How do prokaryotes survive in fluid inclusions in halite for 30 k.y.?

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ABSTRACT

Long-term survival of microorganisms has been demonstrated by prokaryotes cultured from ancient halite, but previous results are controversial. Three genera of non-spore-forming halophilic *Archaea* were cultured from 22–34 k.y. old subsurface halite from Death Valley, California. Primary, brine-filled inclusions in this halite contained prokaryotic organisms in miniaturized starvation-survival forms and dead cells of the algal genus *Dunaliella*. The energy needed for protracted survival of halophilic *Archaea*, including repair of damaged DNA, may have been provided by glycerol and other carbon molecules leaked from *Dunaliella* cells. These results provide further evidence that fluid inclusions in halite are a favorable refuge for long-term survival of microorganisms, and indicate that the original depositional environment influences the distribution and viability of prokaryotes.

INTRODUCTION

Reports of *Bacteria* and *Archaea* cultured from halite crystals, some more than 250 m.y. old, are at the center of the debate about long-term survival of microorganisms on Earth and elsewhere in the solar system (Grant et al., 1998; McGenity et al., 2000; Mormile et al., 2003; Stan-Lotter et al., 1999; Vreeland et al., 2000, 2007). Critics of the evidence for protracted survival of microorganisms in ancient salt formations point to potential recrystallization of halite and late movement of brines and question whether environmental and laboratory contamination can be ruled out (Graur and Pupko, 2001; Hazen and Roedder, 2001; Hebsgaard et al., 2005; Nickle et al., 2002; Willerslev and Hebsgaard, 2005). The ages of reported viable prokaryotes in ancient halite crystals are also at odds with data indicating shorter survival times of bacteria, <1 m.y., established from permafrost (Willerslev and Cooper, 2005; Willerslev et al., 2004). In addition, the bacterium cultured from 250 m.y. old halite of the Salado Formation (*Virgibacillus* sp. Permian strain 2-9-3) forms endospores (Vreeland et al., 2000), but bacteria recovered from permafrost suggest that long-term sustainability is best achieved not by dormancy, but by low-level metabolic activity to repair DNA damage (Johnson et al., 2007). In all cases, little is known about the mechanisms by which ancient microorganisms survive for extended periods inside minerals, such as salt or ice, and from where they obtain energy to perform functions including repair of damaged DNA.

Here we report non-spore-forming halophilic *Archaea* cultured from 22–34 k.y. old halite from the subsurface of Death Valley, California (Fig. 1). All cultured halophiles come from primary halite crystals containing prokaryote cells and algal cells of the genus *Dunaliella*, in situ within primary, brine-filled inclusions. Ancient prokaryotes in fluid inclusions are miniaturized compared to modern cells, which indicates that they are indigenous to the halite and that starvation survival may be the normal response of halophilic prokaryotes to entrapment in fluid inclusions for millennia. We hypothesize that glycerol and other organic compounds leaked from *Dunaliella* cells in fluid inclusions may have been available as carbon sources for prokaryotes to maintain metabolic processes, including DNA repair, for periods of tens of thousands of years. Contamination is improbable because (1) growth of halophilic *Archaea* was only obtained from one particular interval of the Death Valley borehole core where the most abundant prokaryotes were observed in situ within fluid inclusions, (2) cultured *Archaea* are from the *Halorubrum*, *Natronomonas*, and *Haloterrigena* clades, exactly the types expected to be found in halite crystals originally formed in a perennial hypersaline lake, (3) intralaboratory and interlaboratory reproducibility of positive cultures was achieved for this section of the core, and (4) a paired contamination control (rinse from crystal surface) was cultured for each of hundreds of crystals processed, and none of these produced a positive culture.

