

Microbial communities in fluid inclusions and long-term survival in halite

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ABSTRACT

Fluid inclusions in modern and ancient buried halite from Death Valley and Saline Valley, California, USA, contain an ecosystem of “salt-loving” (halophilic) prokaryotes and eukaryotes, some of which are alive. Prokaryotes may survive inside fluid inclusions for tens of thousands of years using carbon and other metabolites supplied by the trapped microbial community, most notably the single-celled alga *Dunaliella*, an important primary producer in hypersaline systems. Deeper understanding of the long-term survival of prokaryotes in fluid inclusions will complement studies that further explore microbial life on Earth and elsewhere in the solar system, where materials that potentially harbor microorganisms are millions and even billions of years old.

INTRODUCTION

Microbes are known to exist in subsurface habitats, such as sub-seafloor sediments and continental and oceanic crust, to depths of up to ~3 km (Parkes et al., 2000; Kerr, 2002; Lin et al., 2006; Onstott et al., 2006). Prokaryotes (single-celled organisms lacking a nucleus and other membrane-bound specialized structures) in these subsurface environments live in water within sediment pores and rock fractures. Most are heterotrophic and depend upon preexisting organic matter around them for metabolism, but some are autotrophic and can use non-photosynthetically derived energy sources (Lin et al., 2006). Other prokaryotes that live in Earth’s subsurface under such so-called “extreme” conditions have been found in ice as old as 120 ka from Antarctica, Greenland, and mountain glaciers, and in permafrost, perhaps as old as 8 Ma (Christner et al., 2000; Miteva et al., 2004, 2005; Bidle et al., 2007; Johnson et al., 2007). Collectively, these discoveries have extended the realm of the biosphere into Earth’s crust and have given hope for finding life beneath the surface of other planets, moons, asteroids, and comets of our solar system where present surface conditions are inhospitable.

The world’s “oldest living organisms” come from another subsurface setting, buried salt deposits. Over the past 50 years, a series of papers have claimed long-term survival of prokaryotes (*Bacteria* and *Archaea*) in these deposits, in some cases for >250 m.y. (Reiser and Tasch, 1960; Dombrowski, 1963; Norton and Grant, 1988; Grant et al., 1998; Stan-Lotter et al., 1999;

McGenity et al., 2000; Vreeland et al., 2000, 2007; Radax et al., 2001; Mormile et al., 2003; Schubert et al., 2010a). Prokaryotes in ancient salt deposits also apparently survived in water, but in this case were confined to brine-filled “fluid inclusions” in the halite itself, isolated from surrounding pore- and fracture-filling waters.

Reports of extreme microbe longevity in salt are controversial. The well-known Permian bacterium from the Waste Isolation Pilot Plant (WIPP) site, Salado Formation, New Mexico, USA (Vreeland et al., 2000), for example, comes from a brine inclusion within a large, diagenetically formed halite crystal. That brine inclusion could have been trapped after the Permian during burial cementation and recrystallization processes (Hazen and Roedder, 2001). Later study of those fluid inclusions, however, shows that they most likely contain evaporated

