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Halophilic *Archaea* cultured from ancient halite, Death Valley, California

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Summary

Halophilic *Archaea* cultured from ancient fluid inclusions in a 90-m-long (0- to 100 000-year-old) salt core from Death Valley, California, demonstrate survival of bacterial cells in subsurface halite for up to 34 000 years. Five enrichment cultures, representing three genera of halophilic *Archaea* (*Halorubrum*, *Natronomonas* and *Haloterrigena*), were obtained from five surface-sterilized halite crystals exclusively in one section of the core (13.0–17.8 m; 22 000–34 000 years old) containing perennial saline lake deposits. Prokaryote cells were observed microscopically *in situ* within fluid inclusions from every layer that produced culturable cells. Another 876 crystals analysed from depths of 8.1–86.7 m (10 000–100 000 years old) failed to yield live halophilic *Archaea*. Considering the number of halite crystals tested (culturing success of 0.6%), microbial survival in fluid inclusions in halite is rare and related to the paleoenvironment, which controls the distribution and abundance of trapped microorganisms. Two cultures from two crystals at 17.8 m that yielded identical 16S rRNA sequences (genus: *Haloterrigena*) demonstrate intra-laboratory reproducibility. Inter-laboratory reproducibility is shown by two halophilic *Archaea* (genus: *Natronomonas*), with 99.3% similarity of 16S rRNA sequences, cultured from the same core interval, but at separate laboratories.

Introduction

Prokaryotes, notably halophilic and halotolerant *Archaea* and *Bacteria*, have been cultured from many ancient salt deposits (Reiser and Tasch, 1960; Dombrowski, 1963; Norton *et al*., 1993; Grant *et al*., 1998; Stan-Lotter *et al*., 1999; McGenity *et al*., 2000; Vreeland *et al*., 2000; 2007; Stan-Lotter *et al*., 2002; Mormile *et al*., 2003; Gruber *et al*., 2004). Mormile and colleagues (2003), for example, isolated *Halobacterium salinarum* from a single fluid inclusion in a 100 000-year-old halite crystal from Death Valley, California, and concluded it was an ancient prokaryote. Another cultured halophilic archaeon, *Halococcus salifodinae,* found in halite from subsurface mines in England, Germany and Austria was interpreted by Stan-Lotter and colleagues (2002) as 'remnants of populations' living in Permian (250–300 million-year-old) brines that once covered western Europe. These studies collectively conclude that prokaryotes can survive in halite for periods of thousands to hundreds of millions of years. Such conclusions have implications for the long-term survival of prokaryotes on Earth and elsewhere in the solar system (Kminek *et al*., 2003; Fendrihan and Stan-Lotter, 2004; Fendrihan *et al*., 2006).

Critics have argued that prokaryotes cultured from ancient halite, such as the bacterium *Virgibacillus* strain 2-9-3 isolated from the Permian Salado Formation of New Mexico (Vreeland *et al*., 2000), may be substantially younger than originally reported (Cooper and Poinar, 2000; Graur and Pupko, 2001; Hazen and Roedder, 2001; Nickle *et al*., 2002; Kminek *et al*., 2003; Willerslev *et al*., 2004a; Hebsgaard *et al*., 2005; Willerslev and Hebsgaard, 2005). For example, the 16S rRNA sequence of strain 2-9-3 is nearly identical (differing by only two out of 1555 aligned, unambiguous nucleotides) to the bacterium *Virgibacillus marismortui* recovered from the Dead Sea (Arahal *et al*., 1999; 2000; Heyrman *et al*., 2003). This suggests to some that *Virgibacillus* 2-9-3 is a laboratory contaminant (Graur and Pupko, 2001).

Hazen and Roedder (2001) challenged the Permian age of the halite crystal and fluid inclusion that housed *Virgibacillus* 2-9-3 using geological arguments. Evidence defending the 250-million-year age of the host halite, including primary sedimentary structures mapped in a salt mine, was then presented by Powers and colleagues (2001). Satterfield and colleagues (2005) found that the disputed fluid inclusions contained evaporated Permian seawater, which supports the antiquity of the fluid inclusions and the host halite. More work, however, is needed before the geological and biological communities

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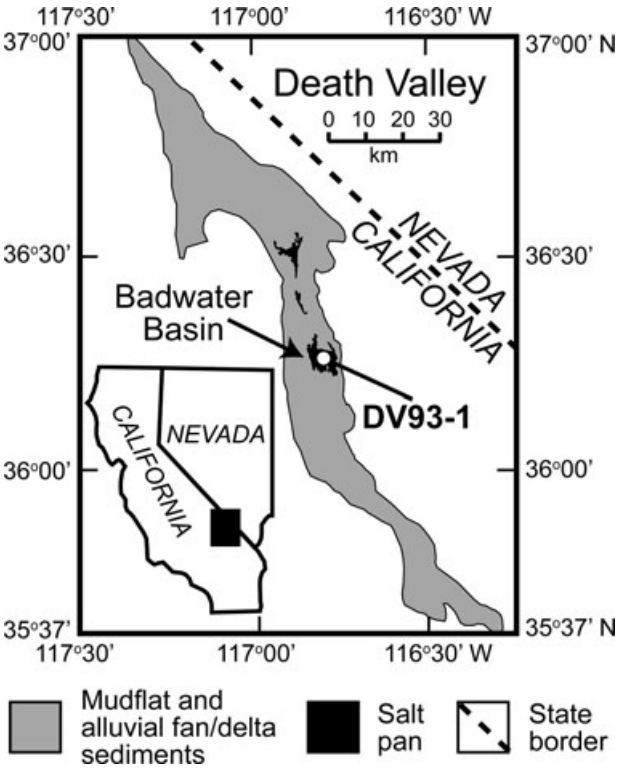

Fig. 1. Map of Death Valley, California. The Death Valley salt core (DV93-1) was drilled in the middle of Badwater Basin.

completely agree that ancient halite harbours viable prokaryotes.

Here we report the isolation of halophilic *Archaea* from ancient halite in a 90-m-long core, Badwater Basin, Death Valley, California (Fig. 1). Halophilic *Archaea* were cultured from ancient halite crystals in which prokaryotes were observed *in situ* within fluid inclusions. Reproducible growth of related taxa of halophilic *Archaea* from the salt core and the absence of growth in paired, surface-sterilization controls provide evidence that the cultured prokaryotes are not contaminants. Successful cultivation of halophilic *Archaea* from specific stratigraphic intervals of the Death Valley core can be interpreted in the context of the original surface environments in which the halite precipitated during the past 100 000 years (Li *et al*., 1996; Lowenstein *et al*., 1999).