GEOLOGIC BACKGROUND

A 90-m-long salt core from Death Valley, California, composed of interbedded halite and mud, contains a 100 k.y. record of ancient lake levels, closed basin environments, and paleoclimates (Lowenstein et al., 1999) (Fig. 1). Halite deposited during a prolonged lake episode, 10–35 k.y. ago, exhibits primary textures diagnostic of crystallization in a density-stratified, perennial brine body (Li et al., 1996). Older halite, 35–60 and ~100 k.y. in age, formed in

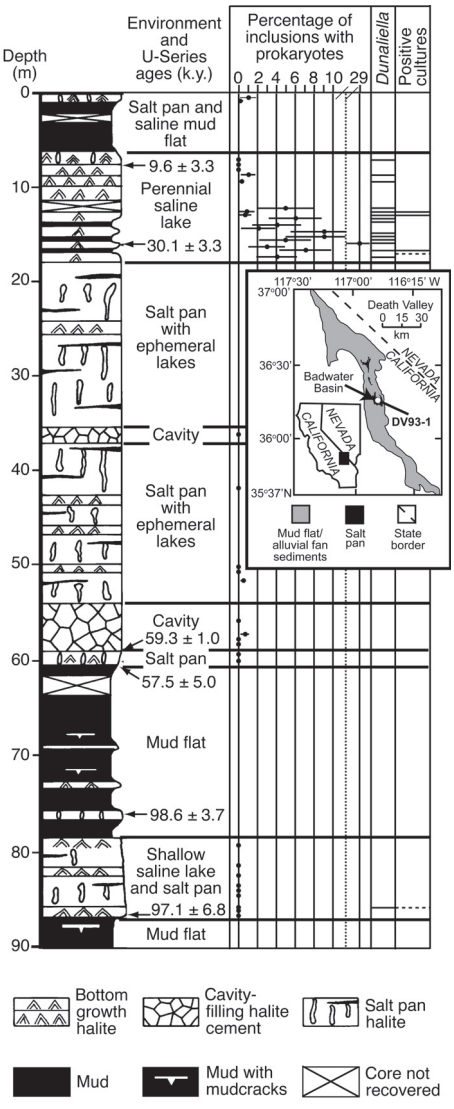

Figure 1. Stratigraphy of Death Valley core DV93–1 from 0 to 90 m (0–100 k.y.) with halite textures, uranium-series ages, and interpreted paleoenvironments; inset map shows location (modified from Lowenstein et al., 1999). Black circles show percentage of fluid inclusions in halite that contain prokaryote cells (left) (error bars indicate one standard error). Horizontal lines mark depths with *Dunaliella* cells in fluid inclusions and positive archaeal cultures (right). Dashed lines at 17.9 m and 85.7 m show depths from which halophilic *Archaea* were cultured by Vreeland et al. (2007) and Mormile et al. (2003), respectively. Note paleoenvironmental control on distribution of prokaryotes, *Dunaliella*, and positive cultures.

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drier salt-pan environments with ephemeral shallow saline lakes, similar to conditions in modern Death Valley, where rare flooding events produce ephemeral (<1 m deep) saline lakes (Li et al., 1996).

RESULTS

Halite crystals from the Death Valley core contain submicron to millimeter-size primary, single-phase aqueous inclusions oriented in bands parallel to growth faces, which demonstrates preservation of brines originally trapped during crystallization in ancient surface environments (Fig. 2A) (Goldstein and Reynolds, 1994). Fluid inclusions surveyed from 38 stratigraphic intervals of the Death Valley core contain relatively small (<1 m diameter) cocci and rare straight rods (<2.5 m long), interpreted as prokaryotes (Fig. 2B) (see the GSA Data Repository[1] for microscopy methods). These prokaryote shapes are colorless under transmitted light and commonly autofluoresce brightly using epifluorescence light microscopy (Fig. 2C). None exhibit birefringence under cross-polarized light, which, along with angular faces or irregular polyhedral morphologies of crystals, helped distinguish putative prokaryotes from minerals. Further study of prokaryote shapes, dissolved and filtered from halite crystals, using environmental scanning electron microscopy (Schubert et al., 2009), confirmed the presence of smooth, small (submicron diameter) cocci (Fig. 2D).

