))

Fluid inclusions in primary gypsum have not been studied as much as in halite but are reported by Sabouraud-Rosset (1969, 1972, 1974, 1976), Attia et al. (1995), and Petrichenko et al. (1997). Primary fluid inclusions in gypsum, as in halite, are normally single phase, aqueous, and arranged in alignment with the growth direction of the gypsum crystal (Figs. 5.12 and 5.13). Primary aqueous inclusions in gypsum are <1 μm to several millimeters in size. They are typically smaller than those found in halite and have a variety of shapes including negative crystals and triangular, pentagonal, or horn-shaped inclusions in two dimensions (Attia et al. 1995). The largest and easiest inclusions to visualize in gypsum occur along crystal growth bands in primary bottom growth crusts, such as those shown in Figs. 5.12 and 5.13. Although detailed studies are lacking, solid minerals, organic matter, and microorganisms (prokaryotes including cyanobacteria and charophytes) have been observed in fluid inclusions in ancient gypsum deposits (Attia et al. 1995; Petrichenko et al. 1997). Secondary fluid inclusions in gypsum are common; they are tabular-shaped, single phase and several tens of microns in size (Fig. 5.13c) (Attia et al. 1995).

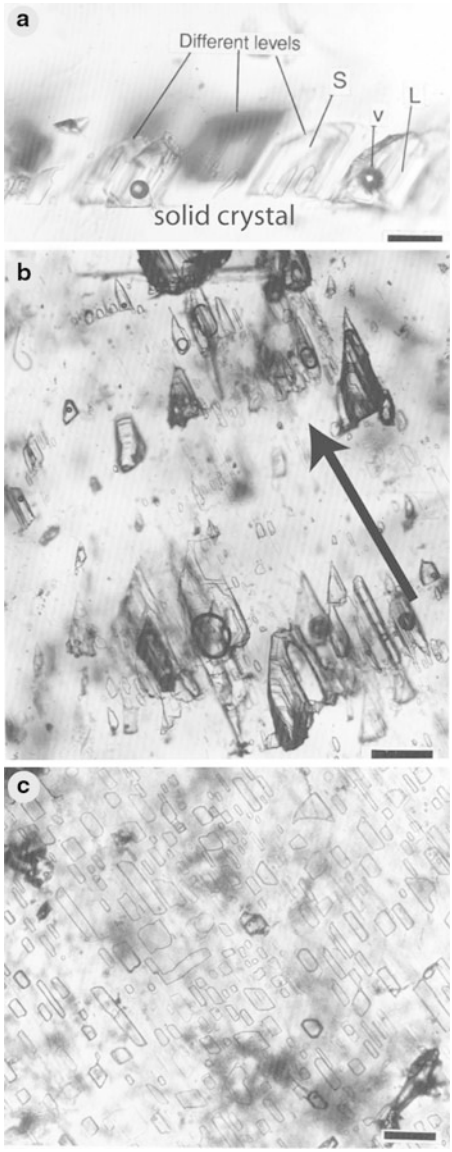

Fig. 5.13 Photomicrographs of fluid inclusions in gypsum crystals, Middle Miocene (~11–16 million years old), Gulf of Suez, Egypt. (Modified from Attia et al. (1995)). **a** Primary fluid inclusions (aqueous, single phase, S, and liquid-vapor, L-V) formed along a common surface. Scale bar is 20 μm. Vapor bubbles, not present in original samples, were produced in the laboratory after freezing and melting experiments. **b** Primary fluid inclusions aligned in rows parallel to the growth direction of the gypsum crystal (*arrow*). Scale bar is 20 μm. **c** Plane of secondary tabular aqueous inclusions along a cleavage plane. Scale bar is 30 μm

