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Research Article

Microscopic Identification of Prokaryotes in Modern and Ancient Halite, Saline Valley and Death Valley, California

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Abstract

Primary fluid inclusions in halite crystallized in Saline Valley, California, in 1980, 2004–2005, and 2007, contain rod- and coccoid-shaped microparticles the same size and morphology as archaea and bacteria living in modern brines. Primary fluid inclusions from a well-dated (0–100,000 years), 90 m long salt core from Badwater Basin, Death Valley, California, also contain microparticles, here interpreted as halophilic and halotolerant prokaryotes. Prokaryotes are distinguished from crystals on the basis of morphology, optical properties (birefringence), and uniformity of size. Electron micrographs of microparticles from filtered modern brine (Saline Valley), dissolved modern halite crystals (Saline Valley), and dissolved ancient halite crystals (Death Valley) support *in situ* microscopic observations that prokaryotes are present in fluid inclusions in ancient halite. In the Death Valley salt core, prokaryotes in fluid inclusions occur almost exclusively in halite precipitated in perennial saline lakes 10,000 to 35,000 years ago. This suggests that trapping and preservation of prokaryotes in fluid inclusions is influenced by the surface environment in which the halite originally precipitated. In all cases, prokaryotes in fluid inclusions in halite from the Death Valley salt core are miniaturized (<1 μm diameter cocci, <2.5 μm long, very rare rod shapes), which supports interpretations that the prokaryotes are indigenous to the halite and starvation survival may be the normal response of some prokaryotes to entrapment in fluid inclusions for millennia. These results reinforce the view that fluid inclusions in halite and possibly other evaporites are important repositories of microbial life and should be carefully examined in the search for ancient microorganisms on Earth, Mars, and elsewhere in the Solar System. Key Words: Ancient prokaryotes—Death Valley—Fluid inclusions—Halite—Saline Valley—Starvation survival. Astrobiology 9, 467–482.

Introduction

The potential for long-term preservation of halophilic and halotolerant prokaryotes in fluid inclusions in halite (NaCl) has long been recognized (Reiser and Tasch, 1960; Dombrowski, 1963, 1966; Norton and Grant, 1988; Norton *et al.,* 1993; Fredrickson *et al.,* 1997; Grant *et al.,* 1998; Stan-Lotter *et al.,* 1999; McGenity *et al.,* 2000; Vreeland *et al.,* 2000, 2007; Radax *et al.,* 2001; Fish *et al.,* 2002; Mormile *et al.,* 2003; Lowenstein, 2008). Fluid inclusions in halite are suitable hosts for preserving microorganisms and DNA because they are sealed systems (Goldstein and Reynolds, 1994) that contain NaCl-rich brine with little or no oxygen. Vreeland *et al.* (2000, 2007) isolated bacteria from fluid inclusions in Permian halite (~250 Ma) and halophilic archaea from Cretaceous (121–112 Ma) and Pleistocene (~34,000-year-old) halite. Mormile *et al.* (2003) isolated *Halobacterium salinarum* from a fluid inclusion in 97,000-year-old halite from Death Valley, California. Fish *et al.* (2002) amplified ancient bacterial and archaeal DNA from various halites 11–425 million years in age.

One aspect of research on ancient microorganisms and DNA that has not been emphasized is microscopic study of the host mineral, halite, and its fluid inclusions. Such studies are needed to establish that microorganisms are indigenous to, and the same age as, the halite in which they are found. Microscopy can also resolve questions about the preservation of primary sedimentary textures, the primary versus secondary nature of trapped fluid inclusions, and the paleoenvironments in which halite precipitated (Hardie *et al.,* 1985; Lowenstein and Hardie, 1985). Moreover, microscopic studies are the most convenient way to evaluate the mode of

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preservation of microorganisms trapped in fluid inclusions in halite and to assess their populations.

This study summarizes the microscopy of ancient halite from a 90 m long borehole core, Death Valley, California, with emphasis on possible prokaryotes trapped in fluid inclusions. Modern halites from Death Valley and nearby Saline Valley, California (Fig. 1), were also examined in order to understand the microscopic characteristics of prokaryotes in fluid inclusions and their abundances. Criteria were established for distinguishing prokaryotes from crystals. Prokaryotes in fluid inclusions in halite from the Death Valley core were directly counted to provide rudimentary information on their abundance and distribution, with respect to age and paleoenvironments. Environmental scanning electron microscopy studies were done to confirm that microbial shapes observed in fluid inclusions in ancient halite were prokaryotes. *In situ* microscopic studies provide key background information for biological work directed at culturing organisms and amplifying DNA from fluid inclusions. The results from this work will also guide future searches for traces of microbial life in chloride and sulfate evaporite deposits on the surface of Mars.

Materials and Methods

Textural analysis that forms the basis for paleoenvironmental interpretations was done on 5×7.5 cm polished thin sections up to 1 mm thick and single crystals and cleavage fragments of halite with a Leica Wild M3Z stereomicroscope. Plan view photographs of thin sections were taken with a Nikon Coolpix 8800 camera (8.0 megapixels). Fluid inclusions and their contents were examined *in situ* via a Zeiss compound microscope (AXIO Imager.A1) at 100–1000× magnification with an AxioCam MRm B&W camera and AxioVision software (Version 4.5). An oil immersion objective (PLAN APO 100×/1.4 OIL) facilitated study of possible prokaryotes in fluid inclusions. We used transmitted and cross-polarized light, and epifluorescence microscopy supplied by an HBO 100 mercury lamp and Chroma Technology Corp. filters to study the autofluorescence of microparticles [DAPI: exciter D350/50 nm, emitter D460/50 nm, beamsplitter 400dclp; Green Fluorescent Protein (GFP): exciter HQ450/50 nm, emitter HQ510/50 nm, beamsplitter Q480lp; and TRITC: exciter HQ545/30 nm, emitter HQ620/60 nm, beamsplitter Q570lp]. Epifluorescence imaging was improved when the diameter of the incoming light path was narrowed. The Max/Min function in the AxioVision software enhanced autofluorescence images of prokaryotes by automatically adjusting minimum and maximum gray values.

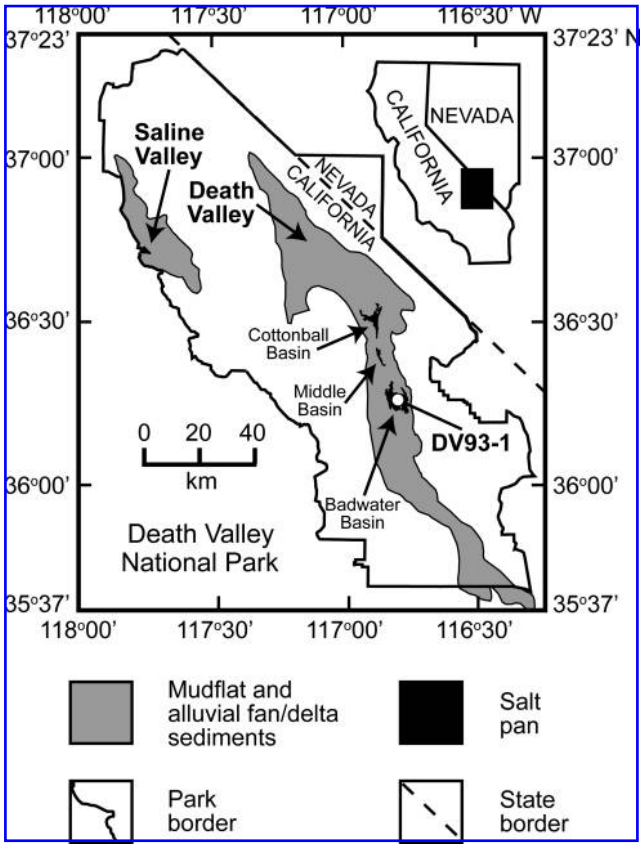


FIG. 1. Map of Death Valley National Park in California and Nevada, including Death Valley and Saline Valley. The Death Valley salt core (DV93-1) was drilled in the middle of Badwater Basin (modified from "Death Valley map," National Park Service, http://www.nps.gov/deva/planyourvisit/upload/DEVAmap1.pdf).

Light microscopy was used to distinguish possible prokaryotes from crystals in fluid inclusions in modern halite (Saline Valley and Death Valley) and ancient halite from the Death Valley salt core. Possible prokaryotes in fluid inclusions in halite were directly counted along random transects of thin sections with use of the 100× oil immersion objective. The volumes of individual fluid inclusions were estimated from measurement of the side lengths of each inclusion and estimation of the third dimension. Fluid inclusions <1 μm in side length were not measured because they were too small to identify possible prokaryotes unambiguously. Fluid inclusion volume estimates together with microorganism counts allowed us to make rough comparisons of relative microorganism populations in modern Death Valley and Saline Valley halite and different segments of the Death Valley salt core.

Microparticles from modern surface brines collected in 2004 in Saline Valley and from the insoluble residue following dissolution of halite crystals from Saline Valley (2004) and the Death Valley salt core were studied with an environmental scanning electron microscope (ESEM, Electroscan Model 2020) to compare prokaryote and crystal shapes. Microparticles from brine and crystals were filtered with a stacked filter set that included two 25 mm Isopore polycarbonate track-etched screen filters (1.2 μm and 5.0 μm, Millipore) and one 25 mm Nuclepore track-etched polycarbonate membrane (0.2 μm, Whatman). Filtering was done in a vacuum filtration system (Wheaton) under a Class IIA laminar flow hood with a HEPA filter (Baker, SterilGARD III) and glassware ashed at 450°C for 8 hours. Halite cleavage fragments (~0.1 g) from modern Saline Valley surface crusts collected in 2004 and at 16.5 m depth (31,000 years old) in the Death Valley core were viewed with a 100× oil immersion objective to confirm that prokaryotes were present in fluid inclusions. These crystals were then surface sterilized in 37% HCl for 1–3 hours. Following surface sterilization, cleavage fragments were placed on top of the filters. Modern surface brines from Saline Valley (100 μl) were pipetted directly onto the filter surface. All liquids used in the following steps (water, glutaraldehyde, and ethanol) were prefiltered with a 0.2 μm pore size filter. For halite samples and brine samples, 30–40 ml of 18.2 megohm water with less than

1 ppb total organic carbon (NANOpure DIamond UV Model #D11911 with Organic Free Deionized Feed kit #D50281) was passed over the filter to dissolve crystals and remove salts. Samples were fixed for 30–60 minutes in 2.5% glutaraldehyde, rinsed with 10–20 ml of 18.2 megohm water, and successively dehydrated in 25%, 50%, and 75% ethanol (10–20 minutes each) followed by 1–6 hours in 100% ethanol. Samples were air dried overnight, mounted to 25 mm aluminum stubs with double-sided carbon tape, and gold coated before examination in the ESEM at 20 kV and 5 torr in wet mode. Electron backscatter images of the filtered samples were studied to compare modern and ancient prokaryotes and distinguish prokaryotes from crystals. The morphology of prokaryotes in electron micrographs may differ from that observed under *in situ* transmitted light because of cell hydration and possible rupture prior to glutaraldehyde fixation.

