Research Paper

Entrapment of Bacteria in Fluid Inclusions in Laboratory-Grown Halite

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ABSTRACT

Cells of the bacterium *Pseudomonas aeruginosa*, which were genetically modified to produce green fluorescent protein, were entrapped in fluid inclusions in laboratory-grown halite. The bacteria were used to inoculate NaCl-saturated aqueous solutions, which were allowed to evaporate and precipitate halite. The number, size, and distribution of fluid inclusions were highly variable, but did not appear to be affected by the presence of the bacteria. Many of the inclusions in crystals from inoculated solutions contained cells in populations ranging from two to 20. Microbial attachment to crystal surfaces was neither evident nor necessary for entrapment. Cells occurred exclusively within fluid inclusions and were not present in the crystal matrix. In both the inclusions and the hypersaline solution, the cells fluoresced and twitched, which indicates that the bacteria might have remained viable after entrapment. The fluorescence continued up to 13 months after entrapment, which indicates that little degradation of the bacteria occurred over that time interval. The entrapment, fluorescence, and preservation of cells were independent of the volume of hypersaline solution used or whether the solutions were completely evaporated prior to crystal extraction. The results of this study have a wide range of implications for the long-term survival of microorganisms in fluid inclusions and their detection through petrography. The results also demonstrate the preservation potential for microbes in hypersaline fluid inclusions, which could allow cells to survive harsh conditions of space, the deep geologic past, or burial in sedimentary basins. Key Words: Fluid inclusions—Geomicrobiology—Halite. Astrobiology 6, 552–562.

INTRODUCTION

DURING THE LAST 75 YEARS, a number of studies have indicated that long-term preservation and survival of microorganisms in ancient halite is a distinct possibility (McGenity *et al.*, 2000). Tilden (1930) documented the presence of cyanobacteria in halite from Kansas, Oklahoma, and Texas. Reiser and Tasch (1960) isolated halophilic microorganisms from Permian-age halite from Kansas. Dombrowski (1963) reportedly extracted species of *Bacillus* and *Pseudomonas* from the Zechstein salt of Europe. More recently, Vreeland *et al.* (2000) reported extracting viable cells from the Salado Formation of southwestern United States. The implication of these studies is

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that the microorganisms survived in a dormant state for 250 million years. These results are controversial and subject to intense debate.

Studies indicate that DNA degrades over time as a result of chemical reactions (Lindahl, 1993) and ionizing radiation (Kminek *et al.*, 2003). In the absence of repair mechanisms, DNA degrades within 400,000 years in permafrost (Willerslev *et al.*, 2004), but may survive much longer, with estimates as much as 109 million years (Kminek *et al.*, 2003), in fluid inclusions in halite.

Critics also contend that cells from ancient halite, such as the Salado Formation, could have been transported into the subsurface in more re-cent times. The cells reported by Vreeland *et al.* (2000) were isolated from halite infilling a dissolution pipe, which critics contend could have formed long after the original deposition of the Salado Formation (Hazen and Roedder, 2001). In fact, analyses by Satterfield *et al.* (2005) indicated that homogenization temperatures and brine chemistries of fluid inclusions within the dissolution pipe are consistent with near-surface precipitation of halite during the Late Permian. In addition, many halophilic microorganisms are susceptible to lysis when suspended in fresh water; therefore, transport of halophilic microorganisms from a surficial source to the subsurface
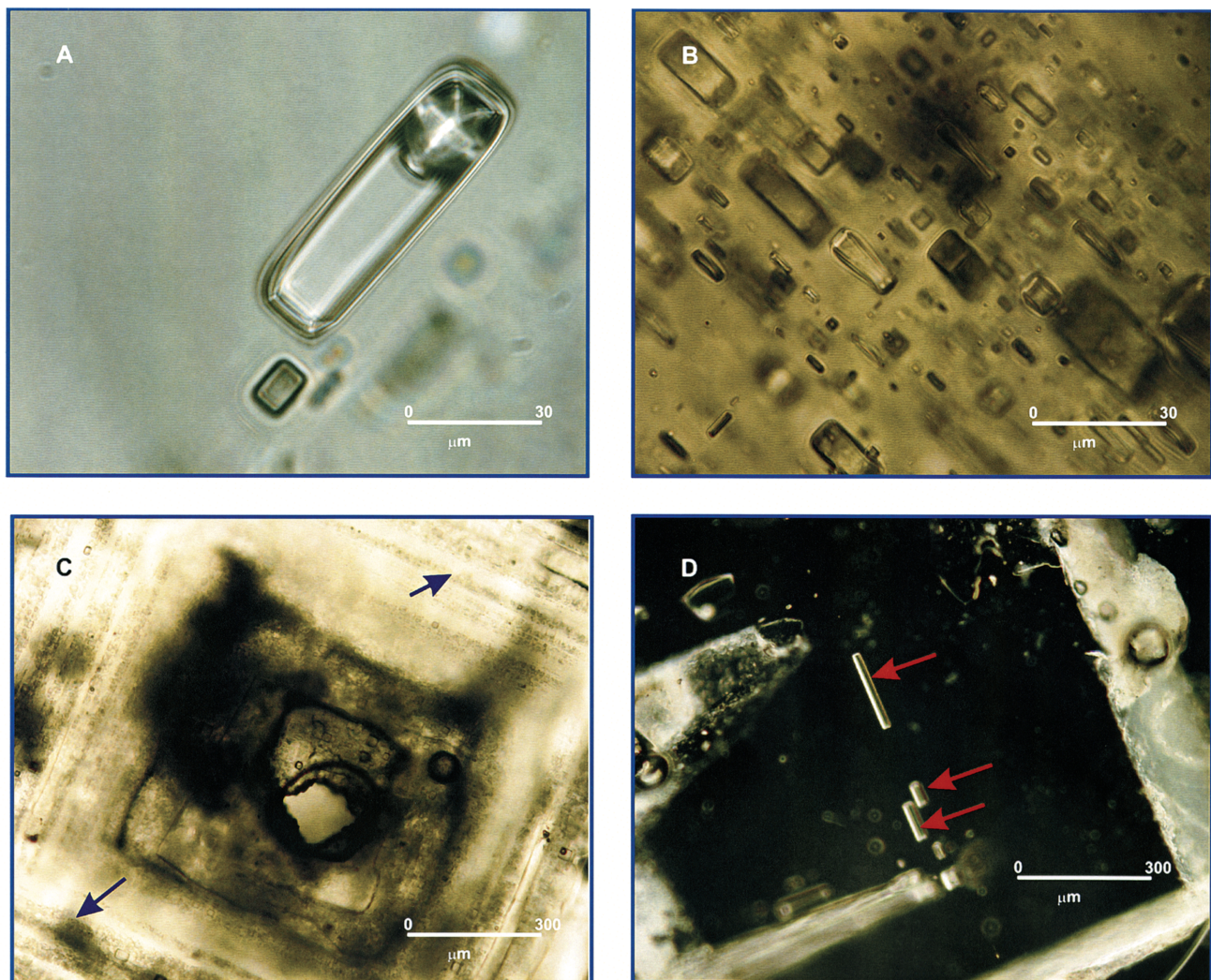