Background on the Death Valley salt core

Halite textures and interpreted paleoenvironments

The Death Valley core, drilled in 1993, consists of layered salts and muds. The top 90 m of the core, dated by U-series methods (Ku *et al*., 1998), records the last 100 000 years of deposition in Badwater Basin (Lowenstein *et al*., 1999) (Fig. 2). The core contains bedded halites (7.7–18.0 m; 10 000–35 000 years old) with textures diagnostic of crystallization at the bottom of a perennial hypersaline lake (Li *et al*., 1996). These textures include vertically oriented crystals of clear bottom growth halite and chevron halite. Clear bottom growth halite contains relatively few fluid inclusions, whereas chevron halite appears cloudy due to bands rich in fluid inclusions, separated by bands with few fluid inclusions. From depths

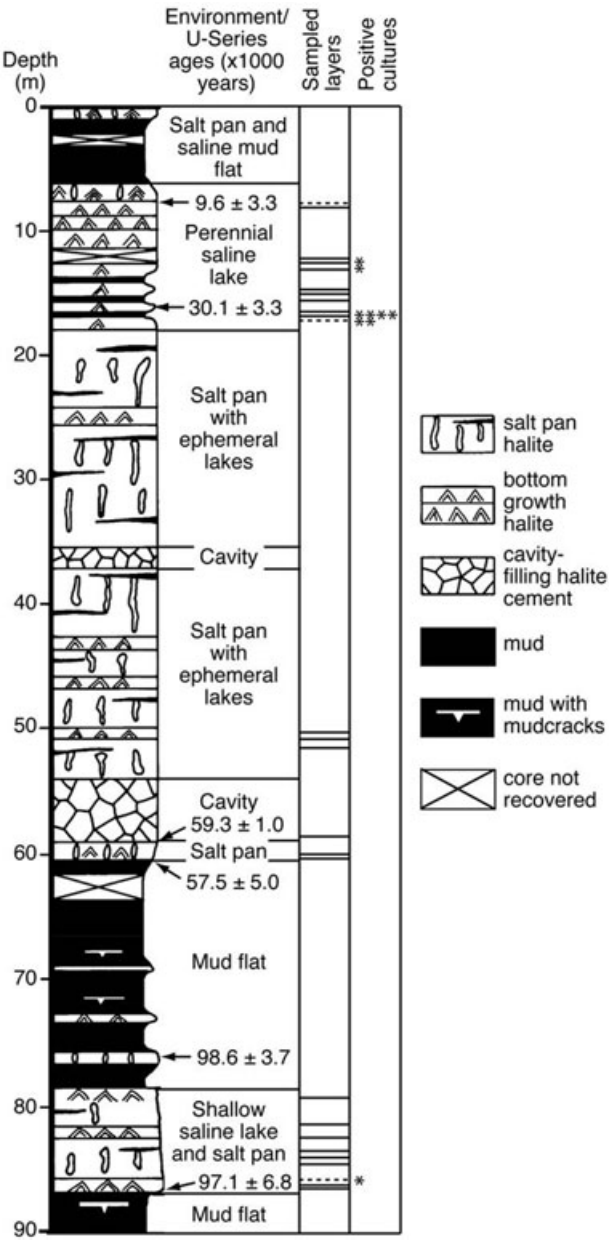

Fig. 2. Stratigraphic column of the Death Valley core from 0 to 90 m (0–100 000 years old) with U-series ages, interpreted paleoenvironments and general halite textures (modified from Lowenstein *et al*., 1999). Horizontal lines show depths of halite samples used for culturing experiments in this study. Dashed lines mark halite layers sampled by Mormile and colleagues (2003) and Vreeland and colleagues (2007). Each asterisk (*) represents a haloarchaeal strain cultured from that layer.

of 18–61 m (35 000–60 000 years old) and 78–87 m (~100 000 years old) (Fig. 2), the core contains halite with a variety of dissolution and cementation textures diagnostic of deposition in salt pan environments (Lowenstein and Hardie, 1985; Li *et al*., 1996). Salt pans are normally dry, but following high inflow events, ephemeral saline lakes develop. Chevron halite crystallizes when lake waters become hypersaline. Typically, during subsequent flooding, such chevron layers are partially to nearly completely dissolved. Voids in porous chevron halite crusts are later filled by halite cements that crystallize from saline groundwaters.

Isolation of halophilic Archaea

Mormile and colleagues (2003) and Vreeland and colleagues (2007) cultured halophilic *Archaea* from the Death Valley core. Mormile and colleagues (2003) isolated *Hbt. salinarum* BBH 001 from a fluid inclusion in chevron halite from a shallow saline lake interval at 85.7 m (~100 000 years old), but no growth resulted from a fluid inclusion in chevron halite from the perennial saline lake interval at 8 m (10 000 years old) (Fig. 2). Vreeland and colleagues (2007) isolated *Natronobacterium* sp. 2-24-1 and *Halobacterium* sp. 2-24-4 from clear bottom growth halite in the perennial saline lake interval at 17.9 m (34 000 years old) (Fig. 2).

Ancient prokaryotes observed in situ

Schubert and colleagues (2009) observed microparticles *in situ* within primary fluid inclusions in halite from the Death Valley core, some of which were interpreted as prokaryotes. Only primary fluid inclusions, trapped during crystal growth, were studied because the timing of their formation is known. Electron micrographs of possible cells from halite that was dissolved and filtered with carbon-free water provided additional evidence that some of these microparticles in the Death Valley core were prokaryotes (Schubert *et al*., 2009). Direct counts of possible prokaryotes in fluid inclusions in halite showed them to be most abundant in the perennial saline lake interval (7.7–18.0 m, Fig. 2), indicating a paleoenvironmental control on prokaryote abundance (Schubert *et al*., 2009).

Results

Sample selection for culturing experiments

Halite deposited in two environments was studied from the Death Valley core: a perennial saline lake interval from 7.7–18.0 m (10 000–34 000 years old) and salt pan intervals from 50.3–86.7 m (55 000–100 000 years old) (Fig. 2). Salient features observed in thin sections from 25 stratigraphic layers confirmed earlier paleoenvironmental interpretations (Li *et al*., 1996; Lowenstein *et al*., 1999) (Table 1) (Figs 3A and B, 4A and B, and 5A and B). Halite crystals cut from the core were examined using a binocular microscope to verify that the crystals did not contain fractures (Figs 3C and 5C–E). Fractured crystals were rejected because the timing of formation of such features, though uncertain, is without exception later than halite crystallization. We selected 517 halite crystals, most with clear bottom growth textures, from 10 layers of the perennial saline lake interval (Table 1). An additional 364 halite crystals with chevron or cement textures were selected from 15 layers in the salt pan intervals (Table 1). We analysed between 12 and 121 halite crystals from each sample layer.