Previous Work on Microscopy of Microorganisms in Fluid Inclusions

Inclusions in halite commonly have a cubic, rectangular prism, or irregular morphology and can trap brine, gas, minerals, microorganisms, and organic debris (Goldstein and Reynolds, 1994; Griffith *et al.,* 2008). Fluid inclusions in halite typically vary in size from submicrometer to millimeter scale. Their size, shape, number, and orientation depend on depositional environment and crystal growth rate (Roedder, 1984; Lowenstein and Brennan, 2001). Primary fluid inclusions formed during crystal growth are emphasized in this study because the timing of entrapment is known. Secondary fluid inclusions are formed along cleavage/fracture planes some time after crystallization, but the timing of formation is commonly not known.

Microorganisms in fluid inclusions in halite have been studied from crystals grown in the laboratory (Norton and Grant, 1988; Fredrickson *et al.,* 1997; Mormile *et al.,* 2003; Fendrihan and Stan-Lotter, 2004; Adamski *et al.,* 2006; Fendrihan *et al.,* 2006) and from samples collected from modern saline environments (Fredrickson *et al.,* 1997). Norton and Grant (1988) studied many types of halophilic archaea trapped in laboratory-grown halite and found them to be viable after 6 months; some remained motile for at least 3 weeks. Adamski *et al.* (2006), Fendrihan and Stan-Lotter (2004), and Fendrihan *et al.* (2006) illustrated that *Pseudomonas aeruginosa* and *Halobacterium salinarum* were trapped in fluid inclusions but not the host crystal matrix during halite crystal growth and that these microorganisms can remain viable for periods of months to years. Fredrickson *et al.* (1997, p 324) found "rare, bacterial-sized particles within primary fluid inclusions" in modern salt crystals collected from Laguna Grande de la Sal, New Mexico.

Direct microscopic observations of microparticles and possible microorganisms from ancient halite crystals have been made by Reiser and Tasch (1960), Tasch (1960), Dombrowski (1963, 1966), Grant *et al.* (1998), Vreeland and Rosenzweig (1999), and Mormile *et al.* (2003). Reiser and Tasch (1960) and Tasch (1960) observed stationary diplococcus-like bodies in crushed salt samples that were interpreted as dead Permian bacteria. Dombrowski (1963, his Fig. 1, pp 455–456) photographed a rod shaped bacterium "embedded in the crystalline structure." Dombrowski (1966, p 215) later described "regularly shaped inclusions, which, by their form and size can be distinctly identified as bacteria and bacterial spores," but no figures were provided. Grant *et al.* (1998) showed autofluorescent material consistent with organic origins in a fluid inclusion in a 20-million-year-old halite crystal. Vreeland and Rosenzweig (1999) showed a rod-shaped bacterium ($>30\times0.5\ \mu$m) with rounded ends on a freshly cut crystal face. Mormile *et al.* (2003, p 1095) showed microparticles "that have similar size and shape of bacteria" in fluid inclusions in 9,600- and 97,000-year-old halites from Death Valley. Bacteria-like microparticles were observed in fluid inclusions in hydrothermal quartz crystals (Bargar *et al.,* 1985; Bargar and Fournier, 1988; Bargar, 1995, 2001). Bargar *et al.* (1985, their Figure 2A, 2B, p 484) showed fluid inclusions with up to hundreds of "micrometer-sized moving particles," which were interpreted as microorganisms on the basis of their size and shape (mostly 1 μm long rods). The movement of the particles was said to resemble Brownian motion (Bargar *et al.,* 1985).

Geological Background

Modern Death Valley and Saline Valley

Death Valley is a narrow, closed basin that now contains three subbasins: from north to south they are Cottonball Basin, Middle Basin, and Badwater Basin (Fig. 1). The normally dry center of Badwater Basin is covered by saline pan evaporites (predominantly halite) and mudflat deposits over an area of several hundred square kilometers. Rare flooding of Badwater Basin occurs when the Amargosa River flows into Death Valley from the south. These flooding events dissolve old surface salt crusts and produce a temporary shallow saline lake less than one meter deep that becomes further concentrated by evaporation (Li *et al.,* 1996). During terminal evaporation of the saline lake, halite forms upward-crystallizing "chevrons" that contain alternating bands of cloudy, fluid inclusion–rich zones and clear zones with fewer fluid inclusions (Lowenstein and Hardie, 1985). Subsequent floods partly, or completely, dissolve halite crusts, which produces dissolution voids and rounded surfaces that truncate crystals. Large crystals of halite cement later form from groundwater brines in the void spaces of salt crusts.

Saline Valley is a closed basin in eastern California (Fig. 1) that contains a saline pan, which is fed by perennial groundwaters (Hardie, 1968). The saline pan, $\sim$10 km^2, is normally dry and underlain by halite but may be partially covered by a shallow brine pool during wet periods. Surface brines and groundwaters in the center of the saline pan are commonly at halite saturation (Hardie, 1968). During the winter of 2004, increased inflow of water caused surface brines to expand in area and deepen so that in March 2004 the brine pool was up to 0.5 m deep. In late March and early April 2004, surface brine salinities rose above $\sim$30%, and halite saturation was reached. At that time, halite crystals nucleated at the air-brine interface and coalesced to form masses of laterally linked crystal rafts, up to 1 m in diameter. Additionally, vertically oriented crystals grew off the bottom of the brine lake.

The Death Valley salt core

Halite crystals examined in this study come from a salt core (DV93-1) drilled in 1993 in Badwater Basin (Li *et al.,* 1996) (Fig. 1). The core was dated by U-series methods (Ku *et al.,* 1998) (Fig. 2). The top 90 m of the core records 100,000 years of

deposition of evaporites and terrigenous muddy sediment in Death Valley (Fig. 2). Previous studies interpreted Death Valley paleoclimates over the past 100,000 years from halite textures, evaporite mineralogy, fluid inclusion homogenization temperatures, shoreline carbonate tufas, and fossil ostracodes (Lowenstein *et al.*, 1998, 1999; Forester *et al.*, 2005).

From 7.7 to 18.0 m depth, the core contains halite with textures diagnostic of crystallization in a perennial saline lake ~10,000 to 35,000 years ago (Li *et al.*, 1996). This perennial lake section also contains ostracodes in interbedded muds, which

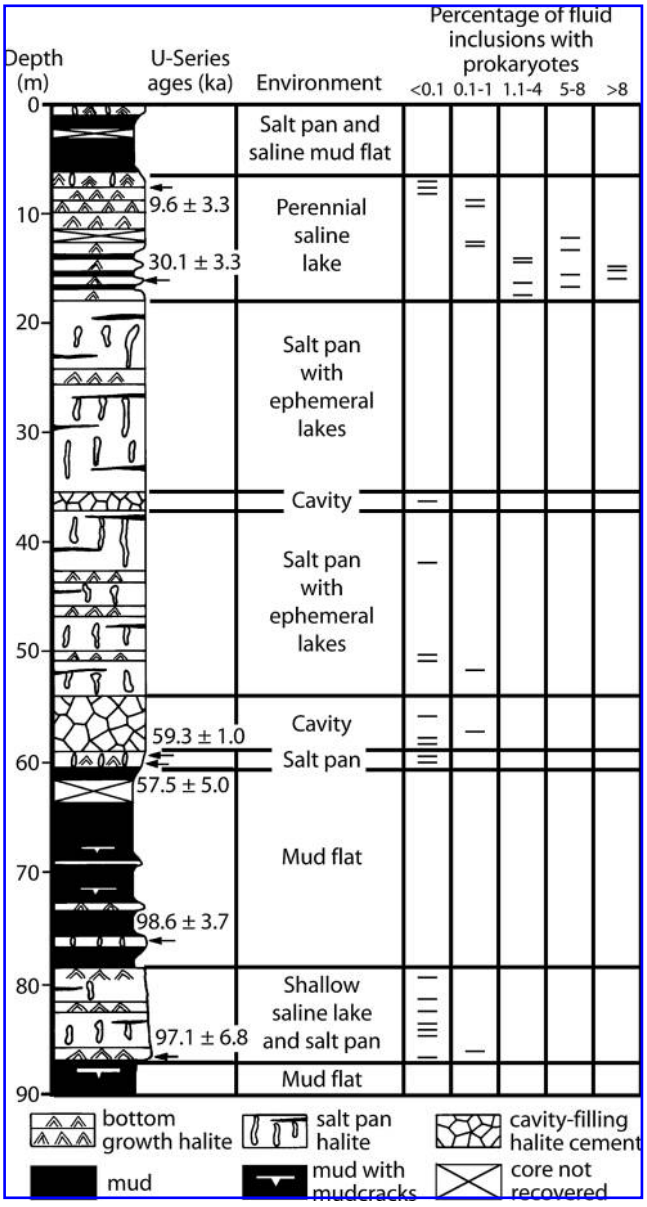


FIG. 2. Stratigraphic column of the Death Valley core from 0–90 m (0–100 ka) with general halite textures, U-series ages, and interpreted paleoenvironments (modified from Lowenstein *et al.*, 1999). The percentage of fluid inclusions that contained prokaryotes for each sample depth shows the stratigraphic and paleoenvironmental control on prokaryote distribution.