FIG. 1. Fluid inclusions in laboratory-grown halite crystals. Note gas bubble in (**A**), and alignment of fluid inclusions with crystal growth zones in (**B**) and (**C**). Also note the uneven distribution of inclusions in (**D**), with large areas of crystal devoid of inclusions. (A) Grown under sterile conditions in experiment 1 (May 2004). (B and C) Grown with *P. aeruginosa* in experiment 1 (May 2004). (D) Grown with *P. aeruginosa* in experiment 2 (September 2004). All images were taken with phase-contrast microscopy.

by infiltrating meteoric water would be unlikely (Stan-Lotter *et al.*, 2004).

Finally, critics contend that revived cells could be a result of contamination during laboratory handling and preparation. The bacterium isolated from the Salado Formation (Vreeland *et al.*, 2000) has been shown to be 99% genetically identical to *Salibacillus marismortui*, a modern strain from the Dead Sea (Maughan *et al.*, 2002). Stringent sterilization techniques and isolation of cells directly from fluid inclusions (Vreeland *et al.*, 2000; Mormile *et al.*, 2003), however, should prevent laboratory contamination.

Most petrographers who examine fluid inclusions in ancient halite have not documented the presence of microorganisms, which again could indicate that revived cells are in fact modern contaminants. However, microscopy techniques used by most fluid inclusion petrographers are dissimilar to techniques used by microbiologists, and may not be optimal for observing entrapped cells. Dombrowski (1963) showed an image of *Bacillus* cells entrapped in the crystal matrix of halite, which would indicate that ancient cells are present in some halite crystals.

Discovery of ancient viable microbial cells is more than just an academic study, but has numerous applications and implications in the search for extraterrestrial microorganisms. Entrapment within fluid inclusions can protect cells against desiccation, biochemical degradation (Mormile *et al.*, 2003), and the effects of radiation (Kminek *et al.*, 2003). Entrapment within fluid inclusions also could provide a mode of transport for microorganisms, both here on Earth and in the vacuum of space. Microscopic crystals and traces of halite have been detected in meteorites collected from Egypt, France, India, and Texas, which indicates transport of halite across the solar system (Stan-Lotter *et al.*, 2004). The Monahans (1998) H5 chondrite contains halite with two-phase (aqueous and gas) fluid inclusions (Zolensky *et al.*, 1999). Evaporite and (or) saline minerals also may exist on Mars, as indicated by recent images and the prospect of past aqueous environments on that planet (Beyer *et al.*, 2000; Squyres *et al.*, 2004; Montgomery and Gillespie, 2005).

To date, few studies have been conducted regarding the process of microbial entrapment in halite and the resulting effects on both cells and crystal growth. Norton and Grant (1988) determined that pure cultures of halophilic Archaea were readily entrapped in fluid inclusions in halite at concentrations greater than or equal to the concentration of cells in the hypersaline solution. They also noted that laboratory-grown halite crystals with entrapped microorganisms had larger and more numerous fluid inclusions than did sterile crystals. Research in salterns indicates possible biomineralization of halite by halophilic microorganisms (Oren, 2002), which could explain the preferential entrapment reported by Norton and Grant (1988).