The distribution of prokaryotes in Death Valley halite was determined microscopically from direct microorganism counts of nearly 7000 fluid inclusions (for methods, see the Data Repository). Prokaryotes occur preferentially in halite crystallized in the perennial hypersaline lake, 10–35 k.y. ago; 15 of the 18 stratigraphic intervals examined from this sequence contained prokaryotes in fluid inclusions (Fig. 1). Several samples contained prokaryotes in at least 5% of fluid inclusions, which indicates a prolific halophile community in the ancient perennial saline lakes of Death Valley with prokaryote numbers comparable to the blooms reported from modern saline systems (Oren, 2002). In contrast, prokaryotes were rarely observed in fluid inclusions from ephemeral saline lake and salt-pan halite, 35–60 and ~100 k.y. in age. This indicates that the original depositional environment at the time of crystallization affected the abundance of prokaryotes trapped in the Death Valley halite.

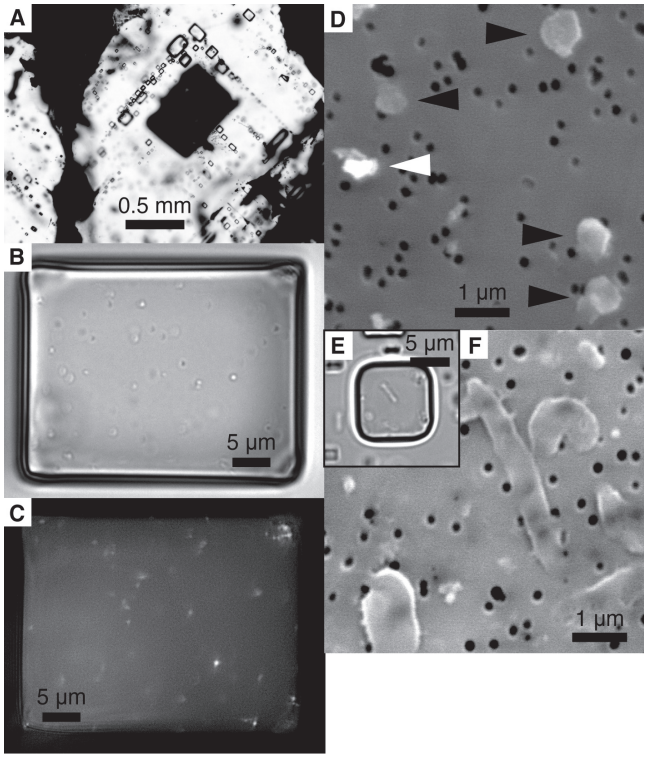

Figure 2. Photomicrographs of fluid inclusions in halite and prokaryotes. A: Halite (white) with irregular, rectangular prism, and cubic fluid inclusions oriented in bands parallel to crystal growth, depth 17.8 m. B, C: Transmitted light and epifluorescence microscopy (green fluorescent protein filter) images of prokaryotes in fluid inclusion (16.5 m; 31 k.y. old). Not all prokaryotes are in the field of focus; Brownian motion caused some prokaryotes to change positions. D, F: Electron micrographs of prokaryotes from halite crystals dissolved on a track-etched polycarbonate membrane (pore size 0.2 m). D: Prokaryotes (black arrows) from depth of 16.5 m are <1 m in size. Crystal (white arrow) shows greater electron backscatter than prokaryotes. F: Modern prokaryotes from Saline Valley, California, halite, crystallized in 2004. E: Transmitted light photomicrograph of prokaryotes in fluid inclusion, modern halite, Saline Valley. Ancient prokaryotes (B–D) are coccoid shaped and smaller than modern prokaryotes (E, F).