Prokaryotes in fluid inclusions: microscopy

Possible prokaryotes in fluid inclusions in halite samples specific to this study were distinguished *in situ* from crystals and other microparticles using the criteria of Schubert and colleagues (2009). Prokaryotes were relatively small cocci (all < 1 μm diameter) and rare, straight and curved rods (1–3 μm long) (Figs 3D, 4C and 5F). These observations are consistent with previous results that most prokaryotes in the Death Valley core are miniaturized compared with modern samples (Schubert *et al*., 2009).

The presence or absence of prokaryotes in fluid inclusions in halite from each of the 25 sampled halite layers was noted (Table 1). Prokaryotes were observed in fluid inclusions in all 10 sampled halite layers from the perennial saline lake interval. In contrast, prokaryotes were observed in fluid inclusions in only two out of 15 halite layers from salt pan intervals, both from chevron halites interpreted to have crystallized in shallow, ephemeral saline lakes. We observed prokaryotes in fluid inclusions in halite from 85.7 m, the same layer from which Mormile and colleagues (2003) isolated *Hbt. salinarum* BBH 001. Halite samples were not available for study from 17.9 m, the layer from which Vreeland and colleagues (2007) isolated *Natronobacterium* sp. 2-24-1 and *Halobacterium* sp. 2-24-4. We did, however, observe prokaryotes in fluid inclusions in adjacent halite layers from 17.8 and 18.0 m.

Culturing results

To test the viability of trapped cells, 881 surface-sterilized halite crystals (Table 1) were dissolved into liquid media (CAB – Casamino Acids Binghamton and PGB – Pyruvate Glycerol Binghamton) designed to culture halophilic and halotolerant microorganisms (Table 2) (see *Experimental procedures*). Growth, identified by a change in the turbidity and colour of the medium, was observed in five

Table 1. Summary of crystals dissolved into nutrient media, for cultivation of halotolerant and halophilic microorganisms.

Depth (m)	Age (× 1000 years)	Halite texture	Total No. of crystals	No. of crystals in each medium[a]				No. of positive cultures	Prokaryotes (*in situ*)
				CAB (0.7 M NaCl)	CAB (1.4 M NaCl)	CAB (3.4 M NaCl)	PGB (4.3 M NaCl)		
Perennial saline lake									
8.1	10	CHEV	67	15	16	16	20	0	+
12.9	22	CBG	67	7	20	20	20	0	+
13.0	22	CBG	121	25	26	26	44	1	+
14.1	25	CBG	24	3	3	7	11	1	+
15.7	29	CBG	48	0	0	0	48	0	+
16.5	31	CBG	33	7	7	7	12	0	+
16.7	31	CBG	34	7	7	7	13	0	+
17.5	33	CBG	31	9	3	9	10	0	+
17.8	34	CBG	73	17	17	17	22	3	+
18.0	34	CBG	19	3	3	3	10	0	+
Overall			*517*	*93*	*102*	*112*	*210*	*5*	
Salt pan with ephemeral saline lakes									
50.3	55	CEM	12	3	3	3	3	0	–
50.8	55	CHEV	16	3	3	3	7	0	–
51.7	56	CHEV	25	6	6	6	7	0	+
58.5	58	CEM	16	3	3	3	7	0	–
60.1	58	CEM	15	2	2	2	9	0	–
60.4	58	CEM	27	5	7	3	12	0	–
79.2	100	CEM	56	13	13	13	17	0	–
81.5	100	CEM	22	4	4	3	11	0	–
82.8	100	CHEV	12	2	2	2	6	0	–
83.9	100	CHEV	22	3	6	6	7	0	–
84.4	100	CEM	33	8	8	8	9	0	–
84.9	100	CHEV	24	5	5	5	9	0	–
85.7	100	CHEV	52	13	13	13	13	0	+
86.7	100	CHEV	16	3	3	3	7	0	–
86.7	100	CEM	16	3	3	3	7	0	–
Overall			*364*	*76*	*81*	*76*	*131*	*0*	
Total for core			*881*	*169*	*183*	*188*	*341*	*5*	

a. Media, modified from Vreeland and colleagues (1984), defined in Table 2.
CHEV, chevron; CBG, clear bottom growth; CEM, cement.
+, prokaryotes observed in fluid inclusions in halite from this depth; –, prokaryotes not observed in fluid inclusions in halite from this depth.

cultures 61–92 days after inoculation (Table 3). All five positive cultures were in PGB media (4.3 M NaCl) and resulted from the dissolution of clear bottom growth halite crystals of the perennial saline lake interval in which prokaryotes had previously been identified in fluid inclusions (13.0–17.8 m; 22 000–34 000 years old) (Table 1) (Figs 3–5). Four cultures were isolated and designated DV427, DV462A, DV582A-1 and DV582B-3 (Table 3). Subculturing attempts to obtain single-colony isolates were not successful for one culture from 17.8 m. For that sample, total DNA was prepared and 16S rRNA genes were amplified by polymerase chain reaction (PCR) and cloned (see *Experimental procedures*). The 16S rRNA genes of two clones were sequenced and designated DV582c2 and DV582c4 (Table 3).

Concurrent with sample inoculation, 808 surface-sterilization control samples were inoculated into nutrient media and none yielded growth (see *Experimental procedures*). To further confirm the effectiveness of the surface-sterilization procedures, 64 halite crystals were surface-spiked with the four Death Valley isolates (16 crystals per isolate), surface-sterilized and then dissolved into growth medium (see *Experimental procedures*). The lack of growth in any of these experiments indicates that

Table 2. Media recipes modified from Vreeland and colleagues (1984).

Component	CAB (g l^{-1})	PGB (g l^{-1})
Casamino acids	7.5	0
Yeast extract	1.0	0
Protease peptone	5.0	0
Sodium citrate	3.0	0
K_2HPO_4	0.5	0.5
$MgSO_4 \cdot 7H_2O$	2.0	2.0
KCl	2.0	4.0
Glycerol	0	2.5
Sodium pyruvate	0	2.5
$(NH_4)_2SO_4$	0	1.0
NaCl	40, 80 or 200	250

CAB, Casamino Acids Binghamton; PGB, Pyruvate Glycerol Binghamton.

Table 3. Summary and comparison of prokaryotes cultured from the Death Valley core.