Many questions remain concerning the processes and effects of bacterial entrapment, including the effect of cell motility and random versus preferential entrapment. Motile cells can move and remain evenly distributed in the solution. Non-motile cells might float or flocculate and sink, depending on the relative density of the cells compared with the hypersaline solution. Hence, cell motility could affect entrapment and preservation. *Pseudomonas aeruginosa* also is capable of attaching to surfaces and forming biofilms (Stewart *et al.*, 1993; Costerton *et al.*, 1995), which could allow for preferential entrapment of the cells.

In this study, we investigated the controls on entrapment and preservation of microorganisms in halite through an experimental approach in which halite was grown in the laboratory in the presence of motile, non-halophilic bacteria. The objectives of the study were as follows:

1. Document petrographic and microscopy techniques that optimize viewing of cells in fluid inclusions. Petrographers have conducted numerous investigations of fluid inclusions in halite, yet few studies have documented the presence of entrapped cells. Microscopy techniques need to be optimized to assess the abundance of entrapped cells in ancient deposits.
2. Evaluate the effect of non-halophilic bacteria on halite crystal growth and the size and shape of fluid inclusions.
3. Determine if motile cells are entrapped, or if attachment to crystal surfaces is necessary for entrapment. Also, determine whether cells become entrapped in the mineral matrix or exclusively in fluid inclusions.
4. Assess the survival and (or) preservation of cells in the fluid inclusions during the year-long experiment.
5. Evaluate whether the concentration of entrapped cells is representative of the concen-

tration of cells in the aqueous solution or whether preferential entrapment occurs.

Petrographers have used fluid inclusions as a method for evaluating paleoenvironments (Goldstein, 2001). Entrapment and preservation of ancient cells in fluid inclusions could further assist researchers in understanding the microbial ecology of ancient hypersaline systems. The results of this study could identify indicators—such as diagnostic patterns of fluid inclusions or concentration and distribution of entrapped cells—that could aid in distinguishing ancient microorganisms from more modern contaminants. Further-

more, diagnostic shapes and sizes of fluid inclusions in ancient deposits could indicate cell entrapment, even if the original cells have since degraded into organic residue. Finally, these results could assist in assessing the preservation potential of bacteria in halite.

MATERIALS AND METHODS

P. aeruginosa, a rod-shaped Gram-negative bacterium, 2–3 μm in length, with a single flagellum, was used in entrapment experiments with laboratory-grown halite. Species of *Pseudomonas* have
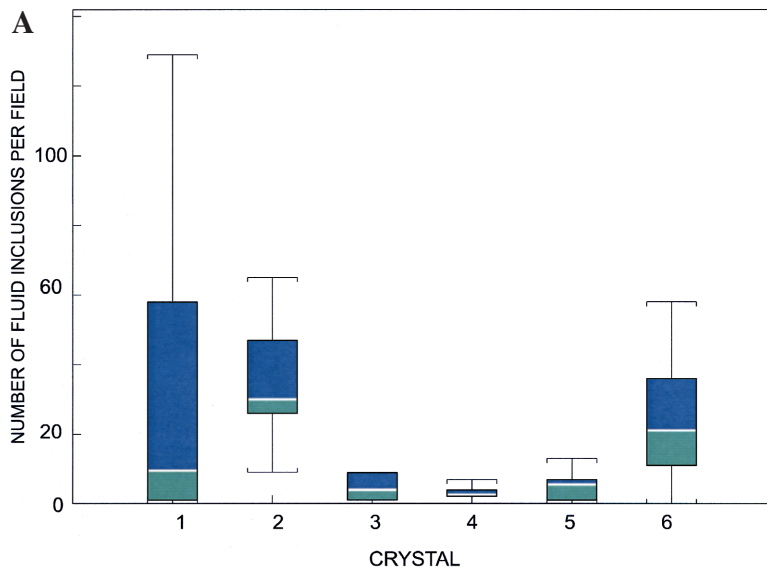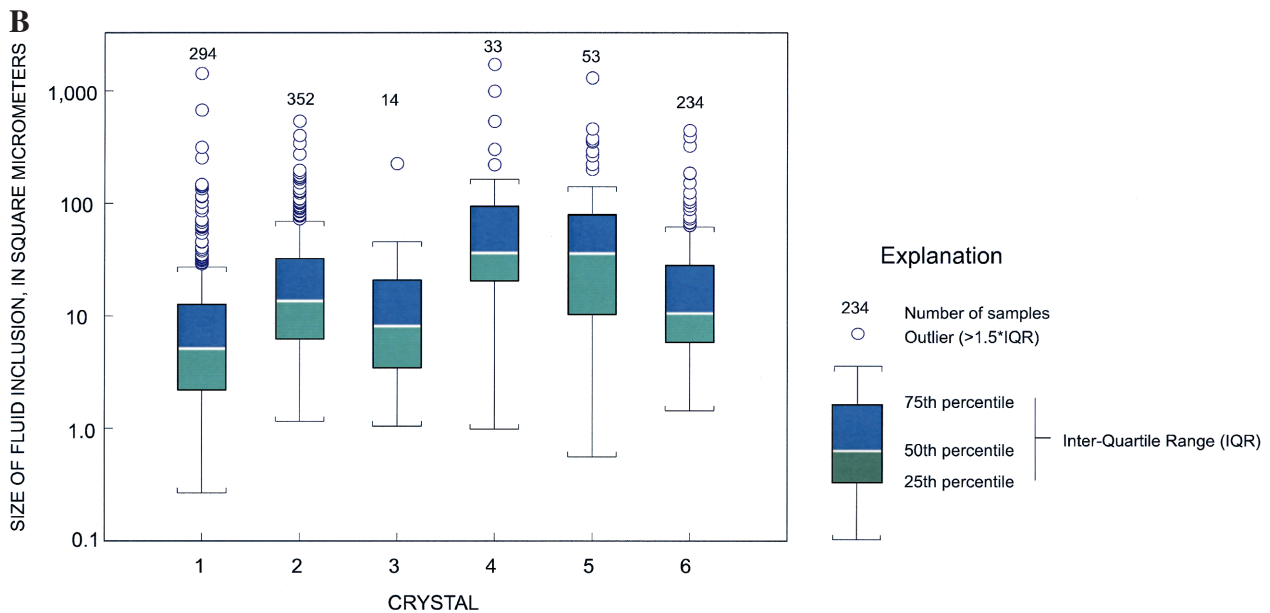