Prokaryotes in fluid inclusions in halite from the Death Valley core are essentially all spherical and smaller (<1 m diameter) than most rod and coccoid shaped halophilic prokaryotes from modern saline environments (Fendrihan and Stan-Lotter, 2004; Norton and Grant, 1988) (cf. Figs. 2B–2D and 2E and 2F). The differences in cell size and shape between modern and ancient prokaryotes resemble the rounding of rod-shaped forms reported from experimental trapping of halophilic *Archaea* in nutrient-free fluid inclusions in halite (Fendrihan and Stan-Lotter, 2004; Norton and Grant, 1988), and, more broadly, the miniaturization of cells widely documented in soils, seawater, and laboratory experiments (Morita, 1997). Miniaturization of cells is an important phase of starvation survival during which prokaryotes respond to nutrient-poor environments (Morita, 1997). We conclude that prokaryotes inside fluid inclusions from Death Valley halite have transformed to smaller cell sizes and coccoid shapes.

To establish whether cells observed in fluid inclusions were viable, halite crystals free of fractures and with primary fluid inclusions were surface sterilized and dissolved into media designed to culture halophilic prokaryotes (Table DR1; for culturing methods, see the Data Repository). The high salinity of the culture media, as much as 4.3 M NaCl, greatly limited the types of prokaryotes that could be cultured, therefore reducing the probability of contamination (Willerslev et al., 2004). Of 881 crystals processed from the Death Valley core, 5 (13.0–17.8 m; 22–34 k.y. old) yielded visible growth within 3 months (Fig. 1; Table 1; Table DR2). A 16S ribosomal RNA (rRNA) sequence was obtained from four of the cultures after subculturing to obtain a single colony isolate (DV427, DV462A, DV582A-1, and DV582B-3) (for DNA sequencing methods, see the Data Repository). Subculturing was not successful for the fifth culture (for unknown reasons). DNA purified from this culture was used as template for PCR (polymerase chain reaction) amplification of the 16S rRNA gene, and sequencing of two cloned PCR products (DV582c2, DV582c4) was done to characterize its identity. All cultured prokaryotes were aerobic and did not form spores.

BLAST (basic local alignment search tool) searches on partial 16S rRNA sequences indicated that the four isolates and two clones were halophilic *Archaea* (Table 1) (for methods, see the Data Repository). The archaeon *Natronobacterium* sp. 2-24-1, independently isolated at a separate laboratory (Vreeland et al., 2007) from a halite crystal in the same hypersaline lake interval of the Death Valley core, was the most

[1]GSA Data Repository item 2009266, details of methods, Tables DR1–DR3, and additional references, is available online at www.geosociety.org/pubs/ft2009.htm, or on request from editing@geosociety.org or Documents Secretary, GSA, P.O. Box 9140, Boulder, CO 80301, USA.

TABLE 1. SUMMARY OF ARCHAEA CULTURED FROM THE DEATH VALLEY CORE

Organism name	Depth (m)	Age (k.y.)	Sequence length (bp)	Closest BLAST match	Sequence similarity	Location and environment of BLAST reference organism
DV427	13.0	22	1117	Uncultured haloarchaeon clone TX4CA_24	95.2%	Former Lake Texcoco, Mexico—Alkaline saline soil*
DV462A	14.1	25	1152	*Natronobacterium* sp. 2-24-1	99.3%	Death Valley salt core (17.9 m)–Perennial saline paleolake†
DV582A-1	17.8	34	1406	*Haloterrigena thermotolerans*	98.6%	Cabo Rojo, Puerto Rico–Solar saltern§
DV582B-3	17.8	34	1406	*Haloterrigena thermotolerans*	98.6%	Cabo Rojo, Puerto Rico–Solar saltern§
DV582c2	17.8	34	1406	*Haloterrigena thermotolerans*	98.7%	Cabo Rojo, Puerto Rico–Solar saltern§
DV582c4	17.8	34	1406	*Haloterrigena thermotolerans*	98.4%	Cabo Rojo, Puerto Rico–Solar saltern§
Natronobacterium sp. 2-24-1†	17.9	34	1322	DV462A	99.3%	Death Valley salt core (14.1 m)–perennial saline paleolake
Halobacterium sp. 2-24-4†	17.9	34	1362	*Halobacterium* spp.	98.6–99.7%	Ancient rock salt of various ages
Halobacterium salinarum strain BBH 001**	85.7	100	1239	*Halobacterium salinarum* DSM 671	100.0%	DSMZ culture collection