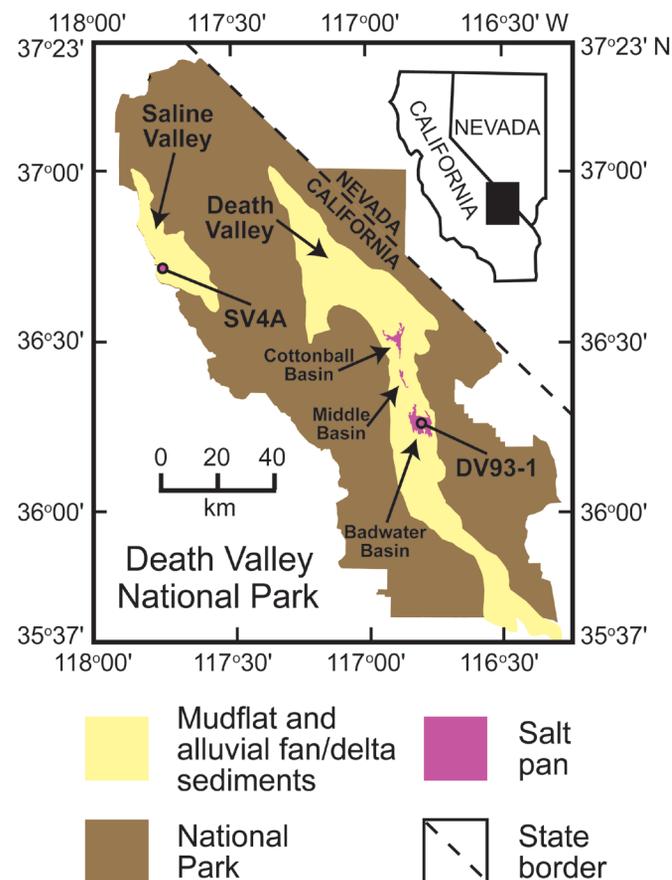


Figure 1. Map of Death Valley and Saline Valley, California, USA, with locations of cores DV93-1 and SV-4A; modified from Schubert et al. (2009a).

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Permian seawater, which supports their 250 Ma age and the antiquity of the trapped bacterium (Satterfield et al., 2005). The strongest criticism of the antiquity of prokaryotes recovered from ancient salt deposits has come from the biological science community, which maintains that deoxyribonucleic acid (DNA) should degrade over time scales far shorter than 250 m.y. in the absence of a repair mechanism (Willerslev et al., 2004; Hebsgaard et al., 2005; Willerslev and Hebsgaard, 2005). In addition, DNA from the Permian bacterium is nearly identical to a modern bacterium, *Virgibacillus marismortui*, sampled from the Dead Sea (Arahal et al., 1999, 2000), which suggests to some that the Permian bacterium is a laboratory contaminant (Graur and Pupko, 2001).

Other reputed ancient *Archaea* occur in bedded halite with primary growth textures and banded arrays of primary fluid inclusions parallel to crystal growth faces, indicating that

the inclusions were trapped during growth of halite from surface brines (Mormile et al., 2003; Vreeland et al., 2007; Schubert et al., 2009a, 2010a). It is now certain that some ancient bedded halite, and the included brines and microorganisms, can remain undisturbed for millions of years (Lowenstein et al., 2001). The problem confronting all studies of prokaryotes trapped in fluid inclusions from ancient halite is understanding how these microorganisms survive for prolonged periods and how they obtain energy to perform necessary functions, such as repair of damaged DNA.

Here we examine microorganisms trapped in fluid inclusions in halite, summarizing results from modern environments (Saline Valley, California, USA) and buried deposits up to 100 ka from Death Valley, California, USA (Schubert et al., 2009a, 2009b, 2010a, 2010b). We also present new, unpublished information from the subsurface salts of Saline Valley, which are up to 150 ka. These modern and Pleistocene deposits contain significant numbers of prokaryotes in fluid inclusions, a small number of which are clearly alive. Microscopy has revealed a remarkable “ecosystem” within fluid inclusions, composed of “salt-loving” (halophilic) prokaryotes and eukaryotes (complex cells containing a nucleus and specialized structures, such as chloroplasts) that may hold key information about long-term survival. We hypothesize that prokaryotes survive inside fluid inclusions for prolonged periods using carbon and other metabolites supplied by members of the trapped microbial community, most notably the single-celled alga *Dunaliella*, an important primary producer in hypersaline systems.

HALOPHILIC MICROORGANISMS IN MODERN HYPERSALINE SYSTEMS

The starting point for evaluating long-term survival of microorganisms in fluid inclusions in salt is to examine modern evaporite systems and the processes by which organisms are preserved in halite there. We illustrate a typical hypersaline environment, Saline Valley, where, under certain conditions, surface brines host prolific numbers of halophilic microorganisms. Saline Valley is a closed-basin saline pan in eastern California that contains surface brines up to 0.5 m deep, fed by groundwaters (Figs. 1 and 2A) (Hardie, 1968; Howe, 1998). A bloom of planktonic halophiles, developed in March 2004, contained one type of photosynthetic autotroph, the single-celled alga *Dunaliella*, and many heterotrophs (prokaryotic *Archaea* and *Bacteria*, that thrived in bright red brines at salinities of 26%–30%, seven to eight and a half times more concentrated than seawater (Fig. 2B). The pink/red brine color is due to the carotenoids (organic pigments, including β -carotene) used by microorganisms for protection from ultraviolet radiation) in halophilic *Archaea* and *Bacteria* and *Dunaliella* (Teller, 1987; Pedrós-Alió et al., 2000; Oren and Rodríguez-Valera, 2001; Oren, 2002b). Wet mounts prepared from Saline Valley brines contained rod- and coccoid-shaped prokaryotes and larger spherical and ellipsoid-shaped cells of *Dunaliella*, some of which were motile one year after collection (Figs. 2C and 2D).