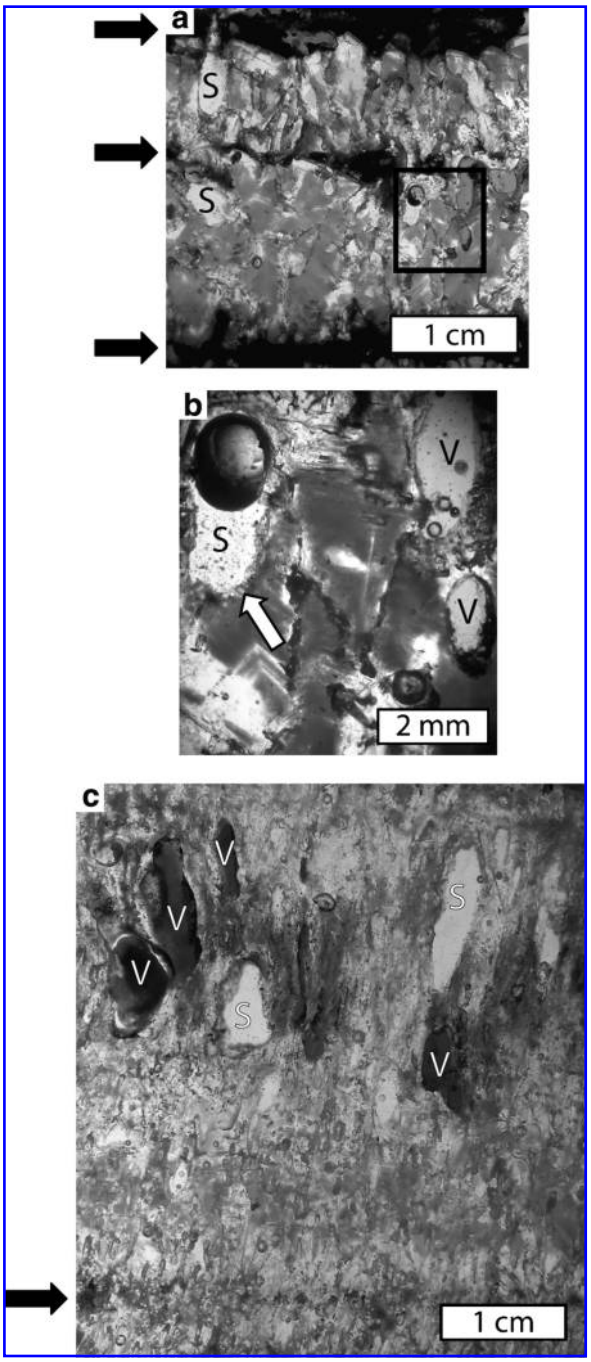


FIG. 3. Thin section photographs of layered deposits collected in 1993 and 1994 from Badwater Basin, Death Valley. (**a**) Thin section contains chevron halite layers, separated by thin dark mud layers (black arrows). Some halite cement (S) crystallized in dissolution voids between chevron halite. (**b**) Photomicrograph of the black box in (a) showing chevron halite, dissolution voids (V) and halite cement (S). A chevron crystal is truncated by the halite cement crystal, and mud lies at the truncation boundary (white arrow). Bubbles present were trapped during thin section preparation. (**c**) Larger dissolution voids, abundant dissolution pipes, and halite cement filling in voids. Thin mud layer is indicated by the black arrow. Chevron halite is less well preserved in (c) than in (a). The halite in (c) comes from below (a) and (b), so it has been recycled for a longer period of time.

Table 1. Distribution of Prokaryotes (Cells) in Fluid Inclusions in Modern Halite from Death Valley and Saline Valley, CA

Sample number	Number of inclusions studied	Median inclusion side length (μm)	Total volume of studied inclusions (μm^3)	Number of inclusions observed with cells	Number of cells observed	Percentage of inclusions with cells[a]	Cell concentration (cells ml^{-1})
Chevron halite, Death Valley (collected in 1993)							
1	371	3	4×10^5	0	0	0	0
2	437	3	8×10^4	0	0	0	0
3	396	3	2×10^5	2	3	0.5 ± 0.7	2×10^7
Overall	*1204*	*3*	7×10^5	*2*	*3*	0.2 ± 0.2	4×10^6
Salt pan halite cement, Death Valley (collected in 1994)							
1	84	5	1×10^6	0	0	0	0
2	84	6	3×10^5	2	2	2 ± 3	7×10^6
Overall	*168*	*6*	1×10^6	*2*	*2*	1 ± 2	2×10^6
Bottom-growth halite, Saline Valley (collected in 2004)							
1	69	5	6×10^4	29	108	42 ± 12	2×10^9
2	140	4	7×10^4	17	27	12 ± 6	4×10^8
3	111	4	7×10^4	14	21	13 ± 6	3×10^8
4	107	5	7×10^4	20	56	19 ± 8	8×10^8
5	71	5	1×10^5	26	149	37 ± 11	1×10^9
6	127	5	4×10^5	19	80	15 ± 6	2×10^8
7	120	6	2×10^5	36	91	30 ± 8	5×10^8
8	121	5	1×10^5	22	42	18 ± 7	4×10^8
9	106	5	5×10^4	22	52	21 ± 8	1×10^9
10	109	4	3×10^4	17	23	16 ± 7	8×10^8
Overall	*1081*	*5*	1×10^6	*222*	*649*	21 ± 2	6×10^8

[a] $\pm$ two standard errors (95% confidence interval).

indicates fresh to saline paleolake waters (Forester *et al.*, 2005). Halites from 18 to 61 m depth (35,000–60,000 years old) and 78 to 86 m depth (~100,000 years old) contain textures (chevrons and clear halite cement filling solution pipes) diagnostic of ephemeral saline lakes and salt pan paleoenvironments (Li *et al.*, 1996). Large cavities filled with coarse halite cement crystals from 35 to 37 m depth (~45,000 years old) and 54 to 59 m depth (55,000–60,000 years old) provide evidence of low water tables and dry conditions (Li *et al.*, 1996). Much of the rest of the core is composed of terrigenous mud deposited on mudflats (Li *et al.*, 1996), which lacks halite and was not examined.

Results

Halite and fluid inclusion microscopy: modern Death Valley and Saline Valley

Surface halite crusts collected from Badwater Basin in 1993 and 1994 consist of halite layers, 1–4 cm thick, composed of millimeter- to centimeter-sized chevron halite crystals and mud layers up to 0.5 cm thick (Fig. 3). Chevron halite crystals are banded with millimeter-thick zones dense with primary fluid inclusions that alternate with clear bands containing fewer fluid inclusions. Chevron halite layers are typically partially dissolved parallel to or perpendicular to layering. Some dissolution voids are cemented by clear halite. Mud occurs between some halite crystals and may also line the bottom of dissolution voids. Primary fluid inclusions in chevrons are typically small, negative cubes and rectangular prisms with median side lengths of 3 μm; clear halite cements have fewer, larger, primary fluid inclusions and median side lengths of 6 μm (Table 1).

Halite crusts in Saline Valley, formed during the evaporative concentration stage of the saline lake, March to April 2004, consist of sunken rafts and vertically oriented crystals. Individual halite crystals, ~1 millimeter to several centimeters in size, contain large numbers of primary fluid inclusions that vary in size from <1 μm to several millimeters, with median fluid inclusion side lengths of 5 μm (Table 1). Primary inclusions occur as negative cubes, rectangular prisms, irregular shapes, and tubes with long axes parallel to crystal growth faces. Fluid inclusions may be concentrated along halite crystal growth bands or, in other cases, may be distributed more evenly in the halite host crystal.

Halite and fluid inclusion microscopy: the Death Valley core

We examined four types of halite: (1) clear bottom-growth halite, (2) chevron halite, (3) salt pan halite cement, and (4) mega cavity-filling halite cement. Salient crystal textures, fabrics, and fluid inclusion microscopy are summarized here. Halite types 1 and 2 formed at the sediment-water interface in hypersaline lakes and halite types 3 and 4 crystallized in the subsurface from groundwaters.

Saline Lake halite. Two types of primary halites identified in the Death Valley core are clear bottom-growth halite and chevron halite (Fig. 4). Most halite from 7.7 to 18 m depth consists of clear, vertically oriented crystals several millimeters to 2 cm in size, which commonly widen upward in a V pattern (Fig. 4a). Primary fluid inclusions in clear bottom-growth crystals are typically oriented in bands parallel to crystal growth faces (Fig. 4b). Inclusions may be cubic,