Organism name/ domain	Depth (m)	Age (years)	Medium/ [NaCl]	Culture colour	Days until growth observed	Sequence length (bp)	Closest BLAST match	Sequence similarity	Location/environment of BLAST reference organism
DV427/ *Archaea*	13.0	22 000	PGB/ 4.3 M	Pink	63	1117	Uncultured haloarchaeon clone TX4CA_24	95.2%	Former Lake Texcoco, Mexico/ Alkaline-saline soil[a]
DV462A/ *Archaea*	14.1	25 000	PGB/ 4.3 M	Pink	72	1152	*Natronobacterium* sp. 2-24-1	99.3%	Death Valley salt core (17.9 m)/ Perennial saline paleolake[b]
DV582A-1/ *Archaea*	17.8	34 000	PGB/ 4.3 M	Pink	87	1406	*Haloterrigena thermotolerans*	98.6%	Cabo Rojo, Puerto Rico/ Solar saltern[c]
DV582B-3/ *Archaea*	17.8	34 000	PGB/ 4.3 M	Pink	61	1406	*Haloterrigena thermotolerans*	98.6%	Cabo Rojo, Puerto Rico/ Solar saltern[c]
DV582c2/ *Archaea*	17.8	34 000	PGB/ 4.3 M	Pink	92	1406	*Haloterrigena thermotolerans*	98.7%	Cabo Rojo, Puerto Rico/ Solar saltern[c]
DV582c4/ *Archaea*	17.8	34 000	PGB/ 4.3 M	Pink	92	1406	*Haloterrigena thermotolerans*	98.4%	Cabo Rojo, Puerto Rico/ Solar saltern[c]
Natronomonas sp. 2-24-1[b]/ *Archaea*	17.9	34 000	CAS[b,d]/ 3.4 M[b]	n.r.	n.r.	1322	DV462A	99.3%	Death Valley salt core (14.1 m)/ Perennial saline paleolake
Halobacterium sp. 2-24-4[b]/ *Archaea*	17.9	34 000	CAS[b,d]/ 3.4 M[b]	n.r.	n.r.	1362	Halobacterium spp.	> 99%	Ancient rock salt of various ages
Halobacterium salinarum strain BBH 001[e]/ *Archaea*	85.7	100 000	HM[f]/ 3.4 M[f]	Red	n.r.	1239	*Halobacterium salinarum* DSM 671 (R1)	100.0%	DSMZ culture collection

a. Valenzuela-Encinas *et al.* (2008).
b. Vreeland *et al.* (2007).
c. Montalvo-Rodríguez *et al.* (2000).
d. Vreeland *et al.* (1984).
e. Mormile *et al.* (2003).
f. Norton and Grant (1988).
n.r., not reported.

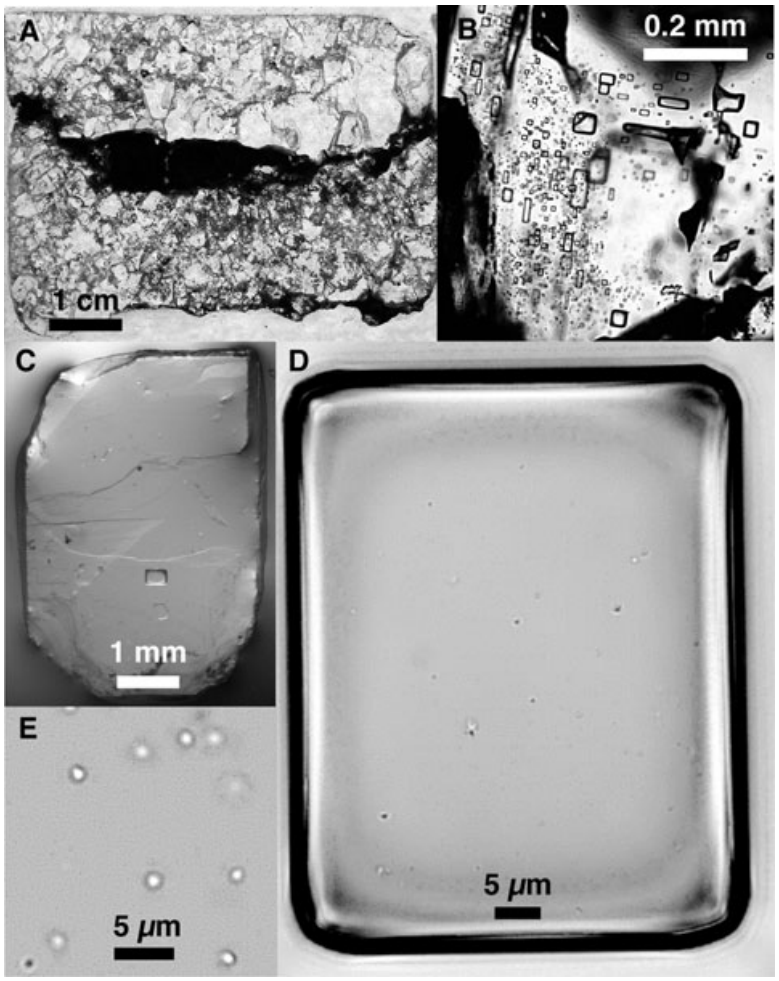

Fig. 3. Photomicrographs of halite 13.0 m (22 000 years old), Death Valley core.
A. Thin section shows clear bottom growth halite, separated by a dark mud layer.
B. Primary fluid inclusions oriented in bands parallel to crystal faces.
C. Halite crystal that yielded DV427. The crystal was cut from the upper halite layer shown in (A).
D. Miniaturized cocci (< 1 μm across) in a fluid inclusion. Not all prokaryotes are photographed in the plane of focus.
E. Wet mount of DV427 in PGB medium (4.3 M NaCl).

the surface-sterilization procedures were effective for these particular strains. Finally, no growth resulted from any of the 364 salt pan halite chevron and cement crystals in the Death Valley core (Table 1). The vast majority of halite crystals from the perennial saline lake section (512 out of 517) also failed to yield growth. These results indicate that growth was rare and not evenly distributed throughout the Death Valley core, which suggests that ancient halite samples were not contaminated by modern microorganisms.

16S rRNA relationships and descriptions of Death Valley strains

Preliminary PCR using archaeal specific primers (arch16Sfs/rP1a) (Table 4) revealed that *Archaea* were cultured from ancient halite. Partial sequencing of the 16S ribosomal RNA (rRNA) gene, BLAST searches (Altschul *et al*., 1990), and construction of a phylogenetic tree (see *Experimental procedures*) confirmed PCR results that only *Archaea* were cultured (Table 3) (Fig. 6).

Table 4. Oligonucleotide primers used in this study.