When surface brines from Saline Valley evaporated to salinities greater than ~30% during March 2004, halite saturation was reached and halite crystals nucleated at the air-brine interface, forming floating masses of linked crystal rafts; vertically oriented crystals also grew off the brine bottom (Fig. 2E). The

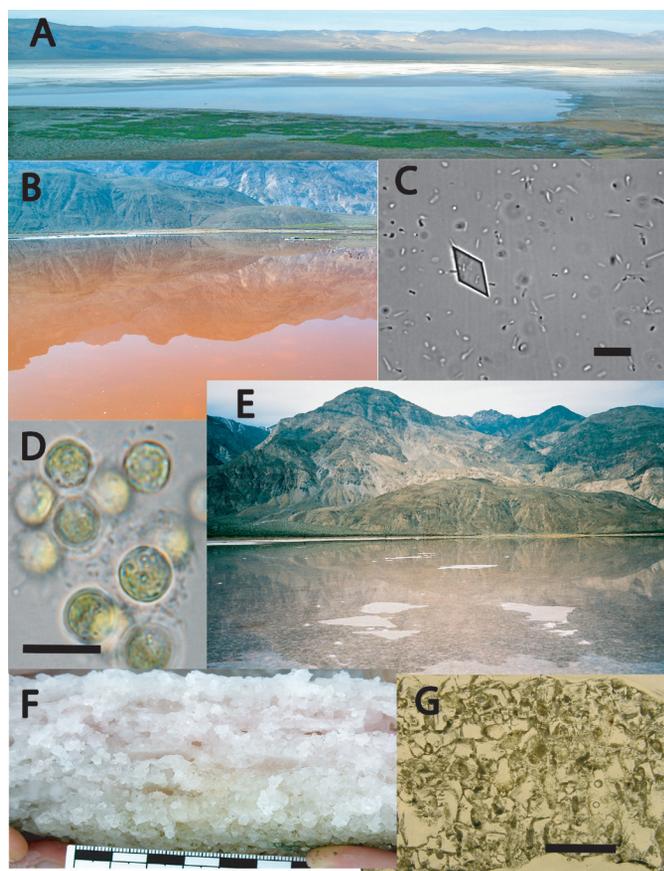


Figure 2. Saline Valley, California, USA, March 2004. (A) Saline pan and surrounding mudflats, with surficial salt crust (white) and shallow saline lake in foreground. (B) Halophile bloom in saline lake. (C) Photomicrograph of wet mount slide prepared from Saline Valley brine (Oct. 2005), with rod- and spherical (cocci) shaped microbes distinct from diamond-shaped crystal of glauberite ($\text{CaSO}_4 \cdot \text{Na}_2\text{SO}_4$). Scale bar is 10 μm . Modified from Schubert et al. (2009a). (D) Photomicrograph of wet mount slide prepared from Saline Valley brine, with spherical green cells of *Dunaliella*. Scale bar is 10 μm . (E) Large rafts (up to 1 m) of laterally linked halite crystals on the brine surface and halite chevrons crystallizing at the brine bottom. (F) Cross section of halite crust formed in 2004, pink from trapped microorganisms. Small divisions on ruler are millimeters. (G) Thin-section photomicrograph of halite crust shown in F. Vertically oriented halite crystals grew upward from the saline lake bottom. Fluid inclusion bands (gray) in some halites outline primary crystal growth directions. Scale bar is 10 mm.

halite crust formed by these processes contained large numbers of brine inclusions trapped during crystal growth, and the salt crust was pink because microbes from the water column were incorporated into the halite inclusions (Figs. 2F and 2G). Individual fluid inclusions housed a community of prokaryotes and *Dunaliella*, the same shape and size as observed in Saline Valley brines (Fig. 3). Microscopic study of >1000 brine inclusions from 10 halite-crust crystals showed that >20% contained prokaryotes (Schubert et al., 2009a). The calculated prokaryote abundance of 6×10^8 microbes/mL of inclusion brine is similar to that reported from red halophile-rich brines in many modern settings (Larsen, 1980; Teller, 1987; Oren, 2002a, 2002b; Pedrós-Alió, 2004). This means that one halite cube from Saline Valley, 1 cm per side, with a typical volume of fluid inclusions of 1% (Roedder and Bassett, 1981) contains six million trapped microbes.