Brine Evolution and Secular Variations in the Major Ion Chemistry of Seawater

Chemical species dissolved in seawater or nonmarine waters on Earth include the major ions Na^+, Ca^{2+}, Mg^{2+}, K^+, SO_4^{2-}, Cl^-, HCO_3^-, and CO_3^{2-}, and minor to trace amounts of various other species including Li^+, Sr^{2+}, and Ba^{2+}. The

major ions in natural waters are concentrated during evaporation until the waters become supersaturated with particular minerals. The types of saline minerals found in evaporite deposits are dependent upon the chemical composition of the parent brines, which in turn, depends upon the chemistry of inflow waters, and the mechanisms by which these waters become brines. The salts formed during evaporative concentration of natural waters at the Earth's surface precipitate in order of increasing solubility. Typically, relatively insoluble calcite ($CaCO_3$) crystallizes first, followed by gypsum ($CaSO_4 \cdot 2H_2O$) and then halite (NaCl). The "bittern salts" composed of K and Mg sulfates (for example polyhalite [$K_2SO_4 \cdot 2CaSO_4 \cdot MgSO_4 \cdot 2H_2O$], kieserite [$MgSO_4 \cdot H_2O$], kainite [$KCl \cdot MgSO_4 \cdot 3H_2O$] and chlorides (sylvite [KCl], carnallite [$KCl \cdot MgCl_2 \cdot 6H_2O$]) form last, when waters become superconcentrated. These late stage bittern or potash salts are unusual because evaporative concentration of natural brines to this degree is rare. Other evaporite minerals include anhydrite ($CaSO_4$) which forms from the dehydration of gypsum, Na-sulfates (mirabilite [$Na_2SO_4 \cdot 10H_2O$], thenardite [Na_2SO_4]), Na-carbonates (trona [$NaHCO_3 \cdot Na_2CO_3 \cdot 2H_2O$], nahcolite [$NaHCO_3$], shortite [$2CaCO_3 \cdot Na_2CO_3$]), and Ca-chlorides (tachyhydrite [$CaCl_2 \cdot 2MgCl_2 \cdot 12H_2O$], antarcticite [$CaCl_2 \cdot 6H_2O$]).

The guiding principle of "chemical divides" is usefully applied to the study of evaporite brines (Hardie and Eugster 1970; Eugster and Hardie 1978; Jones and Deocampo 2004). This concept greatly simplifies understanding the mechanisms by which natural waters evolve during evaporative concentration and mineral precipitation. When brines evaporate, they lose only water and all the dissolved species increase in concentration proportionally. But when minerals precipitate, they form from dissolved species in the brine and thus change the chemistry of the evolving brine. Precipitation of the early, insoluble minerals, such as calcite and gypsum, is important for determining the later brine evolution pathways. For calcite, for example, one mole of Ca^{2+} and one mole of ${CO_3}^{2-}$ are lost from the water for every mole of $CaCO_3$ formed. The equivalents (moles of charge) of Ca^{2+} versus ${CO_3}^{2-} + {HCO_3}^{-}$ in the water at calcite saturation determine whether Ca^{2+} or ${CO_3}^{2-} + {HCO_3}^{-}$ is depleted in the remaining water during precipitation of calcite. If the water has $Ca^{2+} > {CO_3}^{2-} + {HCO_3}^{-}$, for example, seawater, it becomes depleted in ${CO_3}^{2-} + {HCO_3}^{-}$ and enriched in Ca^{2+}, following precipitation of alkaline earth carbonate. If $Ca^{2+} < {CO_3}^{2-} + {HCO_3}^{-}$, then the evolving water will become Ca-depleted and alkaline, enriched in ${CO_3}^{2-} + {HCO_3}^{-}$, such as Mono Lake, California and Lake Bogoria, Kenya, following carbonate mineral precipitation. In the same way, the equivalents of Ca^{2+} and ${SO_4}^{2-}$ in the evaporating water at gypsum saturation determines whether the remaining brine will be enriched or depleted in Ca^{2+} and ${SO_4}^{2-}$ after gypsum precipitates. Seawater and Great Salt Lake waters have ${SO_4}^{2-} > Ca^{2+}$, so they become sulfate-rich, Ca^{2+}-poor brines following gypsum formation, whereas the Dead Sea, with $Ca^{2+} > {SO_4}^{2-}$, becomes depleted in ${SO_4}^{2-}$ after gypsum forms.