Primer	5′ → 3′ nucleotide sequence	Position of primer[a]	Forward or reverse primer
arch16sfs	ATTCCGGTTGATCCTGCC	1 → 18	Forward
arc5ir	CACTYGGAGTCCCCYTATCGCAC	385 → 363	Reverse
archmid	CGAACCGGATTAGATACCC	718 → 736	Forward
arcmidr	CCAATTCCTTTAAGTTTCATCCT	866 → 844	Reverse
aif3	GCCGTCAGCTCGTACCGTGAGG	1000 → 1021	Forward
rp1a	CTACGGCTACCTTGTTACGACTT	1447 → 1425	Reverse

a. Relative to *Halobacterium* sp. NRC-1 16S rRNA gene (NC_002607).

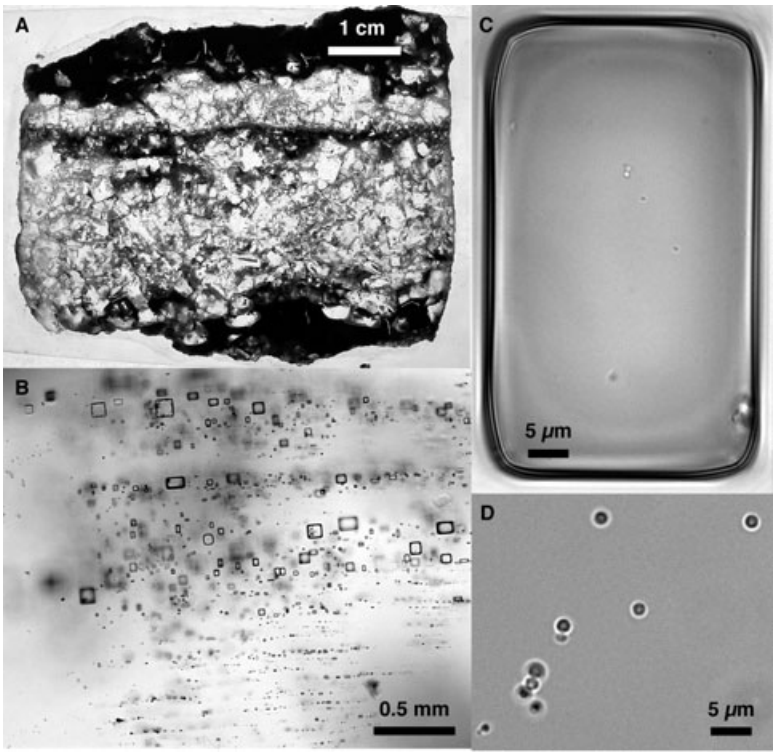

Fig. 4. Photomicrographs of halite 14.1 m (25 000 years old), Death Valley core.
A. Thin section shows clear bottom growth halite, separated by dark mud layers.
B. Primary fluid inclusions oriented in parallel bands.
C. Miniaturized cocci (< 1 μm across) in a fluid inclusion. Not all prokaryotes are photographed in the plane of focus.
D. Wet mount of DV462A in PGB medium (4.3 M NaCl). No photomicrograph was taken of the crystal that yielded DV462A.

Cultures recovered from the Death Valley core include strains that group in the *Halorubrum* (DV427), *Natronomonas* (DV462A) and *Haloterrigena* clades (DV582A-1, DV582B-3, DV582c2 and DV582c4) (Fig. 6). Halophilic *Archaea* previously cultured from the Death Valley salt core include two strains in the *Halobacterium* clade: *Hbt. salinarum* strain BBH 001 (Mormile *et al*., 2003) and *Halobacterium* sp. 2-24-4 (Vreeland *et al*., 2007) (Table 3). *Natronobacterium* sp. 2-24-1, previously cultured from the Death Valley core by Vreeland and colleagues (2007), groups with DV462A and *Natronomonas pharaonis* DSM 2160 (Kamekura *et al*., 1997) (Fig. 6). The high divergence of *Natronobacterium* sp. 2-24-1 from other *Natronobacterium* species (Fig. 6) indicates that it is likely misnamed and will therefore be referred to hereafter as *Natronomonas* sp. 2-24-1.

The 16S rRNA sequence of DV427 was 95.2% (1064/1118 bp) similar to the uncultured haloarchaeon clone TX4CA_24 (EF690579) (Table 3). Clone TX4CA_24 was recovered from alkaline-saline soil from the former lake Texcoco, Mexico (Valenzuela-Encinas *et al*., 2008). DV427 cultures were pink and in wet mounts, cells were coccoid-shaped (~1 μm in diameter) (Fig. 3E).

DV462A proved to be 99.3% (1095/1103 bp) similar to *Natronomonas* sp. 2-24-1 (Table 3). DV462A cultures were pink and in wet mounts, cells were coccoid-shaped (1.5–2.0 μm in diameter) (Fig. 4D).

DV582A-1, DV582B-3, DV582c2 and DV582c4 were 98.4–98.7% similar to a solar saltern isolate, *Haloterrigena thermotolerans*, from Cabo Rojo, Puerto Rico (Montalvo-Rodríguez *et al*., 2000) (Table 3) (Fig. 6). DV582A-1 and DV582B-3 had identical 16S rRNA sequences and were 99.5% (1399/1406 bp) similar to DV582c2 and DV582c4. DV582c2 and DV582c4 were phylogenetically 99.7% (1402/1406 bp) similar to each other and may represent two different strains or possibly multiple rRNA genes of a single strain.

Cultures of DV582A-1, DV582B-3 and DV582c2/DV582c4 were pink. In wet mounts, DV582A-1 and DV582B-3 were relatively long rods (2.5–10 × 0.5–1.1 μm) (Fig. 5G and H). DV582A-1 and DV582B-3 grew well in PGB media with 2.9–5.0 M NaCl (Table 5). DV582A-1 did not grow at 21°C in PGB media, but did grow at 37°C and 45°C; DV582B-3 grew in PGB media at all three temperatures tested (Table 5). Previous fluid inclusion studies from the halite interval that produced DV582A-1 and DV582B-3 indicate halite crystallization temperatures of 23–30°C (Lowenstein *et al*., 1998). When cultured at their optimal NaCl concentration and temperature, DV582A-1 and DV582B-3 grew in PGB media with pH values of 6–9 (Table 6).