Experiments show that prokaryotes (*Archaea* and *Bacteria*) trapped in fluid inclusions in halite from Saline Valley for up to 15 years can be readily cultured when placed in nutrient-rich media. These results are consistent with data from laboratory experiments and other modern surface halite deposits, all of

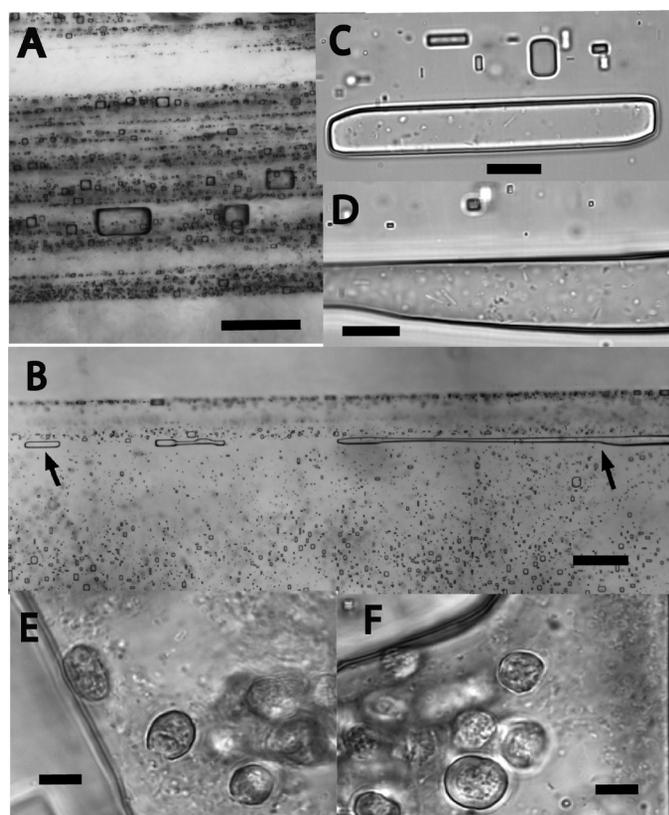


Figure 3. Photomicrographs of fluid inclusions in halite, collected in Saline Valley California, USA, in 2004 and 2005. (A) Horizontal band rich in rectangular prism-shaped brine inclusions, surrounded above and below by bands containing fewer inclusions. Scale bar is 100 μ m. (B) Tubular fluid inclusions and evenly distributed cubic and rectangular prism-shaped inclusions. Arrows point to inclusions shown at higher magnification in C and D. Scale bar is 100 μ m. (C) and (D) Fluid inclusions with rod- and coccoid-shaped prokaryotes. Scale bars are 10 μ m. (E) and (F) Portions of large fluid inclusions in halite with ellipsoidal and spherical cells of *Dunaliella* and numerous smaller prokaryotes. Scale bars are 5 μ m.

which show that prokaryotes can remain alive inside fluid inclusions in halite for many years (Norton and Grant, 1988; Grant et al., 1998; McGenity et al., 2000; Mormile et al., 2003; Adamski et al., 2006; Fendrihan et al., 2006). The next step is to ascertain if prokaryotes remain alive in fluid inclusions following burial.

HALOPHILIC MICROORGANISMS IN BURIED PLEISTOCENE SALT

Borehole cores from Death Valley and Saline Valley, composed of interbedded salt and mud, provide ideal materials for assessing the fate of microbial communities trapped in fluid inclusions in halite in the subsurface for periods of up to 150 k.y. (Fig. 4). The cored sediments contain a dated record of Pleistocene paleoenvironments that varied from saline pans and dry mudflats to deep, perennial lakes (Li et al., 1996; Howe, 1998; Lowenstein et al., 1999). Evaporites accumulated in two settings: (1) bedded halite with abundant primary growth textures formed in perennial saline lakes (i.e., Great Salt Lake, Utah, USA); and (2) massive halite formed in salt pans (i.e., Badwater Basin, Death Valley, USA) (Li et al., 1996; Lowenstein et al., 1999). Microorganisms in fluid inclusions were almost exclusively found in halites deposited in perennial saline lakes in Death Valley (ca. 10–35 ka) and Saline Valley (ca. 20 ka, 75 ka, and 150 ka). Some of these halites have prokaryotes in fluid