The variety of natural waters at the Earth's surface can lead to the formation of many types of brines, but the principle of chemical divides permits easy classification into distinctive groups. Inflow waters with $Ca^{2+} < {CO_3}^{2-} + {HCO_3}^{-}$ precipitate alkaline earth carbonate and evolve into alkaline Na-K-HCO_3-CO_3-SO_4-Cl rich brines from which trona, halite, mirabilite and thenardite may precipitate. Such brines are

found in Mono Lake and Owens Lake California, and Lakes Magadi and Bogoria, Kenya, although sulfate is lost from some of these brines via sulfate reduction. If the inflow waters have $Ca^{2+} > CO_3^{2-} + HCO_3^-$, then Ca^{2+}-rich, carbonate-poor brines form after carbonate mineral precipitation. The resulting brines are Ca-Na-K-Mg-SO_4-Cl-rich. Then, depending on the amount of Ca^{2+} versus SO_4^{2-} in the brine at the point of gypsum precipitation, Ca-Na-K-Mg-Cl-rich brines (Dead Sea, Bristol Dry Lake, California, Qaidam Basin, China) or Na-K-Mg-SO_4-Cl-rich brines (seawater, Great Salt Lake, Death Valley) form.

Seawater, of course, is the most abundant evaporite parent water on Earth and giant marine evaporite deposits are common in the geologic record. As noted previously, it is now known from study of fluid inclusions in halite that the major ion chemistry of seawater has varied over the Phanerozoic Eon (Lowenstein et al. 2001; Horita et al. 2002), in phase with changes in sea floor spreading rates, global volcanism and global sea level. Seawater had high Mg^{2+}/Ca^{2+} and relatively high SO_4^{2-} during the Permian (299–251 Ma), Triassic (251–199.6 Ma) and much of the Cenozoic Era, from 0 to 40 million years ago. In contrast, seawater had low Mg^{2+}/Ca^{2+} ratios and relatively high Ca^{2+} and low SO_4^{2-} concentrations during the Cambrian (542–488 Ma), Silurian (444–416 Ma), Devonian (416–359 Ma), Jurassic (199.6–145.5 Ma) and Cretaceous (145.5–65.5 Ma) periods. Seawater has always had $Ca^{2+} > HCO_3^- + CO_3^{2-}$, except perhaps during the earliest history of Earth, but changes in the amount of Ca^{2+} versus SO_4^{2-} have had a major impact on brine evolution and the formation of marine evaporites. During those times when $Ca^{2+} > SO_4^{2-}$ at the point of gypsum saturation (Cambrian, Silurian, Devonian, Jurassic and Cretaceous), seawater evolved into a Ca^{2+}-rich, SO_4^{2-}-poor brine during evaporative concentration. Marine evaporites from these periods lack $MgSO_4$ salts and contain late stage K-, Mg-, and Ca-chloride salts such as sylvite, carnallite, and tachyhydrite (Lowenstein et al. 2001). When $Ca^{2+} < SO_4^{2-}$ at the point of gypsum saturation, seawater evolved into a SO_4^{2-}-rich brine, as occurred during the Permian, Triassic, and much of the Cenozoic Era. Evaporite deposits of those ages contain $MgSO_4$ salts such as polyhalite, kainite, and kieserite. Such changes in seawater chemistry, now well documented, have had a major impact on the evolution of shell building organisms (Stanley and Hardie 1998), but little is known about the impact of secular variations in seawater chemistry on halotolerant and halophilic marine microbial communities. The detailed changes in seawater chemistry of different ages can be found in Lowenstein et al. (2001, 2005), Horita et al. (2002), Brennan et al. (2004), Satterfield et al. (2005a, b), and Timofeeff et al. (2006), which may be a useful guide for media preparation when attempting to culture halophilic microorganisms from marine evaporites.