Note: BLAST—basic local alignment search tool; DSMZ—Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (German Collection of Microorganisms and Cell Cultures).
*Reported in Valenzuela-Encinas et al. (2008).
†Reported in Vreeland et al. (2007).
§Reported in Montalvo-Rodríguez et al. (2000).
**Reported in Mormile et al. (2003).

similar known relative to isolate DV462A (Table 1). This result shows interlaboratory reproducibility in ancient halite (Stan-Lotter et al., 1999; Willerslev et al., 2004). Isolates DV582A-1 and DV582B-3, cultured from different halite crystals in one layer of the hypersaline lake interval (17.8 m; 34 k.y. old), had identical 16S rRNA sequences (1406 base pairs, bp). Clones DV582c2 and DV582c4, 99.5% (1399/1406 bp) similar to DV582A-1 and DV582B-3, were derived from a third halite crystal in this same layer. Such repeated growth of identical or nearly identical strains from one halite layer in temporally spaced experiments demonstrates intralaboratory reproducibility in recovery of related taxa of microorganisms in samples of ancient halite (Hebsgaard et al., 2005; Stan-Lotter et al., 1999; Willerslev et al., 2004).

Growth of halophilic *Archaea* occurred exclusively from halite in the perennial hypersaline lake interval of the Death Valley core (13.0–17.8 m; 22–34 k.y. old) at depths where prokaryotes were commonly observed in situ in primary fluid inclusions (Fig. 1). We failed to culture halophilic prokaryotes from 364 halite crystals that originally crystallized in ephemeral saline lake and salt-pan environments and contained few verifiable prokaryotes in fluid inclusions. These results indicate that paleoenvironmental conditions during halite crystallization strongly affect the number of cells trapped in fluid inclusions, which, in turn, increases the likelihood of attaining positive cultures.

Contamination of crystal surfaces was improbable because 808 controls from the sterilization protocol failed to produce growth of halophilic *Archaea*. Additional contamination testing involved spiking the surfaces of 64 halite crystals with isolates from the Death Valley core (for culturing methods, see the Data Repository). Spiked crystals were then surface sterilized and dissolved into growth medium. No growth resulted from these crystals, confirming that the strains cultured from the Death Valley core could not survive the surface sterilization methods used in this study.