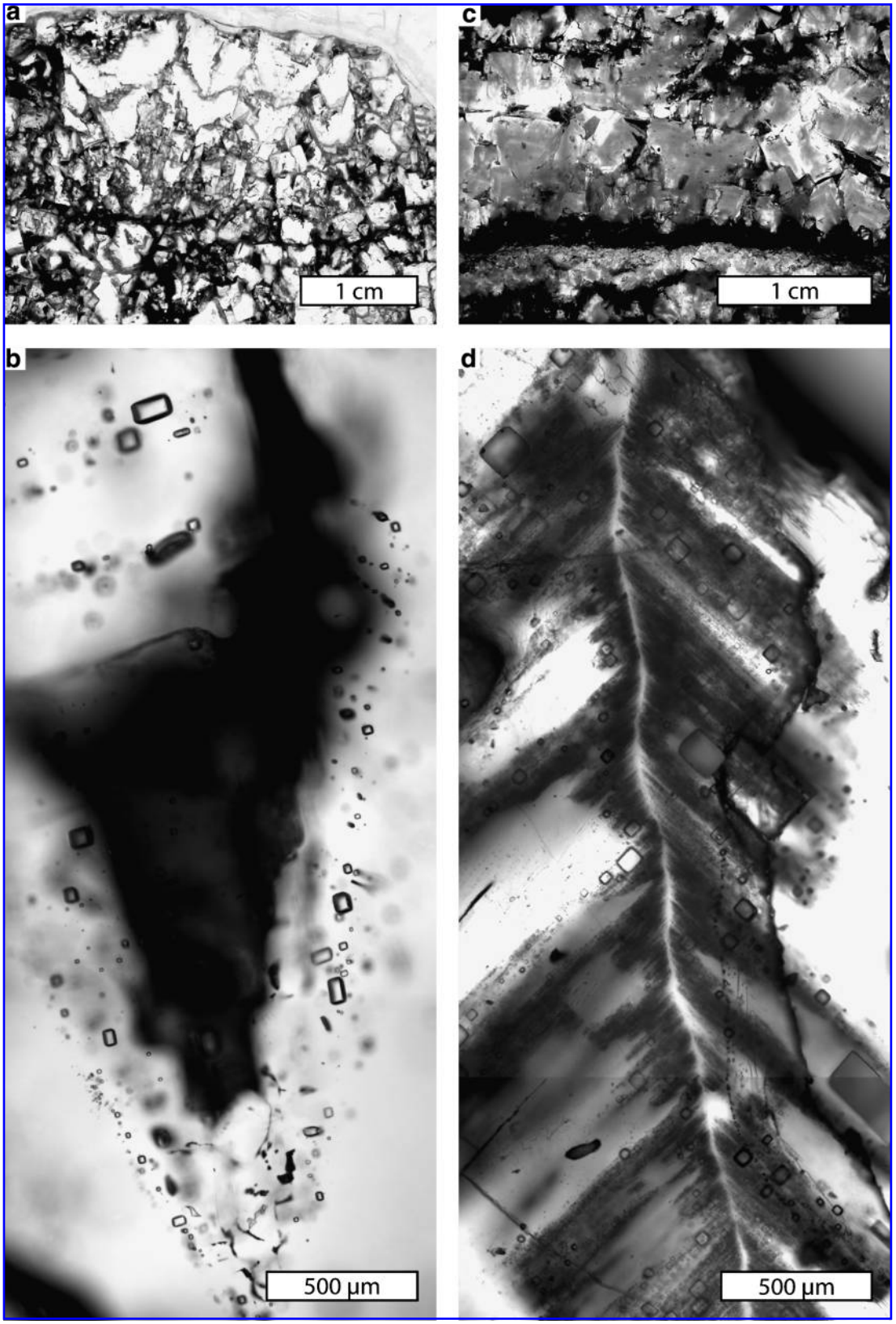


FIG. 4. Photographs and photomicrographs of thin sections of saline lake halite from the Death Valley core. (**a**) Upward widening bottom-growth halite, depth of 15.7 m, displaying vertical growth fabric. (**b**) Clear bottom-growth halite, depth of 15.7 m, with irregular, rectangular, and cubic fluid inclusions oriented in bands parallel to crystal growth. (**c**) Layered chevron halite crystals above a thin mud layer (black), depth of 85.7 m. Chevron halite crystals are cloudy (gray) due to abundant fluid inclusions. (**d**) Chevron halite, depth of 85.7 m, with dense fluid inclusion banding (dark). Clear (white) areas contain fewer fluid inclusions.

Table 2. Distribution of Prokaryotes (Cells) in Fluid Inclusions in Halite from the Death Valley Core

Depth (m)	Age (ka)	Number of inclusions studied	Median inclusion side length (μm)	Total volume of studied inclusions (μm^3)	Number of inclusions observed with cells	Number of cells observed	Percentage of inclusions with cells[a]	Cell concentration (cells ml^{-1})
Clear bottom-growth halite								
8.6	11	188	5	2×10^5	2	11	1 ± 1	6×10^7
12.9	22	58	8	4×10^5	3	23	5 ± 6	6×10^7
13.0	22	119	11	1×10^6	1	65	0.8 ± 2	7×10^7
14.1	25	147	8	5×10^6	1	34	0.7 ± 1	7×10^6
14.2	25	71	11	3×10^6	4	19	6 ± 5	6×10^6
14.6	26	67	9	1×10^6	3	21	4 ± 5	2×10^7
14.7	27	56	9	4×10^5	1	18	2 ± 4	5×10^7
15.7	29	68	13	1×10^6	6	27	9 ± 7	3×10^7
16.5	31	54	15	7×10^5	5	60	9 ± 8	9×10^7
16.6	31	63	14	8×10^5	3	15	5 ± 5	2×10^7
16.7	31	34	23	1×10^6	10	37	29 ± 16	4×10^7
17.5	33	90	9	1×10^6	3	43	3 ± 4	4×10^7
17.8	34	88	11	2×10^6	6	29	7 ± 5	1×10^7
18.0	34	81	8	7×10^5	3	7	4 ± 4	1×10^7
Overall		*1184*	*9*	2×10^7	*51*	*409*	4 ± 1	2×10^7
Chevron halite								
8.1	10	369	3	3×10^5	0	0	0	0
8.2	11	477	3	2×10^5	0	0	0	0
8.3	11	239	3	4×10^5	0	0	0	0
8.7	12	376	4	5×10^5	1	1	0.3 ± 0.5	2×10^6
50.8	55	331	3	2×10^5	0	0	0	0
51.7	56	317	4	4×10^5	2	3	0.6 ± 0.9	8×10^6
82.8	100	320	4	2×10^5	0	0	0	0
83.9	100	193	4	2×10^5	0	0	0	0
84.9	100	554	3	3×10^5	0	0	0	0
85.7	100	526	3	3×10^5	0	0	0	0
86.7	100	379	3	2×10^5	0	0	0	0
Overall		*4081*	*3*	3×10^6	*3*	*4*	0.07 ± 0.08	1×10^6
Salt pan halite cement								
43.8	50	95	6	2×10^6	0	0	0	0
56.3	58	56	8	3×10^6	0	0	0	0
60.1	58	58	11	6×10^5	0	0	0	0
60.4	58	54	8	2×10^6	0	0	0	0
79.2	100	20	12	4×10^6	0	0	0	0
81.5	100	52	5	7×10^5	0	0	0	0
84.4	100	41	8	7×10^5	0	0	0	0
86.8	100	24	11	8×10^7	0	0	0	0
Overall		*400*	*8*	9×10^7	*0*	*0*	*0*	*0*
Mega cavity-filling halite cement								
35.6	44	220	3	1×10^5	0	0	0	0
50.3	55	123	4	2×10^5	0	0	0	0
57.3	58	304	3	1×10^5	2	5	0.7 ± 0.9	5×10^7
58.0	58	423	3	3×10^6	0	0	0	0
58.5	58	88	5	6×10^6	0	0	0	0
Overall		*1158*	*3*	9×10^6	*2*	*5*	0.2 ± 0.2	6×10^5
Core		*6823*	*4*	1×10^8	*56*	*418*	0.8 ± 0.2	4×10^6

[a] $\pm$ two standard errors (95% confidence interval).

rectangular prisms, or irregular in shape and are relatively large, with median side lengths of 9 μm (Table 2). The second type of primary halite, chevron halite (Fig. 4c), differs from clear bottom-growth halite by its cloudy appearance due to numerous fluid inclusions and its submillimeter scale fluid inclusion rich and poor bands (Fig. 4d). Fluid inclusions in chevron halite are typically cubes and rectangular prisms and are commonly small (median side length of 3 μm) (Table 2). The greater number of fluid inclusions in chevron halite versus clear bottom-growth halite probably indicates more rapid crystallization of the former.

Salt pan halite. Salt pan halite textures are characterized by (1) microcrystalline mosaics, (2) patchy remnants of chevrons, and (3) pockets of mud and coarse clear halite that fill cavities (Fig. 5a). Salt pan halites lack well-preserved vertical growth fabrics, and layering is absent or discontinuous (Lowenstein and Hardie, 1985). Microcrystalline halite,

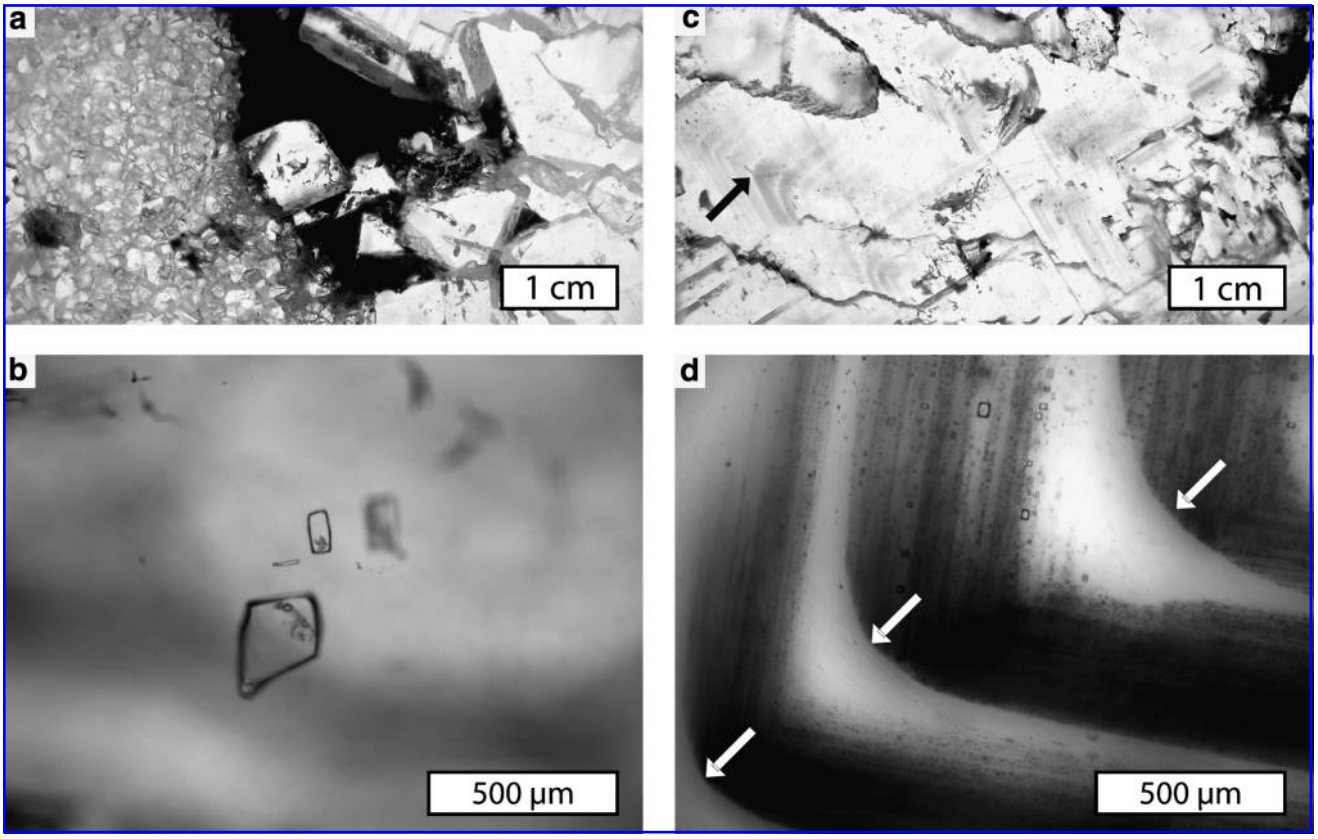


FIG. 5. Photographs and photomicrographs of thin sections of salt pan halite and mega cavity-filling halite cement from the Death Valley core. (**a**) Clear salt pan halite cement crystals (right), microcrystalline efflorescent halite (left), and mud (center), depth of 56.3 m. (**b**) Saline pan halite cement, depth of 79.1 m, with large, isolated, irregularly shaped fluid inclusions. The low abundance of fluid inclusions suggests slow crystallization. (**c**) Large cavity-filling halite cement crystals, depth of 57.3 m. Fluid inclusion bands (arrow) indicate crystal growth to the left. (**d**) Mega cavity-filling halite, depth of 58.5 m, with wide fluid inclusion bands rounded by dissolution (arrows). Dark areas contain abundant fluid inclusions and light areas contain few fluid inclusions. Note the relatively small size of fluid inclusions.