FIG. 2. Number (A) and size of fluid inclusions (B) in laboratory-grown halite crystals: crystal 1, experiment 1, inoculated, allowed to desiccate; crystal 2, experiment 1, inoculated, not desiccated; crystals 3 and 4, experiment 2, inoculated; and crystals 5 and 6, experiment 1, sterile.

been recovered from halite and hypersaline environments (Dombrowski, 1963; Oren, 2002). Although it is not a halotolerant species, *P. aeruginosa* is abundant, occurs in a variety of terrestrial and aquatic environments, and could conceivably be transported to hypersaline water bodies by streams and wind. In addition, the non-halophilic *P. aeruginosa* was used in this study as a negative control (Ruxton and Colgrave, 2003) in assessing the effects of motility and understanding the processes of entrapment.

The *P. aeruginosa* strain used in this study was also selected to aid in tracking cell movement and viability. This strain (obtained from T. Beveridge, University of Guelph, Guelph, ON, Canada) had been genetically modified to produce green fluorescent protein (GFP) (Bloemberg *et al.*, 1997), which is naturally produced by the Pacific jellyfish (Yang *et al.*, 1996). The presence of the protein has been used for tracking the movement and assessing the viability of microorganisms, as GFP production stops after cell death (Normander *et al.*, 1999). GFP was useful in observing cells in fluid inclusions and searching for cells in the mineral matrix. The presence of GFP assured us that cultured cells were not contaminants, and the presence or absence of the fluorescing protein was a useful indicator in evaluating degradation of the cells.

P. aeruginosa was grown in Luria broth (Difco, Detroit, MI) and incubated (incubator from Fisher Scientific, Hampton, NH) at 37°C for 24 h. The resulting solution had a cell concentration of 4.5×10^9 cells/ml as determined by microscopic direct count (Roane and Pepper, 2000). Cultured cells were highly motile, exhibiting characteristic run-and-tumble motility. The cells fluoresced bright green as a result of GFP production when exposed to ultraviolet light (395 nm wavelength).

Two separate, but similar, experiments were conducted. In each case, the bacterial culture was added to a sterile solution (1:3 vol/vol) that contained 280 g/L NaCl. Ten milliliters of the inoculated hypersaline solution was put into each of two 10-ml beakers during experiment 1 (April 2004), whereas 200 ml of the inoculated solution was put into each of two 250-ml beakers during experiment 2 (August 2004). In both experiments, the vessels that contained the hypersaline solution were placed under a laminar-flow hood and allowed to evaporate passively at room temperature (~25°C). Control beakers included sterile deionized water and sterile hypersaline solution with and without Luria broth. A beaker of deionized water inoculated with bacterial culture was also used to verify cell survival in the laminar flow hood. Sterile hypersaline solution and deionized water were added to the beakers that contained inoculated hypersaline solution and water, respectively, to prevent complete desiccation. Experiments 1 and 2 lasted 4 and 6 weeks, respectively. After 4 weeks, one of the two beakers from experiment 1 that contained inoculated hypersaline solution was allowed to completely desiccate. Halite was removed from the remaining beakers (experiment 1) and flasks (experiment 2) prior to complete desiccation.

The fluorescence and motility of cells in the NaCl solution were qualitatively assessed biweekly using wet slides made by placing one drop (~30 μl) of solution on a slide with a coverslip. Microscopic enumeration of cells in solution in experiment 2 was determined by microscopic direct count (Roane and Pepper, 2000) of wet slides and reported as cells/ml. Microscopic-scale variations of cell concentrations in the hypersaline solution was assessed by imaging two fields of view and counting the number of cells in each of the 400-μm^2 grid squares digitally overlain onto the imaged fields. Sterile Luria broth was inoculated with *P. aeruginosa* from the hypersaline solutions (1:10 vol/vol) and incubated for 24 h to determine whether *P. aeruginosa* cells remained viable.

Thick sections (~200 μm) for examination of fluid inclusions (Goldstein and Reynolds, 1994) were made by polishing halite crystals with polishing paper (320, 600, 1,000, and 1,500 grit) wetted with isopropyl alcohol. The thick sections were examined using normal condensed transmitted-light illumination, ultraviolet light (395 nm), and phase-contrast light microscopy with a 10× objective [Nikon (Tokyo, Japan) plan fluor], and oil-immersion 60× and 100× objectives (Nikon plan apo; Nikon Eclipse E600 microscope equipped with an ultraviolet source and Nikon DXM1200 digital camera).