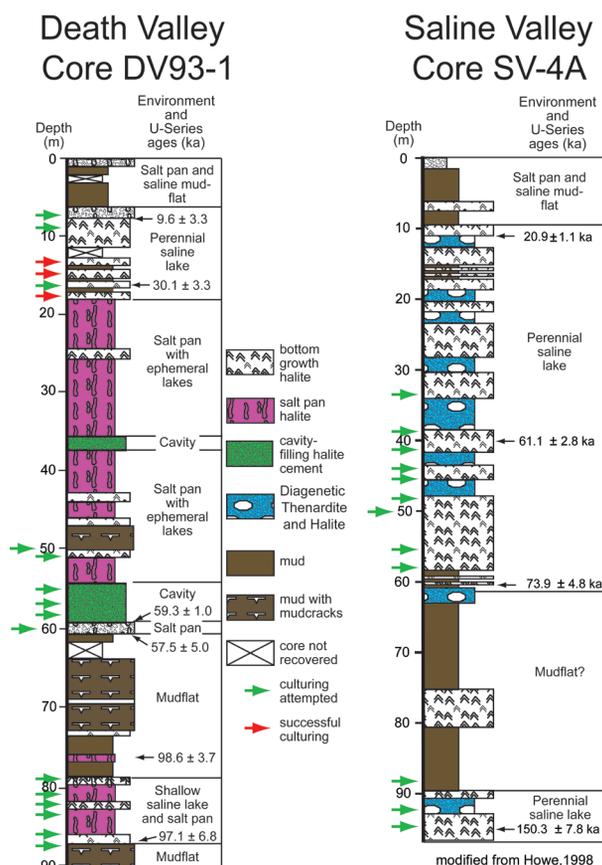


Figure 4. Stratigraphic columns, of cores DV93-1 and SV-4A showing sediment types, uranium-series ages, and paleoenvironments. Modified from Howe (1998) and Lowenstein et al. (1999). Note depths (arrows) where samples were taken for culturing experiments: green—unsuccessful; red—successful.

inclusions comparable in abundance to those found in modern halites formed during the 2004 Saline Valley halophile bloom, which suggests that ancient saline lakes of Death Valley and Saline Valley were at times teeming with microorganisms (Schubert et al., 2009a).

Dunaliella cells trapped in fluid inclusions up to 150 ka may appear virtually the same as those from modern halite (compare Fig. 5A to Figs. 3E and 3F). Remarkably, some ancient *Dunaliella* cells contain a cup-shaped chloroplast and are green and orange, which suggests preservation of pigments, including carotenoids and chlorophyll (Fig. 5B) (Schubert et al., 2010b). Other ancient *Dunaliella* cells, particularly in fluid inclusions in halite from the Saline Valley core, form a “stew” in various stages of disintegration, with cell coats separated from cell contents (Fig. 5F).

Prokaryotes found in buried halites (>10 ka) appear quite different from those trapped in fluid inclusions in modern halite. Ancient prokaryotes are coccoid-shaped and “miniaturized,” with

cell diameters <1 μm (Figs. 5A, 5C, and 5D), much smaller than the straight or curved rods (1–10 μm long, ~0.5–1 μm wide) and coccoid-shaped prokaryotes (typically ~1 μm diameter), of their surface counterparts (Figs. 3C and 3D). 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 widely known that some prokaryotes living under nutrient-poor conditions adjust by changing shape—that is, “rounding” from rod-shaped to coccoid-shaped, and reducing their size (Kjelleberg et al., 1983). We postulate that once trapped inside fluid inclusions for long periods of time, prokaryotes resort to starvation-survival strategies, but the timing and triggering mechanisms are not known. Trapping of halophilic *Archaea* in nutrient-free fluid inclusions in experimentally grown halite also led to rounding and cell-size reduction over periods of weeks to years (Norton and Grant, 1988; Fendrihan and Stan-Lotter, 2004), but more research on starvation-survival of prokaryotes in fluid inclusions is clearly needed.