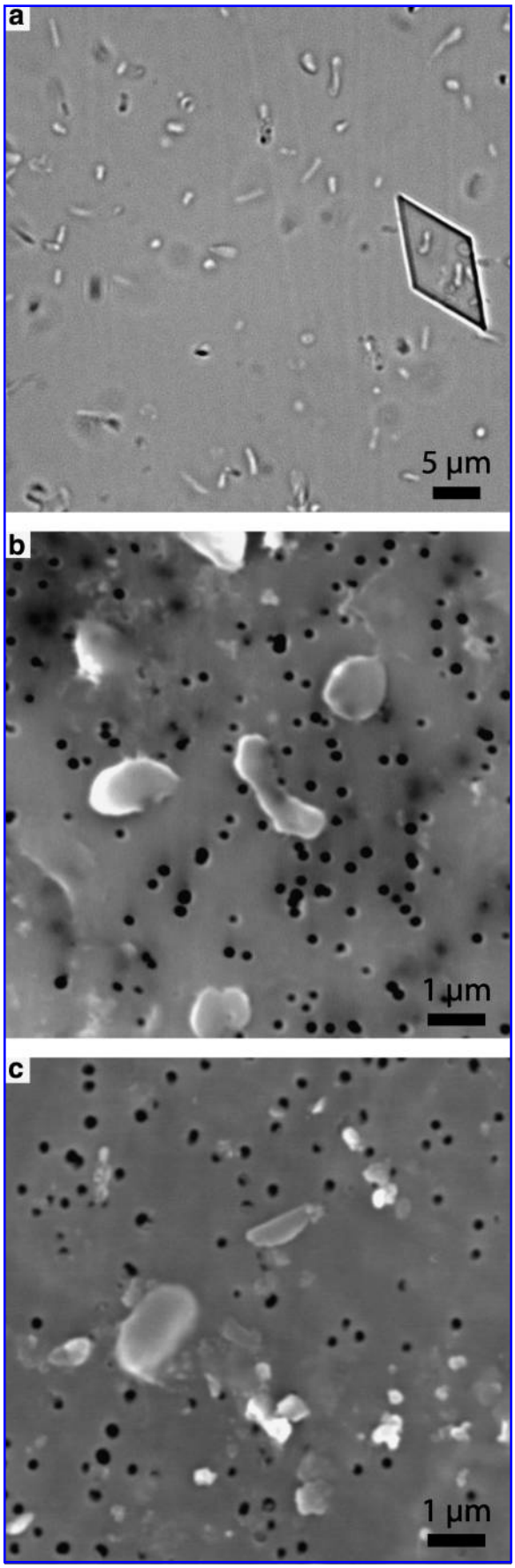
interpreted as efflorescent halite crust formed from evaporation of groundwater brines to dryness, is composed of small, submillimeter-sized crystals that form a poorly layered, discontinuous, equigranular mosaic (Fig. 5a). Pockets of mud and coarse, centimeter-sized halite crystals that crosscut layering are interpreted as cements formed from groundwater brines in dissolution cavities of porous crusts. Primary fluid inclusions in these cements are typically either aligned in bands parallel to crystal faces or isolated in the halite (Fig. 5b). The median side length of fluid inclusions from saline pan halite cement is 8 μm (Table 2). Fluid inclusions in coarse cement crystals are the least uniform in size, and inclusions larger than 100 μm are relatively common.

Mega cavity-filling halite cement. Large dissolution cavities filled with halite cement and insoluble mud from depths of 35–37 m and 54–59 m are evidence that water-table drawdown in Death Valley allowed undersaturated water to percolate through surface salt pan crusts (Li *et al.*, 1996). Cavities are filled with large, fluid inclusion banded halite crystals that are oriented away from cavity walls (Fig. 5c, 5d). Primary fluid inclusions in cavity-filling halite cement are generally small, with a median side length of 3 μm (Table 2).

Microorganism microscopy

Here, we describe the size, shape, and optical properties of possible prokaryote cells *in situ* in fluid inclusions in halite and distinguish them from other microparticles, particularly crystals. We also describe possible prokaryote microparticles from modern brines and those dissolved from halite crystals onto filter membranes, which were observed with an ESEM. Once prokaryotes are positively identified and differentiated from crystals, their abundance and distribution in fluid inclusions in halite can be determined and interpreted in a paleoenvironmental context.

Prokaryotes in modern brine and in fluid inclusions from modern halite. Halophile blooms in Saline Valley (for example, March to April 2004) lead to the development of bright red "tomato soup" brines up to 0.5 m deep in which halophilic prokaryotes (archaea and bacteria) and algae (*Dunaliella*) thrive. The pink-red color of the brine is due primarily to the carotenoid pigments in the membranes of halophilic archaea (family Halobacteriaceae) (Teller, 1987; Oren, 2002). Orange-red brines may also be produced by *Dunaliella salina* cells (β-carotene) and halophilic bacteria (carotenoid) (Oren and Rodríguez-Valera, 2001), but archaea probably dominate



the halophile community in Saline Valley as they do elsewhere. Wet mounts and filtered brines from Saline Valley, which were collected in March 2004 and October 2005, contain rod-shaped microbes (1–12 μm long and $<\sim$1–2 μm wide) and fewer cocci ($\sim$0.5–2 μm in diameter) (Fig. 6a, 6b). Prokaryotes in wet mounts are colorless under transmitted light and lack birefringence under cross-polarized light (*i.e.,* they do not appear bright against the dark background of the isotropic microscope slide and brine). Rods were straight or curved (vibrios), commonly of constant width, and had rounded ends. Some microorganisms were motile in freshly prepared wet mounts made from Saline Valley brines, even one year after collection. Prokaryotes in electron micrographs were typically a similar shade of gray as the polycarbonate filter membrane; however, they commonly had a brighter (white) edge (Fig. 6b, 6c).

Halite crystals precipitated from the red brines in Saline Valley grew vertically off the brine bottom and were pink in color because microorganisms from the water column were trapped in the halite. Primary fluid inclusions in halite crystals collected in 1980, 2004, 2005, and 2007, and the solid residue on filter membranes commonly contain curved and straight rods (1–10 μm long, 0.5–1 μm wide), cocci ($<$1–2 μm diameter), disc shapes, and rare paired spherical (diplococci) microparticles (Figs. 6c and 7). These microparticles inside fluid inclusions and filtered from single halite crystals were interpreted as prokaryotes because they were the same size and shape as halophilic archaea and bacteria observed in wet mounts and on filter membranes prepared from modern brines (Figs. 6 and 7). Prokaryote shapes occur exclusively in fluid inclusions, not the crystal matrix, which is consistent with experimental work (Fendrihan and Stan-Lotter, 2004; Adamski *et al.,* 2006; Fendrihan *et al.,* 2006). Prokaryotes in fluid inclusions are colorless under transmitted light and lack birefringence under cross-polarized light (*i.e.,* they do not appear bright against the dark background of the isotropic halite host crystal). Prokaryotes commonly autofluoresced when epifluorescence microscopy and DAPI, GFP, and TRITC filters were used; autofluorescence with each filter varied for different prokaryotes (Fig. 8). Prokaryotes in fluid inclusions displayed random zigzag movement. Smaller prokaryotes moved faster than larger ones, which suggests that the movement is probably Brownian motion, as observed by Bargar *et al.* (1985) for similar movement of microorganism-like particles in fluid inclusions in quartz. Directional movement of prokaryotes, or motility, was not observed in fluid inclusions in halite from Saline Valley. Fluid inclusions in halite from Searles Lake, California, however,

FIG. 6. Images of prokaryotes from brine and dissolved halite crystals collected from Saline Valley in March 2004. (**a**) Photomicrograph of a wet mount showing rod- and spherical-(coccoid) shaped prokaryotes distinct from diamond-shaped crystal of glauberite ($CaSO_4{\cdot}Na_2SO_4$). (**b**) Electron micrograph of rod- and coccoid-shaped prokaryotes from filtered brine on a track-etched polycarbonate membrane. Prokaryotes are distinct from a brighter crystal at top of image. (**c**) Electron micrograph of prokaryotes filtered from a dissolved modern halite crystal. Crystals appear bright (white). The pores of the filter membrane (black circles) in (b) and (c) are 0.2 μm in diameter.