Knowledge that Permian seawater differed chemically from modern seawater, with respect to Mg^{2+} and SO_4^{2-}, for example, helped demonstrate the Permian, 250 million-year-old age of the fluid inclusions from which Vreeland et al. (2000) cultured the bacterium *Virgibacillus* sp. 2-9-3 (Satterfield et al. 2005a). In that study, fluid inclusions in halite cement crystals from the Permian Salado salts, Waste Isolation Pilot Plant in New Mexico, were chemically analyzed for Na^+, Ca^{2+}, Mg^{2+}, K^+, SO_4^{2-}, and Cl^-. It was found that the Permian fluid inclusions have lower

SO_4^{2-} concentrations than modern seawater, but very similar concentrations to fluid inclusions in other Permian halites (Satterfield et al. 2005a), which suggests a Permian age of the fluid inclusion waters. In addition, the Salado fluid inclusions are different in chemical composition from modern potash mine brines and mine weeps in the Salado salts, which demonstrates that the halite cement crystals that housed the bacterium did not precipitate from modern brines in the Salado salts released by fracturing and deformation associated with mining operations. Fluid inclusions have thus helped show that evaporite crystals have retained brines for periods of hundreds of millions of years.

Fluid Inclusion Microthermetry: Paleobrine Temperatures

Fluid inclusions in halite can be used to establish the temperatures of the waters in which the crystals grew. The method, called microthermometry, uses the homogenization temperature of fluid inclusions to infer ancient brine temperatures. Primary single-phase aqueous inclusions in halite at room temperature are required as the starting material. Halite crystals with these inclusions are then cooled in a laboratory freezer or on a fluid inclusion heating-freezing stage in order to nucleate a vapor bubble. The vapor bubble (water vapor at very low pressure) forms because of the volume decrease of the inclusion water that occurs during cooling, which is much greater than the volume change of the solid halite host crystal. Once vapor bubbles are nucleated in fluid inclusions, halite crystals are transferred to a heating-freezing stage mounted to a transmitted light microscope. Crystals and incorporated fluid inclusions are then slowly heated while being observed under the microscope. With warming, the volume of the water in inclusions increases and the vapor bubbles shrink. At some point, called the homogenization temperature, the vapor bubble disappears completely. The homogenization temperature, if from a primary fluid inclusion, is a record of the water temperature at which the crystal originally grew. This information, actual measurements of the water temperatures at which crystals grew and fluid inclusions were trapped, has been used for paleoclimate studies because there is a direct relationship between water temperatures, air temperatures and climate (Roberts and Spencer 1995; Lowenstein et al. 1998, 1999; Benison and Goldstein 1999; Satterfield et al. 2005a, b). Homogenization temperatures can also guide the design of conditions used for culturing ancient microorganisms trapped inside halite.

The Importance of Microscopy

Geomicrobiological studies of ancient evaporites (halite and gypsum) have seen important advances in the last 10 years using *in situ* light microscopy (Mormile et al. 2003; Fendrihan and Stan-Lotter 2004; Adamski et al. 2006; Fendrihan et al.

2006; Benison et al. 2008; Panieri et al. 2008; Schubert et al. 2009a, b, 2010a, b; Lowenstein et al. 2011). A report by Griffith et al. (2008) used transmission electron microscopy to identify cellulose fibers that were obtained from fluid inclusions and solid crystals of the Permian Salado halite of New Mexico.

In situ microscopy is particularly important because the identification of microorganisms within fluid inclusions confirms their authenticity and provides strong evidence that they are the same age as the crystals in which they are found. Microscopic studies are also important for determining the mode of preservation of microbes and understanding their populations. Such studies have recently revealed complex microbial communities in fluid inclusions in modern and ancient halite, including prokaryotes (some alive), eukaryotes (the alga *Dunaliella* and other single celled species), organic material of unknown origin, and inorganic crystals (Figs. 5.9, 5.10 and 5.11) (Schubert et al. 2010b; Lowenstein et al. 2011). Identification of such fluid inclusion ecosystems has led to hypotheses for long-term survival of halophilic *Archaea* via starvation survival and prokaryote miniaturization, as well as possible nutrient sources including glycerol (Schubert et al. 2009a, b; 2010b; Lowenstein et al. 2011).