Long-term survival of miniaturized prokaryotes in fluid inclusions in buried halite from Death Valley and Saline Valley was tested with culturing experiments designed to grow halophilic microorganisms. One procedure used previously by microbiologists involves surface sterilization of a halite crystal, followed by dissolution of that crystal in a liquid medium composed of Na⁺, Cl⁻, inorganic nutrients, and a carbon source (Vreeland et al., 2007; Schubert et al., 2009b, 2010a). During the dissolution process, the Na⁺, Cl⁻, inclusion brines, and trapped microorganisms mixed with the growth medium. Incubation under aerobic conditions for periods of up to 90 days led to the growth of cultures from five halite crystals (13.0–17.9 m; 22 ka to 34 ka) out of ~900 tested from the Death Valley core (Fig. 4) (Schubert et al., 2009b, 2010a). For unknown reasons, no prokaryotes were cultured from >500 halite crystals (12 intervals between 34 and 93 m) up to 150 ka from the Saline Valley core. It is clear from these experiments that cultivation of prokaryotes sampled from fluid inclusions in halite between 10 ka and 150 ka is rare, occurring in only 0.4% of the crystals tested. These results, coupled with the large number of cells observed in situ within fluid inclusions (Fig. 5), suggest that most ancient prokaryotes in halite are dead or viable but nonculturable, or that our culturing conditions were simply not suitable (Amann et al., 1995). Nevertheless, the DNA from the five cultured organisms from the Death Valley core shows that they are halophilic *Archaea* from the genera *Haloterrigena*, *Natronomonas*, and *Halorubrum*, all organisms expected in hypersaline lakes (Schubert et al., 2009b, 2010a).

MECHANISM FOR LONG-TERM SURVIVAL OF PROKARYOTES IN FLUID INCLUSIONS

All *Archaea* from the Death Valley core we have cultured so far came from one stratigraphic interval (Fig. 4) in which prokaryotes and *Dunaliella* were observed in situ within fluid inclusions. Closer inspection of those fluid inclusions, coupled with what is known about the ecology of modern hypersaline systems, has led us to hypothesize a mechanism that may allow prokaryotes to survive inside fluid inclusions for millennia.

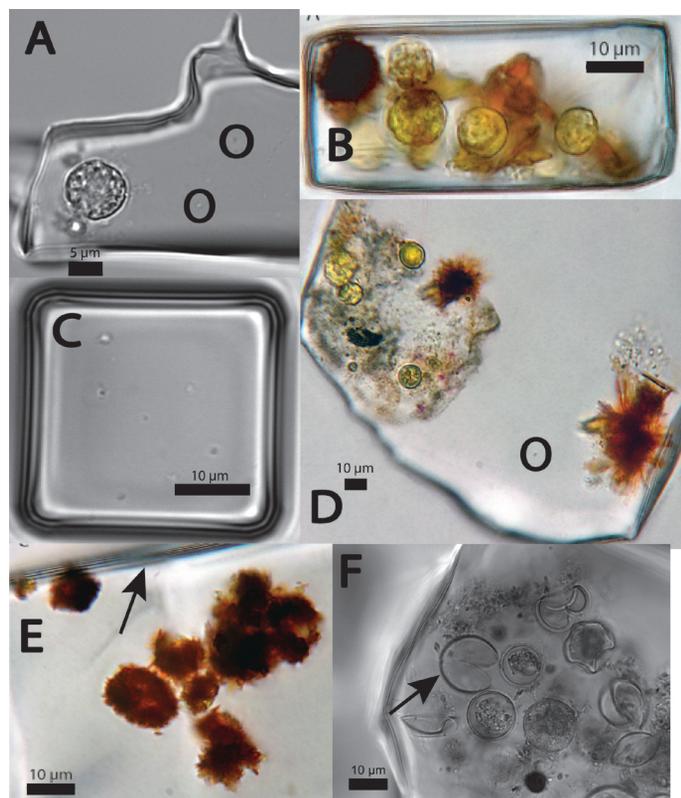


Figure 5. Photomicrographs of fluid inclusions in ancient halite from Saline Valley and Death Valley (Calif., USA) cores. (A) *Dunaliella* cell (left) and miniaturized prokaryotes (circled), in irregularly shaped fluid inclusion, Saline Valley core, 93 m, 150 ka. (B) Light green and orange *Dunaliella* cells suggest preservation of chlorophyll and β-carotene, Death Valley core, 17.8 m, 34 ka. Modified from Schubert et al. (2010b). (C) Miniaturized prokaryotes in cubic fluid inclusion, Death Valley core 16.5 m, 31 ka. Modified from Schubert et al. (2009a). (D) Portion of large fluid inclusion containing yellow-green *Dunaliella* cells and two cells coated with outward radiating crystals of β-carotene (brown). Miniaturized prokaryote is circled. Death Valley core 15.7 m, 29 ka. (E) Portion of fluid inclusion showing *Dunaliella* cells heavily coated with crystalline β-carotene, Death Valley core 15.7 m, 29 ka. Arrow shows the boundary between the fluid inclusion and the host halite crystal. (F) *Dunaliella* cells in various stages of degradation within a large fluid inclusion, Saline Valley core, 44 m, ca. 70 ka. Arrow shows ruptured glycocalyx (cell coat) of one *Dunaliella* cell.