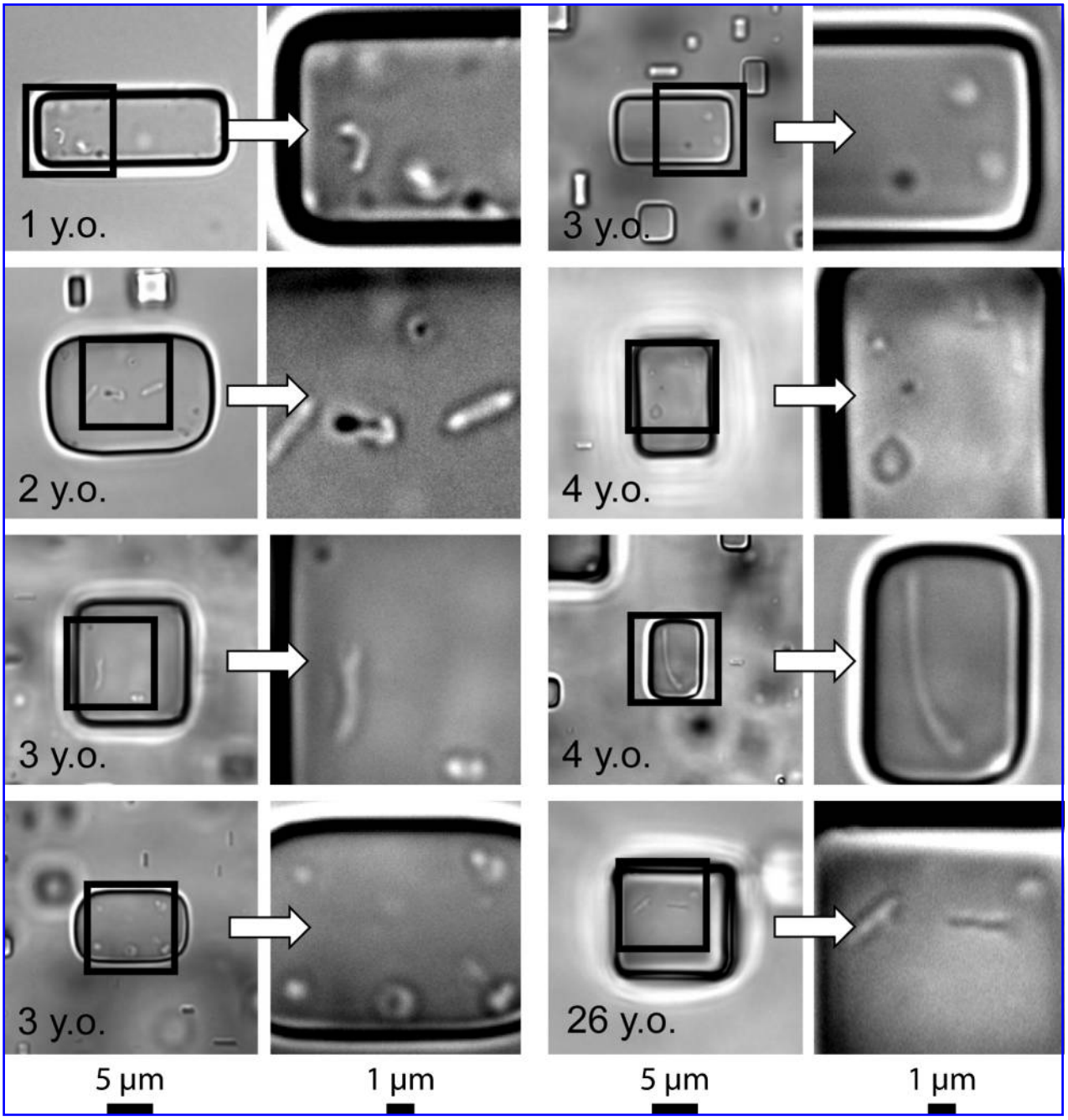


FIG. 7. Transmitted light photomicrographs of prokaryotes trapped in fluid inclusions in halite collected in 1980, 2004, 2005, and 2007 from Saline Valley. Note the variety of sizes and shapes of entrapped microorganisms. Samples range from 1–26 years old (y.o.).

contained motile prokaryotes 15 months after collection of the samples (see Supplemental Movie 1 at www.liebertonline.com/ast).

Ancient prokaryote shapes in fluid inclusions, subsurface halite, Death Valley core. Thin sections and cleavage fragments of halite from 38 stratigraphic intervals of the Death Valley core and the dissolved residues of halite crystals from 16.5 m depth (31,000 years old) were surveyed for prokaryote shapes. Virtually all prokaryote shapes observed *in situ* in fluid inclusions and on filter membranes were cocci and relatively small (<1 μm diameter) compared to most prokaryotes observed from modern brines and halite crystals (compare Figs. 6, 7, and 9). Extremely rare, small (<2.5 μm long), straight rods were also observed. All prokaryote shapes were dull gray in electron micrographs, though they were commonly brighter along their edges. Prokaryote shapes were colorless under transmitted light, and none exhibited birefringence under cross-polarized light. Most prokaryote shapes in ancient halite autofluoresced when epifluorescence microscopy with DAPI, GFP, and TRITC filters (Fig. 10) were used. Prokaryote shapes typically showed random zigzag movement in fluid inclusions suggestive of Brownian motion (see Supplemental Movie 2 at www.liebertonline.com/ast).

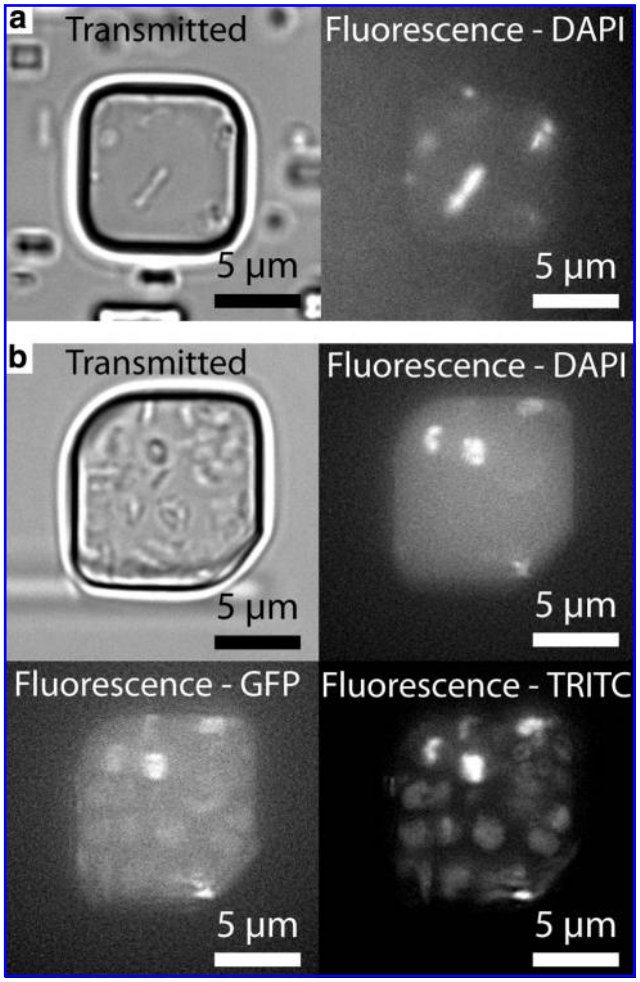


FIG. 8. Photomicrographs of prokaryotes in fluid inclusions in modern halite from Saline Valley using transmitted light and epifluorescence microscopy. (**a**) Prokaryotes in a fluid inclusion in a halite crystal collected in 2004 (4 years old) under transmitted light and epifluorescence (DAPI filter; exposure time of 1251 milliseconds). These prokaryotes autofluoresced more strongly using the DAPI filter than the GFP or TRITC filters (not shown). (**b**) Prokaryotes in a fluid inclusion in a halite crystal collected in 2007 (1 year old) under transmitted light and epifluorescence microscopy (DAPI, GFP, and TRITC filters; exposure times of 1352 milliseconds). Some prokaryotes autofluoresced brightly using the DAPI filter; however, most did not autofluoresce. The most prokaryotes autofluoresced using the TRITC filter.

Crystals in fluid inclusions in halite and on filter membranes: the Death Valley core

Fluid inclusions in halite from the Death Valley core contained a variety of crystals, several microns or smaller in size, which could potentially be confused with prokaryote shapes. Crystals, however, unlike most microorganisms, were typically angular in shape with regular faces. Observed crystals were acicular, bladed, tabular, and platy, and of a variety of polygonal shapes (including triangles, quadrilaterals, and hexagons). Some crystals were rounded or irregular in outline. In electron micrographs, many crystals were typically bright relative to prokaryotes because of the abundance of heavy elements (Figs. 6b, 6c, and 9b). Many crystals in inclusions were colorless under transmitted light; others were reddish orange. Crystals in fluid inclusions were commonly birefringent under cross-polarized light. Most crystals autofluoresced for all filters tested; however, crystals usually autofluoresced more brightly than prokaryotes when the TRITC filter was used. Small crystals, in some cases, exhibited Brownian motion in fluid inclusions, but larger crystals appeared stationary.

Saline minerals in the Death Valley core include halite, gypsum ($CaSO_4{\cdot}2H_2O$), glauberite ($Na_2SO_4{\cdot}CaSO_4$), and thenardite (Na_2SO_4) (Li *et al.*, 1997). Mud layers contain clay (illite and smectite), quartz, feldspar, calcite, and dolomite (Li *et al.*, 1996). Gypsum and glauberite were identified in fluid inclusions on the basis of their crystal habits and birefringence (Fig. 11). Gypsum crystals, colorless under transmitted light and birefringent under cross-polarized light, were distinguished by their six-sided (hexagonal) crystal structure and platy habit. Glauberite crystals were differentiated from gypsum by their diamond-shaped crystal habit.

Criteria for distinguishing crystals from prokaryote shapes. Crystals and prokaryote shapes in fluid inclusions in halite may be distinguished on the basis of size, morphology, and optical and electron backscatter properties. Crystals are commonly larger than prokaryote shapes and occur in a variety of sizes. Crystal characteristics include angular or irregular polygonal morphologies (crystal faces), elongated shapes with non-uniform widths along long axes and rectilinear terminations, rectangular prisms, birefringence, and relatively high electron backscatter due to elements of relatively high atomic number. Prokaryote cell characteristics in fluid inclusions and on filter membranes include smooth shapes, curved rods or straight rods of uniform thickness, coccoid shapes, no corners or edges, relatively uniform sizes, no birefringence, and relatively low electron backscatter. Edge effects commonly cause edges and corners to appear brighter than their adjoining surfaces in electron micrographs. It should be noted that square- and triangular-shaped halophilic archaea and bacteria previously identified (Walsby, 1980; Horikoshi *et al.*, 1993) could complicate the distinction between prokaryotes and crystals. Microorganisms with these angular shapes and lacking birefringence, however, were not observed in fluid inclusions in modern halite from Saline Valley or from the Death Valley core. Observed prokaryote shapes never exhibited birefringence, which made this property an especially important criterion for distinguishing prokaryotes from crystals. Although crystals and prokaryotes commonly both exhibited autofluorescence, when studied in the same field of view with the same exposure times, crystals commonly autofluoresced more brightly than prokaryotes, especially when the TRITC filter was used. On the basis of the criteria given above, the microbial shapes in fluid inclusions and on filter membranes in ancient halite from Death Valley are interpreted as prokaryotes.