Transmitted and epifluorescence microscopy, using a 100X oil immersion objective, and environmental scanning electron microscopy (environmental SEM), were combined to assess microbial populations in subsurface halite from Death Valley (Schubert et al. 2009a, b; 2010a, b; Lowenstein et al. 2011). *In situ* microscopy was used to document prokaryotes, eukaryotes, and associated biomolecules within fluid inclusions. Examination of nearly 7,000 fluid inclusions from Death Valley halite showed that microorganisms occur almost exclusively in halites deposited in perennial hypersaline lakes that existed 10,000–35,000 years ago, which shows that trapping and preservation of prokaryotes in fluid inclusions in halite is influenced by the surface environment in which the halite originally precipitated. Some of these halites have prokaryotes in fluid inclusions comparable in abundance to those found in modern hypersaline systems (2×10^7 microbes/ml). The same fluid inclusions contained cells of the alga *Dunaliella*, some green or orange in color, and with a cup-shaped chloroplast, which suggests preservation of intact pigments, such as chlorophyll and carotenoids (Schubert et al. 2009b; 2010b; Lowenstein et al. 2011). In contrast, prokaryotes found in Death Valley halites (>10,000 years old) appear quite different from those trapped in fluid inclusions in modern halite. Ancient prokaryotes are coccoid-shaped and miniaturized, with cell diameters <1 μm, much smaller than the rod (1–10 μm long, ~0.5–1 μm wide) and coccoid-shaped prokaryotes (typically ~1 μm diameter) typical of modern surface brines. The differences in size and shape between modern and ancient prokaryotes trapped in fluid inclusions resemble the starvation-survival forms reported for prokaryotes living in soils and in the ocean (Novitsky and Morita 1976; Morita 1982, 1997; Grant et al. 1998). It is well known that some prokaryotes living under nutrient-poor conditions adjust by reducing their size and changing shape by rounding from rod to coccoid (Kjelleberg et al. 1983). Similarly, it appears that once trapped inside fluid inclusions, prokaryotes resort to starvation-survival strategies, but the timing and triggering mechanisms are not known.

Raman spectroscopy is ideal for the study of biomolecules and other species generated through biological processes (such as CH_4 and CO_2) in fluid inclusions because it is an *in situ*, non-destructive technique capable of characterizing solid and liquid materials within cells or free in fluid inclusions (Wopenka and Pasteris 1993; Burruss 2003). Using a laser-excitation source focused through an optical microscope and into a fluid inclusion, spatial resolution on the micron scale is possible (Burruss 2003). Most covalently-bonded solids, liquids and dissolved species may be identified on the basis of Raman peak positions: peak intensities (or areas) provide information on relative concentrations of species in the analytical volume. Until recently, analysis of many organic and biological materials with Raman was limited owing to the strong fluorescence induced by some visible-wavelength laser excitation. However, in recent years the application of near-infrared and UV lasers has shown considerable promise for analyzing a wide range of biological samples (Petry et al. 2003), and there is now a large database of Raman spectra of biological molecules, including nucleic acids, amino acids, metabolites, and others such as β-carotene and chlorophyll DeGelder et al. 2007) available to interpret the Raman spectra. *In vivo* measurements of individual live cells of the alga *Dunaliella* yielded strong spectra for chlorophyll *a* and β-carotene (Heraud et al. 2007), and Fendrihan et al. (2009) identified C_{50} carotenoid compounds from single cells of halophilic *Archaea* in fluid inclusions in laboratory-grown halite. Organic compounds such as glycerol, that are soluble in water, also produce Raman spectra with characteristic peaks (Mudalige and Pemberton 2007), as do dissolved covalently bonded gases such as CO_2 and CH_4 (Burruss 2003).

Microbiological Considerations

Cultivation experiments and efforts to extract DNA have used three techniques: (1) dissolution of surface sterilized crystals, (2) grinding surface sterilized gypsum crystals to powder, and (3) microdrilling into crystals and extracting individual inclusion fluids with a syringe. The preferred technique depends upon the particular samples and minerals involved and the goal of the experiments.