Fluid inclusions were analyzed in six laboratory-grown halite crystals that were produced as follows: crystal 1, experiment 1 (4 weeks), inoculated with *P. aeruginosa*, allowed to desiccate; crystal 2, experiment 2 (4 weeks), inoculated with *P. aeruginosa*, not allowed to desiccate; crystals 3 and 4, experiment 2 (6 weeks) inoculated with *P. aeruginosa*; and crystals 5 and 6, experiment 1 (4 weeks), sterile. Ten fields of view (imaged with the 100× oil objective) were examined for each crystal, except crystal 3, for which only five fields

FIG. 3. *P. aeruginosa* **cultured from NaCl-saturated aqueous solution.** Genetically modified cells produce GFP, and fluoresce under ultraviolet light (395 nm). GFP is light sensitive, and fluorescence decreases during imaging, which could explain the lack of fluorescence of some cells.
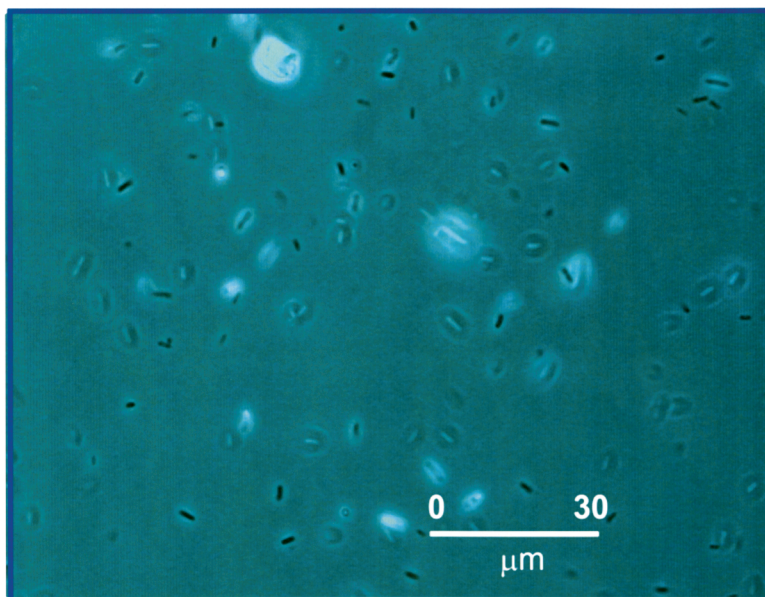
were examined because of the small number of inclusions present. Each field of view was 13,500 μm^2 in area. The number and size (perimeter and area) of fluid inclusions in each field of view were measured using analySIS software (version 3.2, Soft Imaging System, Münster, Germany). The non-parametric Kruskal–Wallis test (S-Plus 2000) was used to determine if the median number and size of fluid inclusions were significantly (α level = 0.05) different between the crystals.

Cells in fluid inclusions were positively identified by their size, morphology, and fluorescence. Microscopy techniques (bright field and phase contrast) and settings (stage diaphragm and light intensity) were varied to determine optimal conditions for observing microorganisms in fluid inclusions without the benefit of fluorescence. Optimal settings for light intensity were determined by the objective used (for the Nikon E600 microscope the 10× requires setting 1 in bright field and Ph1 in phase contrast; 60× requires 2 and Ph2, respectively; and 100× requires 3 and Ph 3, respectively). To view cells, we found that the substage diaphragm should be almost entirely closed. The actual setting of the diaphragm was dependent on the thickness and clarity of the halite crystal. Alternatively, the inclusions should be viewed using phase-contrast microscopy in order to distinguish cells from the hypersaline solution.

The concentration of cells trapped in fluid inclusions was determined by direct count using phase-contrast microscopy. The number of cells was counted in each of 21 randomly selected in-

clusions. The volume of each inclusion was determined by multiplying the area by a depth estimated to be equal to the width. Finally, the number of cells was extrapolated and reported as cells/ml. The concentration of cells in the fluid inclusions was statistically compared to the concentration of cells in solution using the Wilcoxon rank sum test (S-Plus 2000).

Attempts were made to culture cells from laboratory-grown halite crystals. During experiment 2, cultures were made by dissolving the halite [surface-sterilized with ethanol (Reiser and Tasch, 1960)] in Luria broth and incubating at 37°C for 24 h. One month after the end of experiment 2 (October 2004), culturing of entrapped cells was attempted by dissolving the halite in deionized water, filtering the solute, then putting the filter in a Petri dish with Luria broth, and incubating as above. Both culturing experiments included sterile controls.

Halite crystals from each experiment were continually examined microscopically, and the entrapped cells were imaged through June 2005. The cells were examined microscopically for motion and fluorescence.

RESULTS

Fluid inclusions

Halite crystals formed in each experiment after 2–4 weeks of evaporation. In general, crystals

from experiment 2 (6 weeks) were larger than those from experiment 1 (4 weeks). Many of the crystals contained abundant fluid inclusions. Fluid inclusions exhibited a negative crystal shape (Fig. 1) and were square or rectangular in two dimensions. A total of 981 fluid inclusions were counted and measured, which ranged in size from 0.3 to 1,700 μm^2, with a median of about 13 μm^2.