Distribution of prokaryotes and paleoenvironmental interpretations

The distribution of prokaryotes in fluid inclusions in halite was determined microscopically from direct microorganism counts and estimates of individual fluid inclusion sizes. The

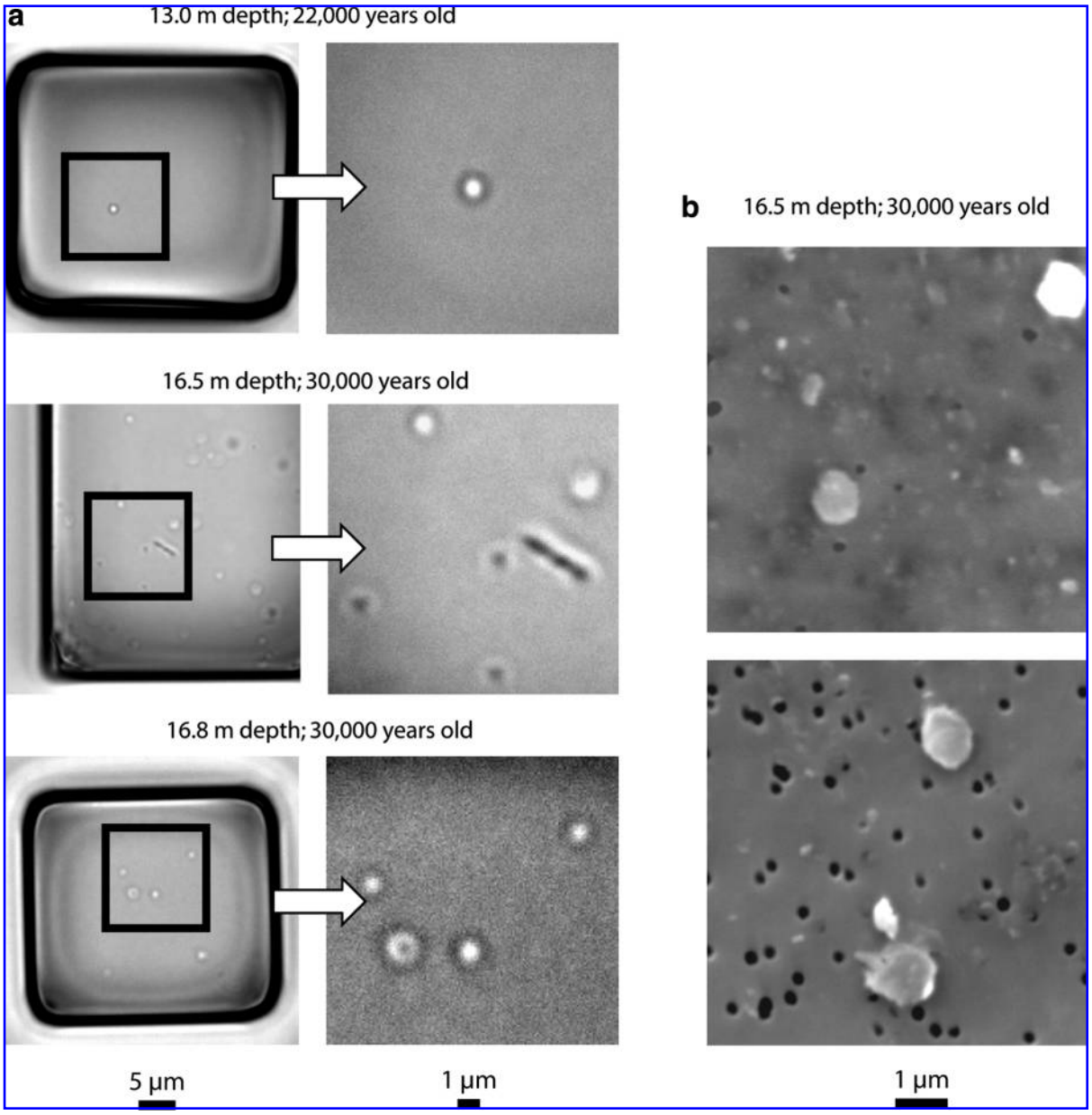


FIG. 9. Images of ancient prokaryote shapes from the Death Valley salt core. (**a**) Transmitted light photomicrographs of ancient prokaryotes trapped in three fluid inclusions in clear bottom-growth halite (perennial saline lake interval) from the Death Valley salt core. Nearly all prokaryote shapes were cocci (<1 μm diameter), but rare rods (<2.5 μm long) were observed. Note that not all prokaryote shapes are in the field of focus at one time. (**b**) Electron micrographs of ancient prokaryotes on a track-etched polycarbonate membrane (pore size 0.2 μm). Prokaryote sizes and shapes are similar to those observed *in situ* in fluid inclusions.

number of prokaryotes in fluid inclusions in pink halite crystals formed during the March 2004 halophile bloom in Saline Valley provided a modern analogue of a biologically productive saline environment (Table 1). Microorganism counts in primary fluid inclusions in modern chevron halite and clear halite cement collected from Badwater Basin, Death Valley, were done for comparison with fluid inclusions from the same halite types in the Death Valley core (Table 1). The distribution of prokaryotes in fluid inclusions in halite from the Death Valley core was determined for 38 stratigraphic intervals divided by halite textures and fabrics into clear bottom-growth halite and chevron halite (both saline lake deposits), and salt pan halite cement and cavity-filling halite cement (both groundwater deposits) (Table 2, Fig. 2).

Microorganism counts in fluid inclusions from ten halite crystals precipitated in Saline Valley in March 2004 show that 222 out of 1081 brine inclusions examined contained prokaryotes, with a total cell count of 649 (Table 1). Prokaryotes were observed in every crystal examined and were present in 21% of all fluid inclusions surveyed. Measurement of the volume of each of these inclusions, together with prokaryote counts, gives the number of prokaryotes (649) in a total volume of fluid inclusion brine: $\sim 1 \times 10^6\ \mu m^3$. Although the total volume of fluid inclusion brine surveyed was by necessity small,

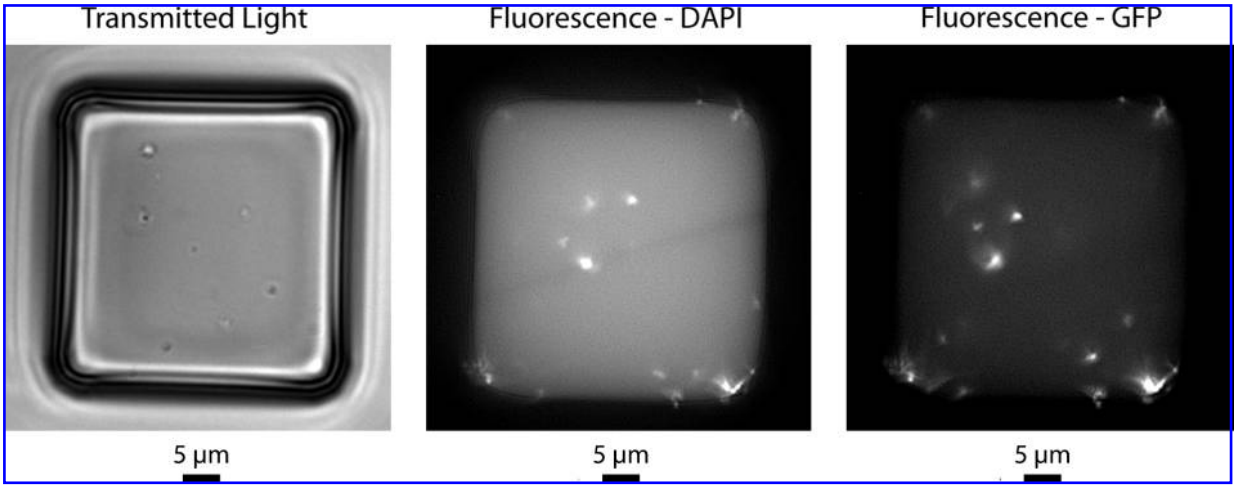


FIG. 10. Photomicrographs of prokaryote shapes trapped in a fluid inclusion in clear bottom-growth halite from 16.5 m depth (30,000 years old) using transmitted light and epifluorescence microscopy. The same fluid inclusion is shown in all three pictures. These microbial shapes, interpreted as prokaryotes, autofluoresced brightly using the DAPI and GFP filters (exposure times of 1107 milliseconds). The prokaryotes also autofluoresced using the TRITC filter; however, the exposure time (5730 milliseconds) was too great for a photomicrograph to be taken of the moving prokaryotes (not shown). Brownian motion of prokaryote shapes causes them to appear in different positions within the same fluid inclusion.

the high concentration of prokaryotes (2×10^8 to 2×10^9 cells ml^{-1} and an overall value of 6×10^8 cells ml^{-1}) is a useful reference for comparison with results from the Death Valley core.

Modern chevron halite from Death Valley, in contrast, contains many fewer prokaryotes in fluid inclusions. Only three prokaryote cells in two fluid inclusions were observed from a total of 1,204 fluid inclusions (volume of 7×10^5 μm^3) surveyed from modern Death Valley chevrons (Table 1). Similarly, salt pan halite cements from Death Valley contain relatively few prokaryotes—only two fluid inclusions out of 168 examined (volume of 1×10^6 μm^3) contained a total of two prokaryote cells (Table 1). The overall cell concentrations in chevrons and salt pan halite cements from modern Death Valley were 4×10^6 cells ml^{-1} and 2×10^6 cells ml^{-1}, respectively. The volume of fluid inclusions surveyed from modern Death Valley halite (chevrons and halite cements) is similar to the volume of inclusions examined from Saline Valley halite, so it is safe to conclude that fluid inclusions in modern Saline Valley halite contain far more prokaryotes than fluid inclusions in modern Death Valley halites.

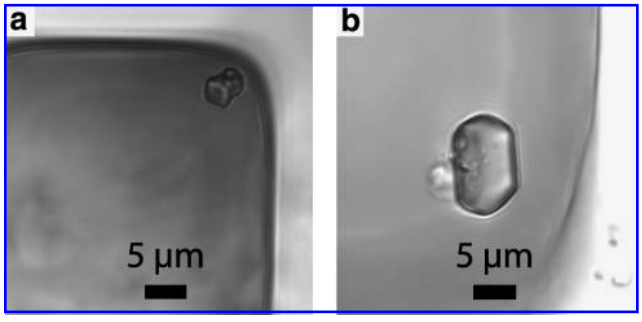


FIG. 11. Transmitted light photomicrographs of crystals trapped in fluid inclusions in halite from the Death Valley salt core. (**a**) Two diamond-shaped, colorless glauberite crystals from 86.7 m depth. (**b**) A hexagonal, colorless gypsum crystal from 86.7 m depth.