We tested the survival of Death Valley strains DV582A-1 and DV582B-3 following short-term entrapment in fluid inclusions in halite (see *Experimental*

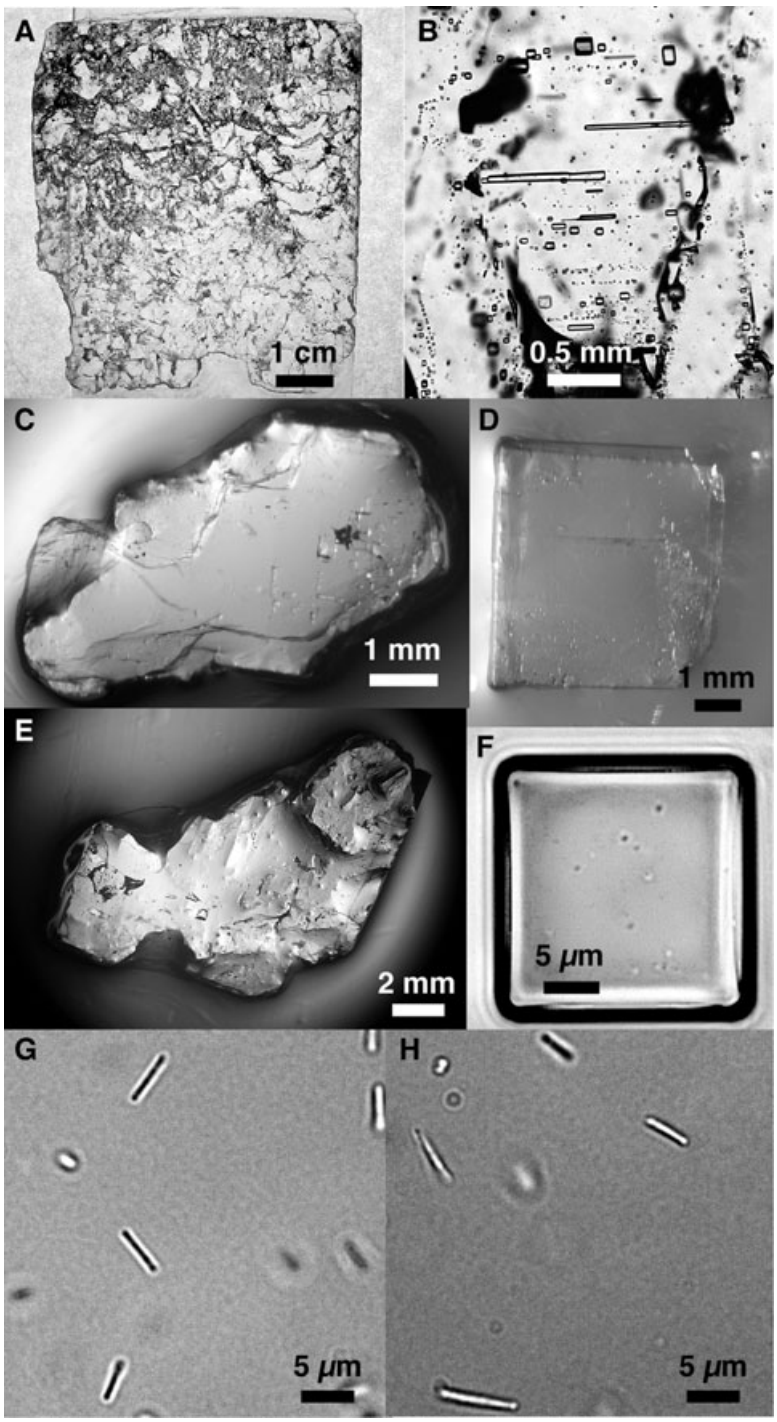

Fig. 5. Photomicrographs of halite 17.8 m (34 000 years old), Death Valley core.
A. Thin section shows clear bottom growth halite.
B. Primary fluid inclusions parallel to crystal growth faces of an upward widening bottom growth halite crystal. Some fluid inclusions have elongate, tubular shapes.
C. Crystal that yielded DV582A-1.
D. Crystal that yielded DV582B-3.
E. Crystal that yielded DV582c2 and DV582c4.
F. Miniaturized cocci (< 1 μm across) in a fluid inclusion. Not all prokaryotes are photographed in the plane of focus.
G. Wet mount of DV582A-1 in PGB medium (4.3 M NaCl).
H. Wet mount of DV582B-3 in PGB medium (2.9 M NaCl). No wet mounts were available for clones, DV582c2 and DV582c4.

procedures). In brief, DV582A-1 and DV582B-3 were washed in sterile NaCl brine to decrease nutrients and the brine containing the isolates was then evaporated, forming at least one halite crystal. After 74 and 474 days, halite crystals with included isolates were surface-sterilized and dissolved into growth medium. Both strains were successfully cultured following this short-term entrapment.

Discussion: evidence against laboratory contamination

Studies designed to culture cells from ancient materials must demonstrate that growth did not result from laboratory-based or sample-based contamination. Laboratory-based contamination results from laboratory personnel, reagents and tools (Hebsgaard *et al*., 2005).