The vast majority of the fluid inclusions were single phase; only seven two-phase (gas and liquid) inclusions were positively identified in crystals from experiment 1 (Fig. 1A). The gas bubbles ranged from 16 to 374 μm^2 in area, or from 4% to 42% of the area of the fluid inclusion. Inclusions from experiment 2 contained only liquid and microbial cells.

The distribution of the fluid inclusions was highly variable. In some parts of the halite crystals, the fluid inclusions appeared to have a random distribution in three dimensions (Fig. 1D). In other parts of the crystal, fluid inclusions were concentrated in concentric growth zones separated by inclusion-free zones (Fig. 1B and C). Fluid inclusions were absent from large areas of some crystals, particularly in crystal 3 (Fig. 1D).

Results of statistical analysis indicate that the number and size of fluid inclusions were significantly different between experiments 1 and 2, and between inoculated and sterile crystals. However, the variation within each crystal was as great as the variation between the crystals (Fig. 2). For example, the median size of fluid inclusions in four crystals (sterile and inoculated) from experiment 1 ranged from 5 to 36 μm^2, whereas the median sizes in the two crystals from experiment 2 were 8 and 36 μm^2.

Bacteria

Green fluorescent cells were common in the solution of all beakers inoculated with *P. aeruginosa*, but absent from the sterile solutions. The concentration of *P. aeruginosa* cells in the hypersaline solution ranged from 0 to 8.8×10^9 cells/ml with a median concentration of 3.8×10^9 cells/ml, as indicated by direct microscopic count. *P. aeruginosa* appeared to be viable in the hypersaline solutions during the duration of both experiments as indicated by bright fluorescence. In relatively fresh water, *P. aeruginosa* moves in a series of runs and tumbles. In contrast, the cells in the hypersaline solutions exhibited a twitching-type motion, possibly due to the high density of the so-

lution adversely affecting cell motility. Figure 3 shows cells cultured during experiment 2 by inoculating sterile Luria broth with *P. aeruginosa* from hypersaline solution. The Luria broth became turbid after incubation for 24 h, which indicated log-phase growth. Sterile control samples had no growth. These results demonstrate that *P. aeruginosa* remained viable in hypersaline solutions for at least 6 weeks. Nearly all of the cells shown in Fig. 3 exhibited fluorescence; however, fluorescence decreased upon exposure to ultraviolet light during imaging. Furthermore, the fluorescence was dim in comparison with the visible light necessary for imaging.

Microscopic analysis indicated that bright, green fluorescing *P. aeruginosa* cells were common in numerous fluid inclusions in the halite crystals from experiments 1 and 2, but were absent from crystals grown in sterile solutions. Cells in the inclusions were difficult to see under normal condensed illumination transmitted-light microscopy, but were more easily viewed by closing down the iris diaphragm so that very little light escaped the substage or by using phase-contrast microscopy (Fig. 4A). The cells were approximately 2-μm-long rods and did not appear to have lysed or be misshapen as a result of immersion in the hypersaline solution and subsequent entrapment. All of the entrapped cells appeared to be within fluid inclusions, and none was identified within the crystal matrix.

Entrapped cells did not appear to be attached to the walls of the fluid inclusions but were moving freely in solution. Cells in the inclusions exhibited a twitching motion, similar to cells in the hypersaline solution, but were necessarily more stationary and restricted in their movement. The motion could be cell motility or Brownian motion caused by thermal gradients inside the fluid inclusion. The motion continued even if the only light source was ultraviolet.

The thick sections were examined to determine the percentage of large fluid inclusions that contained cells. In crystals 2, 3, and 4, every large (greater than 35 μm^2) inclusion examined contained cells. About 9% of the inclusions were less than or equal to 2 μm^2 (approximately the size of a single cell) and probably could not have contained any cells because of their small size. Cells were also present in every large inclusion in the outermost growth zone of crystal 1. However, an assemblage of large inclusions in the center of crystal 1 appeared to be devoid of cells.

For example, cells were absent in all nine inclusions (33–930 μm^2 in area) in one field of view (total area 37,000 μm^2), which is particularly anomalous considering the ubiquitous presence of cells in all the other large fluid inclusions.

The number of entrapped cells ranged from 2 to 20 in each of 21 fluid inclusions with volumes ranging from 215 to 33,000 μm^3. Concentrations of entrapped cells ranged from 3.9×10^8 to 2.8×10^{10} cells/ml, with a median concentration of 1.8×10^9 cells/ml (Table 1). Statistical analysis indicated that the median concentration of cells in the fluid inclusions was significantly less than the concentration of cells in solution ($P = 0.01$).

Cells in inclusions from experiments 1 and 2 continued to twitch and fluoresce when examined in April (Fig. 4C) and June 2005, as much as 13 months after entrapment. Attempts to culture cells from the inclusions were inconclusive. The only culture that contained positive growth had non-fluorescing cells, which could indicate contamination or that the entrapped cells lost their ability to produce GFP through mutation. Bloemberg *et al.* (1997) indicated that the GFP-bearing plasmid is stable in 92% of *P. aeruginosa* cells and exerts minimal metabolic burden on the cells grown on Luria broth. However, the minor burden of GFP production could be a significant factor for cells experiencing the hostile (anaerobic, nutrient-limited hypersaline solution) conditions in the fluid inclusions. Mutants that lose the ability to produce GFP could have an advantage in survival. In any event, *P. aeruginosa* remained viable in the hypersaline solution for several weeks, which is remarkable for a non-halotolerant species, and was positively identified by the presence of GFP within fluid inclusions in the halite. Therefore, our results indicate that even non-halophilic cells could potentially be preserved in ancient halite deposits.