Successful revival of prokaryotes trapped within ancient crystals of halite using cultivation techniques is reported in nine publications since 1999 (Stan-Lotter et al. 1999, 2002; Vreeland et al. 2000, 2007; Mormile et al. 2003; Gruber et al. 2004; Schubert et al. 2009b, 2010a; Gramain et al. 2011). Halites used for culturing ancient prokaryotes range from hand samples, obtained from underground mines and borehole cores, hundreds of grams in weight (Stan-Lotter et al. 1999, 2002), to individual crystals (Vreeland et al. 2007; Schubert et al. 2009b, 2010a; Gramain et al. 2011), to single fluid inclusions within a crystal (Vreeland et al 2000; Mormile et al. 2003). Many of these studies screened samples to target primary halite crystals with primary fluid inclusions (Mormile et al. 2003; Vreeland et al. 2007; Schubert et al. 2009b, 2010a; Gramain et al. 2011). Work was performed in clean laboratory conditions, under a laminar flow hood, using sterilized equipment. The most rigorous treatments

to decontaminate crystal surfaces involve immersion of individual halite crystals in concentrated sodium hydroxide (NaOH) and hydrochloric acid (HCl) (Rosenzweig et al. 2000; Vreeland et al. 2000, 2007; Schubert et al. 2009b, 2010a). Once crystals were surface sterilized, they were dissolved in growth media containing high salt concentrations and a carbon source such as yeast extract, casein-derived amino acids, pyruvate, or glycerol. The two studies that targeted individual fluid inclusions in halite used a microdrill to breach the inclusion cavity (Vreeland et al. 2000; Mormile et al. 2003). The inclusion brine was then removed with a micropipette and inoculated into growth medium. Contamination by younger organisms is an important concern in any study claiming to revive ancient prokaryotes and therefore reports of ancient microorganisms in halite should be viewed as controversial.

All prokaryotes cultured from ancient halite are halophilic *Archaea*, with the exception of the halotolerant bacterium *Virgibacillus* sp. 2-9-3 reported from the Permian Salado salts of New Mexico (Vreeland et al. 2000). A number of haloarchaea have been cultured from Permian-Triassic (200–300 million-year-old) halites in England, Germany, and Austria. One of these, *Halococcus salifodinae*, isolated from geographically separated areas, was interpreted by Stan-Lotter et al. (1999) as the trapped microbial remains of marine brines that once covered western Europe. The genus *Halobacterium* is the most widely cultured ancient halophilic archaea (Mormile et al. 2003; Gruber et al. 2004; Vreeland et al. 2007; Gramain et al. 2011). Schubert et al. (2009b, 2010a) cultured halophilic *Archaea* from 5 halite crystals (22,000 to 34,000 years old) out of 881 tested from the Death Valley core, showing the rarity of microbial survival in fluid inclusions. The five halophilic *Archaea* are from the genera *Haloterrigena*, *Natronomonas,* and *Halorubrum*. Supporting evidence showing that these halophilic *Archaea* were not contaminants included: (1) well-preserved primary halite and fluid inclusions (Fig. 5.7) sampled only from interior sections of the Death Valley core, (2) *in situ* microscopic confirmation that prokaryotes existed in fluid inclusions in all halite crystals that yielded growth (Fig. 5.10), (3) intra-laboratory reproducibility, in which repeated growth of related taxa of halophilic *Archaea* (*Haloterrigena*) was achieved for one interval, and, (4) inter-laboratory reproducibility, in which two halophilic *Archaea* (DV462A and *Natronomonas* sp 2-24-1) with 99.3 % similarity of DNA from the 16S rRNA gene, were cultured at separate laboratories from different halite crystals of the same cored interval (Schubert et al. 2010a). Schubert et al. (2009b, 2010b) hypothesized that glycerol and other metabolites leaked out of *Dunaliella* cells supplied heterotrophic prokaryotes trapped in fluid inclusions with the carbon and energy sources required for their prolonged survival. Support for this hypothesis comes from the fact that all five halophilic *Archaea* cultured from fluid inclusions in Death Valley halite were isolated in media containing glycerol as a carbon source.