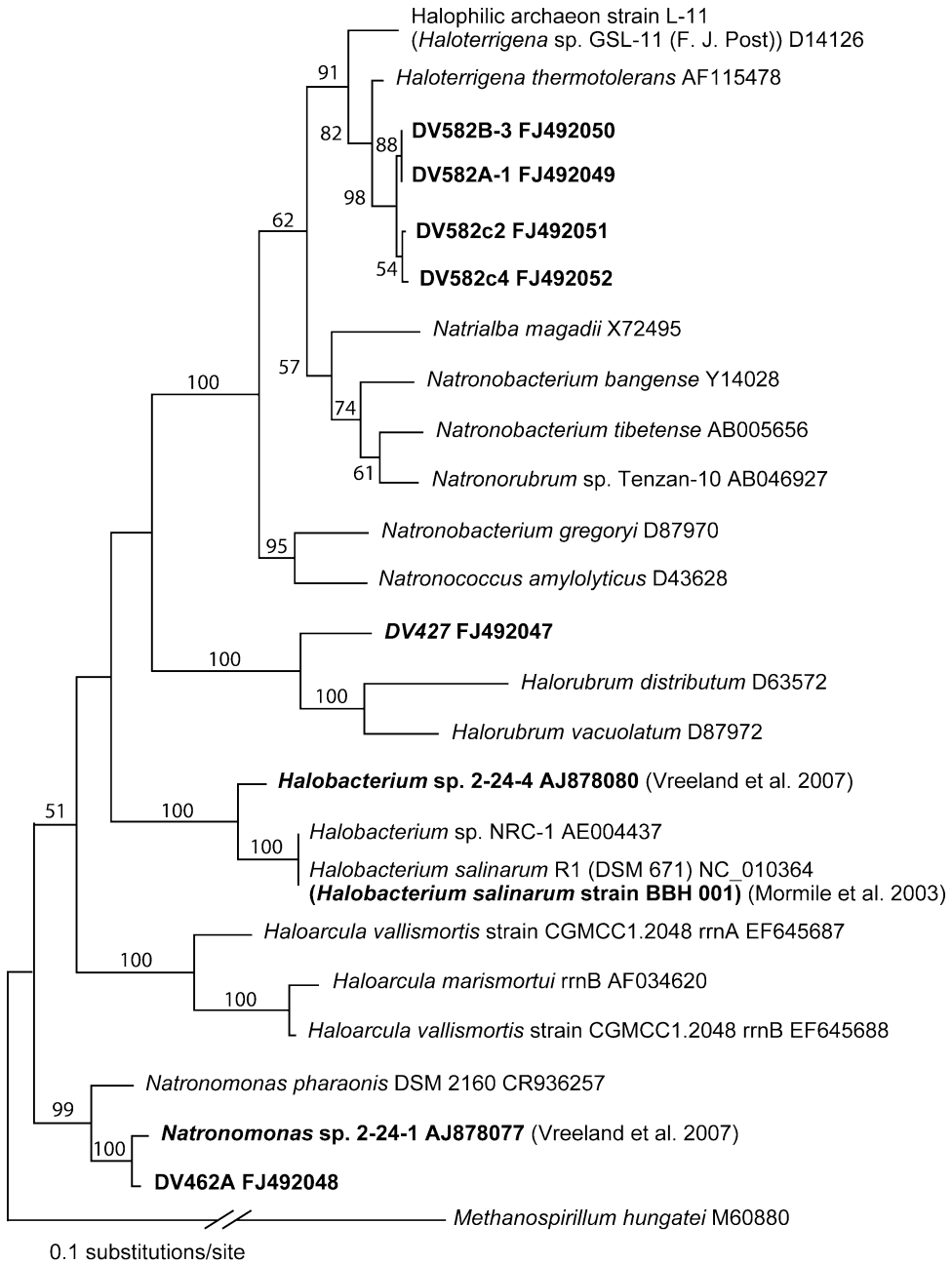

Fig. 6. Maximum likelihood tree for 16S rRNA sequences of all the Death Valley *Archaea* cultured to date from the Death Valley core (bold) and other taxa in the order *Halobacteriales* (Wright, 2006). Bootstrap values (200 replicates) are shown for branches with > 50% bootstrap support. Outgroup is *Methanospirillum hungatei* (family *Methanospirillaceae*).

Sample-based contamination results primarily from core drilling or re-colonization of the sample with organisms from the modern environment (Willerslev *et al*., 2004b; Hebsgaard *et al*., 2005). We address specific issues raised by Willerslev and colleagues (2004b) and Hebsgaard and colleagues (2005) regarding laboratory and environmental contamination and discuss advances made towards authenticating viable cells cultured from ancient salt samples. Support that the halophilic *Archaea* cultured from ancient halite in the Death Valley core are not contaminants includes: (i) well-dated samples, (ii) work conducted in a clean, isolated environment with sterile equipment and reagents; surface sterilization of crystal surfaces demonstrated by negative controls, (iii) use of specific media, (iv) microscopic confirmation that prokaryotes existed in fluid inclusions in all halite crystals that yielded growth, (v) facies-dependent pattern of microbial survival, (vi)

Table 5. Summary of sodium chloride concentrations and temperature conditions required for growth.

	2.9 M NaCl	3.6 M NaCl	4.3 M NaCl	5.0 M NaCl
		DV582A-1		
21°C	–	–	–	–
37°C	++	++	++	++
45°C	+	++	++[a]	++
		DV582B-3		
21°C	++	+	+	–
37°C	+++	+++	++	++
45°C	+	++	+++[a]	++

a. Growth conditions used to determine optimal pH; see Table 6
Cultured in PGB media containing NaCl concentrations as noted.
+++, abundant growth; ++, moderate growth; +, poor growth; –, no growth after 3 months.

intra-laboratory reproducibility and (vii) inter-laboratory reproducibility.

Well-dated samples

Halite crystals selected from the Death Valley core came from bedded, undeformed strata with well-preserved, primary, depositional textures (Figs 3A and B, 4A and B, and 5A and B) and known ages (Li *et al*., 1996; Ku *et al*., 1998; Lowenstein *et al*., 1999). This indicates that the fluid inclusions and associated organisms formed contemporaneously with halite precipitation (Lowenstein and Brennan, 2001). Environmental contaminants associated with drilling activities were avoided by sampling halite only from interior sections of the core.

Laboratory conditions and surface sterilization of halite

Ancient halite samples must be rigorously decontaminated before testing them for viable organisms (Vreeland *et al*., 2000; Hebsgaard *et al*., 2005). A disinfected Class IIA laminar flow hood provided a physically isolated work area for surface sterilization and inoculation of growth media (see *Experimental procedures*). We sterilized halite by immersing each crystal in 10 M NaOH for 5 min (see *Experimental procedures*). It was earlier demonstrated that placing halite in 10 M NaOH for only 1 min reduced the number of non-spore-forming microorganisms on crystal surfaces to undetectable levels (from 1.1×10^7 cells ml^{-1} to < 10 cells ml^{-1}) (Rosenzweig *et al*., 2000).

Surfaces of halite crystals spiked with Death Valley isolates DV427, DV462A, DV582A-1 and DV582B-3 further tested the sterilization techniques (see *Experimental procedures*). The lack of growth from these surface contamination experiments demonstrates that the strains isolated from the Death Valley core cannot survive on crystal surfaces treated with 10 M NaOH. This implies that the viable cells cultured from ancient halite in the Death Valley core came from the interior of the crystals. In summary, evidence for sterility is provided by 808 controls, 876 out of 881 halite crystals from the Death Valley core, and 64 surface-spiked (and then surface-sterilized) halite samples, which all failed to yield growth.

Specific media

The use of specific media decreases the risk of laboratory-based contamination (Willerslev *et al*., 2004b). All growth occurred in media containing 250 g NaCl l^{-1} (4.3 M NaCl), designed to culture halophilic microorganisms. The high salinity of this media will select against many laboratory contaminants that cannot tolerate hypersaline conditions.

In situ *microscopy of prokaryotes in fluid inclusions in halite*

Prokaryotes have been observed *in situ* in primary fluid inclusions in halite (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; Schubert *et al*., 2009). Such observations establish that cells are indigenous to, and the same age as, the halite in which they are found (Schubert *et al*., 2009). Prokaryotes were observed in fluid inclusions in all five halite layers of the perennial saline lake interval from which halophilic *Archaea* were cultured (Figs 3D, 4C and 5F) (Table 1). We also observed prokaryotes in fluid inclusions from the halite layer from which Mormile and colleagues (2003) cultured *Hbt. salinarum* (85.7 m; ~100 000 years old). These observations of prokaryotes in fluid inclusions show that cells occurred in samples that yielded positive cultures in the Death Valley core.