DISCUSSION

Petrographers who work with halite typically have not documented the presence of microbial cells in fluid inclusions, most likely because of the common use of bright condensed transmitted light illumination at high magnifications (Goldstein and Reynolds, 1994). Entrapment of GFP-producing *P. aeruginosa* in laboratory-grown halite crystals allowed us to identify cells within

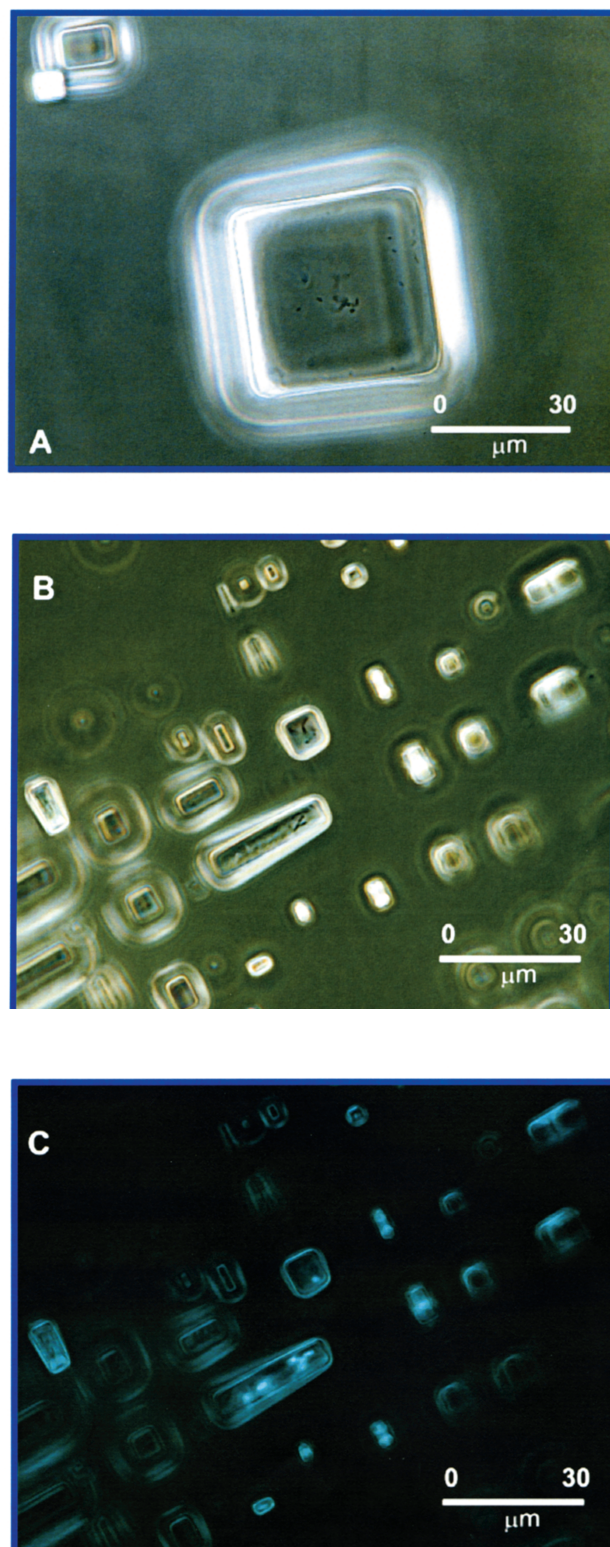**FIG. 4.** *P. aeruginosa* entrapped in fluid inclusion in laboratory-grown halite crystals: **(A) imaged using phase-contrast microscopy, (B) imaged with phase-contrast microscopy, and (C) same field of view as (B), imaged with ultraviolet light (395 nm).** Bright green spots in (C) are fluorescing cells of *P. aeruginosa*, as imaged in April 2005, nearly a year after the cells were entrapped.

TABLE 1. CONCENTRATION OF ENTRAPPED CELLS, AS DETERMINED BY DIRECT COUNTS OF CELLS IN FLUID INCLUSIONS

| Halite crystal | Fluid inclusion | Fluid inclusion dimensions | | | | Number of cells | Concentration (cells/ml) |
		Length (μm)	Width (μm)	Area (μm^2)	Volume (μm^3)		
1	1	22	21	460	9,500	6	6.3×10^8
1	2	57	7	390	2,600	2	7.6×10^8
1	3	47	14	640	8,600	11	1.3×10^9
1	4	17	11	190	2,100	5	2.4×10^9
1	5	12	9	110	950	4	4.2×10^9
1	6	15	11	160	1,800	8	4.6×10^9
2	7	19	16	320	5,100	2	3.9×10^9
2	8	32	32	1,000	33,000	15	4.5×10^8
2	9	12	17	200	3,400	2	5.9×10^8
2	10	29	16	450	7,000	12	1.7×10^9
2	11	32	11	360	4,100	8	2.0×10^9
2	12	9	5	43	220	6	2.8×10^{10}
3	13	23	15	360	5,500	10	1.8×10^9
3	14	15	14	210	3,000	17	5.6×10^9
3	15	22	11	240	2,600	20	7.6×10^9
4	16	36	16	570	9,200	8	8.7×10^8
4	17	42	16	670	10,600	10	9.4×10^8
4	18	58	11	640	7,000	11	1.6×10^9
4	19	41	9	390	3,600	8	2.2×10^9
4	20	18	7	130	920	4	4.3×10^9
4	21	8	9	70	620	3	4.9×10^9
Median concentration							1.8×10^9