Prokaryotes in fluid inclusions occur preferentially in certain stratigraphic intervals of the Death Valley core, particularly clear bottom-growth halite at depths of 8–18 meters (Table 2, Fig. 2), which suggests a paleoenvironmental control on microbe entrapment. All 14 stratigraphic intervals of clear bottom-growth halite examined from this perennial saline lake sequence contained prokaryotes in fluid inclusions. Approximately 4% of the 1,184 total inclusions surveyed contained at least one microorganism. In one interval (16.7 m, 31,000 years old), 10 out of 34 fluid inclusions examined contained prokaryote cells; and, in seven intervals, prokaryote cells were found in at least 5% of the fluid inclusions examined (Table 2, Fig. 2). The total volume of fluid inclusions surveyed was small (2×10^7 μm^3); cell concentrations, however, are similar for all stratigraphic intervals of clear bottom-growth halite (6×10^6 to 9×10^7 cells ml^{-1} and an overall value of 2×10^7 cells ml^{-1}).

Ancient chevron halite rarely contained prokaryotes in the fluid inclusions examined. Only three out of 4,081 fluid inclusions surveyed (volume of 3×10^6 μm^3) from 11 stratigraphic intervals of chevron halite in the Death Valley core contained prokaryote cells (Table 2). Salt pan halite cements from the Death Valley core did not contain prokaryotes in any fluid inclusions examined, from a total of 400 fluid inclusions in halite from eight stratigraphic intervals (volume of 9×10^7 μm^3) (Table 2). Two fluid inclusions out of 1,158 studied (volume of 9×10^6 μm^3) from mega cavity-filling halite cement in the Death Valley core contained a total of five prokaryote cells.

Discussion

Paleoenvironmental control on the preservation of prokaryotes in halite

The survey of fluid inclusions in halite from the Death Valley core demonstrates that ancient prokaryotes are preferentially found in clear bottom-growth halite precipitated in a perennial saline lake in Death Valley 10,000–35,000 years

ago. Several halite intervals of the perennial saline lake section contain relatively large percentages of fluid inclusions with prokaryotes (9%, 9%, and 29% at 15.7, 16.5, and 16.7 m, respectively) that approach the abundances found in the modern halite formed during the Saline Valley 2004 halophile bloom. The relatively large number of prokaryotes in the halite between 8 and 18 m in the Death Valley core suggests a prolific halophile community in the ancient perennial saline lakes of Death Valley. We speculate that slow crystallization of clear halite in biologically productive saline lakes may have allowed large numbers of prokaryotes to be trapped in fluid inclusions. This result is not surprising, considering the high prokaryote cell concentrations reported from many modern saline lakes (Larsen, 1980; Oren, 2002). The Dead Sea, for example, has been colored red at times when halophilic archaea and bacteria reached levels of 1.9×10^7 to 3.5×10^7 cells ml^{-1} (Oren, 2002), which is comparable to the overall cell concentrations estimated for clear bottom-growth halite (8–18 m).

Fewer prokaryotes are trapped in fluid inclusions in ancient halite formed in shallow, ephemeral saline lakes (chevron halite) and from groundwater brines (salt pan and mega cavity-filling halite cements). The relatively small number of prokaryotes in fluid inclusions from modern and ancient Death Valley chevron halite suggests that large halophile communities were not present when chevron crystals precipitated. One possibility is that the shallow saline lakes from which chevrons formed may have been short lived (Li *et al.*, 1996), which did not allow time for large halophile communities to develop. The high density of fluid inclusions in chevron halite indicates rapid crystal growth during periods with high evaporation rates (Lowenstein and Hardie, 1985). It is thus possible that large populations of halophilic prokaryotes were not present in brines during the brief periods when chevrons precipitated. Whatever the explanation, the probability of trapping prokaryotes suspended in the water column in fluid inclusions in halite was lower during rapid crystallization of chevron halite from ephemeral water bodies than during slower crystallization of clear bottom-growth halite in a perennial saline lake.

Halite cements are interpreted to have crystallized below the surface from groundwater brines (Li *et al.*, 1996). Study of diagenetic processes in modern closed basins has shown that voids in halite crusts are typically cemented within the first 10 m of burial and most visible pore spaces are filled within the first 45 m of burial (Casas and Lowenstein, 1989). The scarcity of prokaryotes in salt pan halite cement in the Death Valley salt core indicates that the saline groundwaters from which the cements formed did not support large numbers of microorganisms.

We conclude that the environment in which halite crystallized exerts the primary control on the distribution of prokaryotes in fluid inclusions from the Death Valley core.

Miniaturization of ancient cells

Prokaryotes in fluid inclusions in halite from the Death Valley core are essentially all spherical, $<1\,\mu$m in diameter (Fig. 9), and smaller than most prokaryotes in fluid inclusions in modern halite from Saline Valley collected in 1980, 2004, and 2005 (Fig. 7). Rod-shaped prokaryotes in fluid inclusions in halite collected from Saline Valley are common and typically 2–4 μm long, whereas the extremely rare rods in fluid inclusions from the Death Valley core are $<2.5\,\mu$m long. The differences in size and shape between modern and ancient prokaryotes in fluid inclusions described above resemble the "rounding" of rod-shaped forms described by Norton and Grant (1988) for various halobacteria after several weeks of entrapment in nutrient-free fluid inclusions in laboratory-grown halite. The formation of spheres from original rod shapes was also documented by Fendrihan and Stan-Lotter (2004) for *Halobacterium salinarum* after entrapment in nutrient-free fluid inclusions in laboratory-grown halite for periods of weeks. These changes in size and shape undergone by prokaryotes after entrapment in fluid inclusions appear to be similar to the transformations observed in microorganisms when they are in a state of "starvation-survival" (Morita, 1982, 1997). Starvation survival has been documented for microorganisms in many environments, including soils and marine surface waters, and has been verified by laboratory studies (Novitsky and Morita, 1976; Morita, 1982; Amy and Morita, 1983; Kjelleberg *et al.*, 1983; Kjelleberg and Hermansson, 1984; Moyer and Morita, 1989; Amy *et al.*, 1993; Bass *et al.*, 1998). Laboratory experiments have shown that, in nutrient-poor environments, some microorganisms change shape (rod to coccus) and reduce in size, "miniaturize," over periods of hours to months by fragmentation, that is, cell division without growth, and reduction in size of fragmented cells (Kjelleberg *et al.*, 1983). Upon returning to nutrient-rich environments, miniaturized cells are viable but not always culturable (Amy and Morita, 1983; Amy *et al.*, 1993; Kieft *et al.*, 1997; Bass *et al.*, 1998).

On the basis of the relatively large sizes of rod- and coccoid-shaped prokaryotes observed in fluid inclusions in 1- to 26-year-old halite and the miniaturized cells in fluid inclusions greater than 10,000 years old described above, we suggest that miniaturization has taken place over long periods of time. Unfortunately, the youngest halite in the Death Valley core is 10,000 years old, so the timing of miniaturization of cells is not known with any certainty. The transformation to smaller cell sizes and coccoid shapes apparently occurs much more slowly inside fluid inclusions in halite than in the laboratory studies cited above, perhaps because the composition of natural brines trapped in fluid inclusions is different from the nutrient-free media used in the crystal growth experiments of Norton and Grant (1988) and Fendrihan and Stan-Lotter (2004). Although we do not know the status of miniaturized prokaryote cells in fluid inclusions, we speculate that they are now, or once were, in a starvation state in response to the loss of nutrients from fluid inclusions over long periods of time. Miniaturized cells in fluid inclusions in the Death Valley core are also strong evidence that the prokaryotes in fluid inclusions are not modern contaminants. It is also possible that miniaturization of prokaryote cells occurred before entrapment in fluid inclusions in halite and these miniaturized cells are all that remain of the prokaryote community in the Death Valley core. More research is needed to understand the timing and details of miniaturization of halophilic microorganisms in fluid inclusions in halite.

Conclusions

Prokaryotes in fluid inclusions in ancient halite from Death Valley, probably halophilic and halotolerant archaea and bacteria, were positively identified *in situ* and distinguished from

crystalline microparticles with criteria that include size, shape, and optical properties. Prokaryotes are most common in fluid inclusions in halite that formed in perennial saline lakes 10,000–35,000 years ago (clear bottom-growth halite). Halite formed in shallow, ephemeral lakes (chevrons) and groundwaters (salt pan cements and cavity-filling cements) trapped few prokaryote cells in fluid inclusions. We conclude that the paleoenvironment in which halite precipitated is the most important factor in determining the number of prokaryotes trapped in fluid inclusions in halite in the Death Valley core. These results concur with glacial ice studies in which viability of ancient microorganisms, on timescales of ~12,000 years, is related to climate and not to age because ice from cooler, wetter periods contained more viable microorganisms than ice from warmer, drier times (Christner *et al.,* 2000).

Ancient prokaryotes in fluid inclusions are relatively small and spherical, which suggests miniaturization of cells. Miniaturization may be a survival strategy for prokaryotes trapped in fluid inclusions in halite for long periods. Miniaturization of prokaryotes in fluid inclusions in halite appears to take longer in natural samples than in experimental studies that involve halophilic microorganisms trapped in fluid inclusions in halite (Norton and Grant, 1988; Fendrihan and Stan-Lotter, 2004), but more work is needed to constrain miniaturization times.

The *in situ* identification of prokaryote cells in fluid inclusions in ancient halite from Death Valley should provide additional impetus to search surface sulfate and chloride salts on Mars for signs of life (Squyres *et al.,* 2004; Gendrin *et al.,* 2005; Osterloo *et al.,* 2008). The salts on Mars formed from ancient brines that may have been trapped as fluid inclusions in saline minerals. If ancient surface habitats with hypersaline water existed on Mars, then efforts to find possible preserved microbial life should be focused on the study of fluid inclusions in salt crystals that formed from these martian surface brines. This study shows that prokaryotes are preferentially found in fluid inclusions in ancient halite formed in perennial hypersaline lakes. Presumably, perennial hypersaline lakes would have also been favorable environments for microbial life on Mars. Salt deposits from such perennial lake environments, if they exist, should receive the highest consideration on future Mars missions.

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Author Disclosure Statement

No competing financial interests exist for Brian Schubert, Tim Lowenstein, and Michael Timofeeff.

Abbreviations

ESEM, environmental scanning electron microscope; GFP, Green Fluorescent Protein.

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