Ancient DNA from halite and gypsum has been extracted, purified, amplified, and sequenced (Radax et al. 2001; Fish et al. 2002; Park et al. 2009; Panieri et al. 2010; Gramain et al. 2011). Halite samples from underground mines and borehole cores from the Permian-Triassic of Germany and Austria were found to contain haloarchaeal DNA similar to *Halobacterium, Halorubrum, Haloferax, and Halogeometricum* (Radax et al. 2001). Haloarchaeal and bacterial DNA fragments were

recovered by Fish et al. (2002) from primary crystals in halite deposits between 11 and 425 million years old from Poland, Thailand and the United States. These studies, like all others, amplified the 16S rRNA gene, followed by cloning and sequencing. Park et al. (2009) similarly sequenced haloarchaeal DNA related to the modern genera *Haloarcula*, *Halorubrum and Halobacterium*, from halites 23, 121 and ~419 million years old. Panieri et al. (2010) extracted and amplified the oldest known cyanobacterial DNA from gypsum crystals of the late Miocene (Messinian, 5.8–5.9 million years old) from the northern Apennines, Italy. Those samples are unusual because they contained microbial filaments trapped within the solid portions of primary gypsum crystals. Sampling for DNA in that case was accomplished by surface flaming using ethanol, followed by grinding of the gypsum into a powder, from which DNA was extracted (Panieri et al. 2010). Finally, Gramain et al. (2011) detected DNA from the genus *Halobacterium* from primary fluid inclusions in halite cements from the Pliocene (>1.8 million years old) subsurface halite of the Salar Grande, northern Chile. It should be noted that recent testing of surface sterilization protocols by Gramain et al. (2011) and Sankaranarayanan et al. (2011) has shown that many of the methods used in previous cultivation studies are not fully effective in destroying DNA attached to halite crystal surfaces. These studies both indicate the need for longer soak times, 20 min in each of bleach (6 % sodium hypochlorite), NaOH, HCl and ethanol (Gramain et al. 2011), or 15 min in each of NaOH, HCl, bleach or HCl and bleach (Sankaranarayanan et al. 2011). Whatever the method used to study ancient microorganisms and DNA, Gramain et al. (2011) and Sankaranarayanan et al. (2011) have shown that surface sterilization using a combination of concentrated HCl and bleach are required to completely remove potentially contaminating surface-bound DNA.

Conclusions

A community of microorganisms (bacteria, archaea, algae) has been found in ancient fluid inclusions in halite and gypsum. Syndepositional evaporites, that formed at or soon after the time of deposition, should be the focus for future geomicrobiological studies because they are expected to be the richest source of microorganisms and biomaterials. But within the class of syndepositional evaporites, there are only a small number of deposits formed in particular environments, such as perennial saline lakes and lagoons, that have been found to contain appreciable numbers of microorganisms (Panieri et al. 2008; Schubert et al. 2009a, b, 2010b). Other types of syndepositional evaporites, such as those formed in desiccated saline pans, contain little biomass and are thus less useful for geomicrobiological studies.

More work combining sedimentology, microscopy, geochemistry, and microbiology is needed to understand fluid inclusion ecosystems that are millions of years old. This includes more complete documentation of the suite of microorganisms that existed at the time of inclusion formation, regardless of whether they are viable. Biomaterials (DNA, chlorophyll, cellulose, carotenoids) and inorganic materials (major

elements, nutrients) associated with microorganisms in fluid inclusions also merit further study because they may hold the key for understanding the mechanisms by which prokaryotes survive for long periods inside fluid inclusions. Such knowledge is vital as studies further explore the evolution of microbial communities over geological time and the preservation of life within Earth's crust and elsewhere in the solar system where materials that potentially harbor microorganisms are millions and even billions of years old.

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