fluid inclusions and evaluate the optimal petrographic techniques for viewing cells. The results of this study indicated that microorganisms are most observable in fluid inclusions with near-complete closure of the iris diaphragm on the substage or with phase-contrast microscopy. Petrographic analysis of ancient halite should include these techniques if the objective is to view entrapped cells.

The presence of non-halophilic bacteria does not appear to affect the size, shape, or number of fluid inclusions; hence, results did not indicate a diagnostic pattern or size range of inclusions in halite that would signify the presence of motile microbes in a sample. Though variations in the size, shape, and number of fluid inclusions were large, they were likely related to environmental factors other than the presence of bacteria. Non-halophilic motile microorganisms apparently contain no mechanism that affects the growth of halite crystals or the occurrence of fluid inclusions. These results are in contrast to those of Norton and Grant (1988), which indicated that the number and size of fluid inclusions are affected by the presence of halophilic Archaea.

We hypothesize that processes such as biomineralization of halite by halophilic Archaea could explain the discrepancy between our findings and those of Norton and Grant (1988). Biomineralization appears to be important in the production of halite from salterns, and is related to cell multiplication and mechanisms for maintaining intracellular osmotic balances (Castanier et al., 1999). Halophilic Archaea maintain osmotic balance by accumulating K^+ and removing Na^+ ions from the cytoplasm (Javor, 1989). The removal of Na^+ ions from the cytoplasm can cause solutions that surround the cells to become supersaturated and, subsequently, precipitate halite (Oren, 2002).

We found that non-halophilic motile microorganisms were readily entrapped in fluid inclusions that were large enough to contain them. The process of entrapment is uncertain. Cells were observed to float freely in the fluid inclusions, and were not identified within the crystal matrix, which indicates that cell attachment to the halite surface was not needed for entrapment. The large range in concentrations of entrapped cell is consistent with, and at the same spatial scale as, the documented variations in cell concentration in the hypersaline solution; therefore, entrapment could result from crystal growth around a solution that contains a dispersion of cells. The me-

dian concentration of entrapped cells (1.8×10^9 cells/ml) was statistically less than the concentration of cells in the hypersaline solutions (3.8×10^9 cells/ml), which could indicate a slight negative selection against entrapment, an observation worthy of further investigation.

Many factors can affect the accuracy of determining cell concentrations of fluid inclusions, including the difficulty of counting motile cells and errors in estimating fluid inclusion depths. For example, if the actual depths of fluid inclusions are half of the estimated depths, the median concentrations of cells in fluid inclusions and cells in the hypersaline solution would be nearly identical, which would indicate no preferential entrapment or exclusion from the fluid inclusions. Additional study is needed to better determine the rate and concentration of cell entrapment.

The results of this study also demonstrate the preservation potential of hypersaline fluid inclusions of even non-halophilic bacteria, as indicated by the intact cells and continued fluorescence of the GFP. As with other proteins, GFP production ceases after cell death and is subject to degradation by a variety of reactions such as hydrolysis and racemization (Mitterer, 1993). The continued fluorescence indicates that GFP continued to be produced (the cells remained viable) or, more likely, the cells were not viable but significant degradation had not occurred. In either case, the hypersaline solutions in fluid inclusions have a high potential for preserving even non-halophilic cells deposited with the halite. Halophilic microorganisms, which have a number of mechanisms for surviving in extreme conditions in hypersaline environments, could be better adapted for long-term survival within fluid inclusions in salt crystals.

Based on the results of this study and previous studies, fluid inclusions in ancient halite should have trapped cells in concentrations comparable to the large microbial populations (Pedros-Alio, 2004) in hypersaline environments. Even with the possible slight negative selection against entrapment, the concentration of motile cells in fluid inclusions could provide a good approximation of the concentration of cells in the original hypersaline environment, assuming diagenetic processes such as dissolution, and heat and pressure from burial have not removed or destroyed the cells. Estimates of cell concentrations should be based on a large number of fluid inclusions in numerous thick sections to minimize the effects of spatial variations in cell populations and anomalous sterile regions, as we observed in crystal 1. In this laboratory experiment, 21 fluid inclusions were examined. In natural systems, the variations in cell concentrations could be much larger, and many more inclusions would need to be examined.

The high rates of entrapment and high preservation potential of cells in halite fluid inclusions have significant implications for the discovery of microbial life in ancient extraterrestrial materials. Additional research regarding the survival of cells in halite under various conditions associated with harsh environments such as those in space or burial in sedimentary basins is warranted. However, extraterrestrial halite should be an excellent starting point in an astrobiological search.

ABBREVIATION

GFP, green fluorescent protein.

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