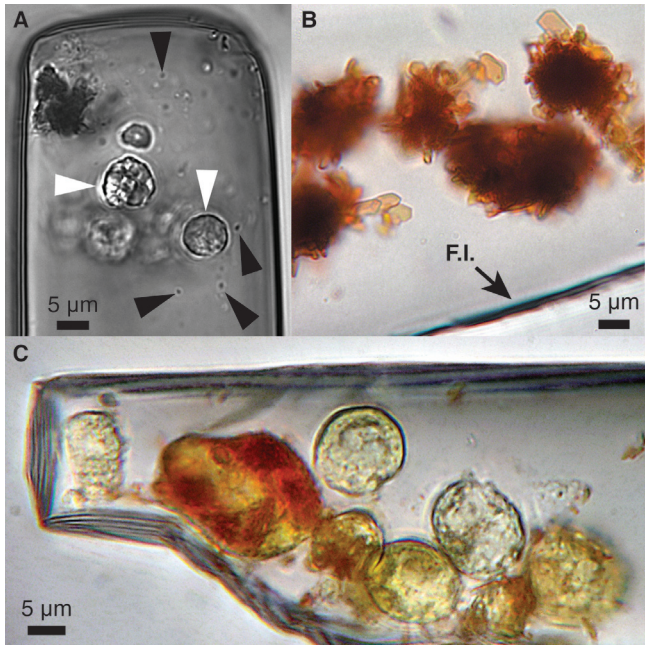

Figure 3. Black and white and color photomicrographs of *Dunaliella* cells in fluid inclusions in halite. A: *Dunaliella* cells (white arrows) and prokaryote cells (black arrows) from 17.8 m (34 k.y. old). Not all prokaryote cells are labeled with arrows. β-carotene crystals cover one *Dunaliella* cell in upper left. Not all *Dunaliella* cells and prokaryote cells are in the plane of focus. B: Crystalline β-carotene encrusting and protruding from Dunaliella cells from 14.5 m (26 k.y. old). Note fluid inclusion boundary (F.I.). C: Dunaliella cells from 17.8 m (34 k.y. old), some encrusted with β-carotene.

PROKARYOTE SURVIVAL MECHANISMS

The genus *Dunaliella* contains the only eukaryotic green algae documented in extremely saline lakes (Borowitzka, 1981). It has long been recognized that glycerol, produced by *Dunaliella* (up to 6–7 M) to maintain osmotic equilibrium with its saline environment, may be used by prokaryotes as a carbon source for metabolic processes in saline lake systems (Elevi Bardavid et al., 2008). *Dunaliella* cells were commonly observed in fluid inclusions in halite from the Death Valley core (Fig. 3); their distribution completely overlapped the location of prokaryotes and viable halophilic *Archaea* (Fig. 1). Halite from each sample that yielded viable halophilic *Archaea* contained prokaryotes and *Dunaliella* together within individual fluid inclusions. We hypothesize that glycerol leaked from *Dunaliella* cells may have supplied the carbon for halophilic *Archaea* to survive long-term entrapment in fluid inclusions. Support for this hypothesis includes the

following. (1) The halophilic *Archaea* isolated from halite crystals in the Death Valley core were capable of growth in a medium containing glycerol as the sole carbon source (culturing methods; see Table DR1 and the Data Repository). (2) *Dunaliella* cells associated with prokaryotes discharged intracellular materials into fluid inclusions. This is illustrated by the solid β-carotene protruding from *Dunaliella* that leaked from cells and crystallized upon coming into contact with fluid inclusion brines, where it is insoluble (Fig. 3). Abundant β-carotene associated with *Dunaliella* is not surprising considering that species of this alga, including *D. salina* and *D. bardawil*, produce β-carotene in response to high light, high salinity, and/or low nutrient concentration (Ben-Amotz et al., 1982; Borowitzka and Borowitzka, 1989; Loeblich, 1982). If β-carotene escaped from *Dunaliella* cells, then water-soluble glycerol is also likely to have leaked from the algae into aqueous fluid inclusions, where it could have provided carbon for prokaryotes to maintain metabolic processes. (3) The carbon from glycerol provided by one *Dunaliella* cell is enough to allow one prokaryote cell to repair macromolecular damage for 12 m.y. (carbon availability calculation; see the Data Repository).

CONCLUSIONS

Fluid inclusions in halite are an ideal environment for long-term survival of certain microorganisms because the saline environment is known to decrease the probability that DNA will be damaged by depurination (Lindahl, 1993; Lindahl and Nyberg, 1972), and with burial microbes are removed from sunlight. Reproducible growth of non-spore-forming halophilic *Archaea* from ancient halite indicates that dormancy is not required for long-term survival. Rather, miniaturized prokaryotes may be better suited to long-term survival because their low-level metabolic activity and small size preserve the finite nutrients available in a fluid inclusion. Glycerol, produced by associated *Dunaliella* cells, may provide the energy required for these prokaryotes to maintain metabolic processes in fluid inclusions for extended periods.

ACKNOWLEDGMENTS

We are grateful to Joanne Pfeil for assistance with sequencing. This project was supported by National Science Foundation Biogeoscience grant EAR-0433802.

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Manuscript received 11 June 2009
Manuscript accepted 30 June 2009

Printed in USA