Modern hypersaline environments near halite saturation contain a productive but relatively simple community of planktonic microorganisms, with *Dunaliella* the only primary producer and a number of different heterotrophic *Archaea* and subordinate *Bacteria* (Pedrós-Alió et al., 2000; Elevi Bardavid et al., 2008). Much is still unknown about the prokaryotes because these ecosystems are dominated by nonculturable microbes (Oren, 2002b). Regardless, it has long been postulated that the heterotrophic community of prokaryotes in these extreme environments obtains most of its carbon requirements from glycerol, a sugar alcohol with the chemical formula $C_3H_5(OH)_3$ (Borowitzka, 1981; Elevi Bardavid et al., 2008). Glycerol is produced in large quantities by *Dunaliella* because it is used for osmoregulation to reduce the chemical potential gradient of H_2O and to prevent the loss of water from cells. In fact, *Dunaliella* may have concentrations of 6–7 M glycerol in their cytoplasm to counteract the chemical gradients (Elevi Bardavid et al., 2008). This glycerol apparently leaks out of healthy *Dunaliella* cells into surrounding brines or may enter brines following death and disintegration (lysis) of the cells (Elevi Bardavid et al., 2008). In either case, glycerol constitutes a major source of carbon for the prokaryote community in modern hypersaline systems (Borowitzka, 1981; Elevi Bardavid et al., 2008). We hypothesize that the same relationships hold true inside fluid inclusions and that glycerol and other metabolites leaked out of *Dunaliella* cells have supplied associated heterotrophic prokaryotes with the carbon and energy sources required for their prolonged survival. Close inspection shows that *Dunaliella* commonly occur with prokaryotes in fluid inclusions (Figs. 5A and 5D). Some *Dunaliella* are in various stages of disintegration, indicating leakage of biomaterials, including glycerol, from cells into the surrounding brine (Fig. 5F). Other *Dunaliella* contain a crust of crystalline β -carotene on their exteriors (Figs. 5B, 5D, and 5E) (Schubert et al., 2009b, 2010b). β -carotene is produced by certain species of *Dunaliella*, so finding it precipitated outside the cell is direct evidence that intracellular materials have leaked into fluid inclusions. Solid crystals apparently formed as a crust on *Dunaliella* cells because β -carotene is insoluble in water and thus crystallized when extruded from cells. Glycerol, however, is soluble in water and thus would be completely dissolved in fluid inclusion brines, where it would be available for heterotrophic microorganisms. Support for our “glycerol” hypothesis comes from the five halophilic *Archaea* revived from fluid inclusions in Death Valley halite, all of which were cultured in media containing glycerol as a carbon source (Schubert et al., 2009b, 2010a). Two of these strains grew in media containing glycerol as the only carbon source; the other three are yet to be tested.

CONCLUSIONS

Although we are beginning to understand the community of microorganisms inside modern and ancient fluid inclusions, much more needs to be learned about how they survive. Miniaturized prokaryote cells suggest starvation-survival, despite the availability of carbon. We do not know why prokaryotes in fluid inclusions miniaturize, what factors trigger miniaturization, and what functions miniaturized cells are able to perform in fluid inclusions (e.g., repair of DNA and cell membranes) (Grant et al., 1998; Johnson et al., 2007).

Alternatively, prokaryotes may form spores and survive for long periods in a dormant state, as has been claimed for the bacterium cultured from the Permian fluid inclusion by Vreeland et al. (2000). But none of the halophilic *Archaea* cultured from the Death Valley core formed endospores, nor do any *Archaea*. We thus need to learn more about long-term survival of spore-forming prokaryotes as well as miniaturized forms trapped in fluid inclusions. Such knowledge will be vital as studies further explore deep life on Earth and elsewhere in the solar system, where materials that potentially harbor microorganisms are millions and even billions of years old.

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