Paleoenvironmental controls on archaeal survival

Growth of halophilic *Archaea* was exclusively from one interval in the Death Valley core, 13.0–17.8 m, from halite originally deposited in a perennial saline lake. None of the 364 halite crystals from salt pan intervals yielded growth. These results suggest that the surface environment at the time of deposition influenced the

Table 6. Summary of pH values required for growth at optimal NaCl concentrations and temperature.

	pH				
	6	7	8	9	10
DV582A-1	++	+++	+++	+	–
DV582B-3	++	+++	+++	+	–

Cultured at 45°C in PGB media with 4.3 M NaCl.
+++, abundant growth; ++, moderate growth; +, poor growth; –, no growth after 3 months.

occurrences of culturable cells in halite from the Death Valley core. Perennial saline lakes may be highly productive environments with large numbers of microorganisms (Larsen, 1980; Oren, 2002). Halite in the Death Valley core that formed in such hypersaline lakes crystallized relatively slowly at the brine bottom, allowing time for prokaryotes suspended in the water column to be captured inside fluid inclusions (Schubert *et al*., 2009). Primary halite crystals and fluid inclusions from this perennial saline lake interval are well preserved, without any noticeable changes after crystallization. Slow crystallization of halite at the bottom of a perennial brine body and good preservation of primary textures and fluid inclusions make the halite from 13.0 to 17.8 m in the Death Valley core ideal for trapping prokaryotes and allowing them to survive for millennia. In contrast, salt pan halites formed in ephemeral lakes, which may not develop prolific halophile communities. These halites are normally dissolved during subsequent flooding, so preservation of primary halite is rare, and thus salt pan deposits are typically dominated by halite cements formed diagenetically from groundwater brines (Lowenstein and Hardie, 1985). Rapid crystallization of halite in ephemeral lakes and recycling during subsequent flooding produce salt pan halite deposits with fewer trapped prokaryotes in fluid inclusions and therefore lower probability of prokaryote growth (Schubert *et al*., 2009). The paleoenvironmental controls on prokaryote occurrence and growth described above support our interpretations that cultured strains are not laboratory- or sample-based contaminants.

Intra-laboratory reproducibility

Intra-laboratory reproducibility of results is required to authenticate cultures from ancient samples (Hebsgaard *et al*., 2005). We repeatedly achieved growth of related taxa of halophilic *Archaea* from one core interval. Specifically, sequences obtained from three different halite crystals from 17.8 m indicated that two isolates (DV582A-1 and DV582B-3) and two 16S rRNA clones (DV582c2 and DV582c4) were related to the genus *Haloterrigena* (*Archaea*). Isolates DV582A-1 and DV582B-3 were identical to each other; DV582c2 and DV582c4 were 99.5% similar to DV582A-1 and DV582B-3. The three crystals that yielded these four sequences were dissolved into PGB medium over a period of 147 days during which time 650 crystals from other core depths were also dissolved into nutrient medium (including 191 crystals dissolved into PGB medium). The only samples to yield sequences related to the *Haloterrigena* genus, however, were halite crystals from 17.8 m. It is unlikely that the intra-laboratory reproducibility described above can be attributed to laboratory-based contamination.

Inter-laboratory reproducibility

Inter-laboratory reproducibility, independent replication by another laboratory, is essential in all studies that attempt to culture viable cells because it excludes laboratory-based contamination (Willerslev *et al*., 2004b; Hebsgaard *et al*., 2005). The very low culturing success and heterogeneous distribution of cells in the Death Valley core, however, complicate independent replication. Despite these inherent difficulties, DV462A (this study) and *Natronomonas* sp. 2-24-1 (Vreeland *et al*., 2007) were cultured independently at separate laboratories using two different halite crystals from the same facies and are 99.3% similar to each other. These results show inter-laboratory reproducibility in recovery of related taxa of halophilic *Archaea* in geologically similar samples of ancient halite.

Conclusions

This study and earlier reports (Mormile *et al*., 2003; Vreeland *et al*., 2007) demonstrate that halophilic *Archaea* have remained viable in the subsurface of Death Valley on timescales of 10 000–100 000 years. Four isolates and two clones, representing three genera of halophilic *Archaea*, were cultured from five, primary halite crystals out of 881 tested from the Death Valley core (culturing success of 0.6%). Considering the large numbers of prokaryotes observed *in situ* within fluid inclusions (Schubert *et al*., 2009) and the number of crystals tested, we conclude that survival of halophilic prokaryotes in ancient fluid inclusions is rare. All cultured halophilic *Archaea* came from fluid inclusions in halite from the perennial saline lake interval of the Death Valley core (13.0–17.8 m; 22 000–34 000 years old). The same halites contained miniaturized prokaryotes *in situ* within primary, brine-filled inclusions. We conclude that halite formed in perennial hypersaline lakes has a better probability of preserving live halophilic microorganisms than halite formed in other environments, such as saline pans.

Control samples showed that the protocol for the surface sterilization of halite was effective. Reproducible growth of related *Haloterrigena* strains from three halite crystals, depth of 17.8 m, in one laboratory shows intra-laboratory reproducibility. DV462A and *Natronomonas* sp. 2-24-1, 99.3% similar, were isolated independently at two laboratories from the same facies of the Death Valley core, which demonstrates inter-laboratory reproducibility.

The following strategies will guide future studies seeking to culture halophilic prokaryotes from ancient halite. (i) Samples from layered halite deposits with primary crystallization textures and primary fluid inclusions are required. (ii) Prokaryotes should be microscopically identified *in situ* within fluid inclusions, before

culturing experiments are performed. (iii) Halite formed at the bottom of perennial saline lakes or lagoons is the most likely to have well-preserved primary crystals and fluid inclusions, and large numbers of halophilic prokaryotes within fluid inclusions, all of which enhance culturing success. (iv) Halite with textures indicating dissolution, cementation or re-crystallization, typical of salt pan environments, is less likely to have large numbers of living prokaryotes in fluid inclusions. (v) Reproducible growth of related taxa of halophilic prokaryotes from fluid inclusions in halite is possible, even with low culturing success. (vi) Carefully designed surface-sterilization controls are needed to show that cultured prokaryotes are not contaminants.

Experimental procedures

Sample preparation

Large format thin sections (5 × 7.5 cm) from 25 halite layers in the Death Valley core were studied with a Leica Wild M3Z stereomicroscope to document halite textures and confirm paleoenvironmental interpretations (Table 1). Single crystals were cut from freshly broken, interior sections of the core using a single-edged razor. Halite crystals with conspicuous fractures were discarded. Selected halite crystals had numerous primary, single-phase (liquid) inclusions (Figs 3B, 4B and 5B). Halite crystals were cleaved and mounted on microscope slides, and then examined for prokaryotes in fluid inclusions using a Zeiss microscope (AXIO Imager.A1) at 1000× magnification. An oil immersion objective (PLAN APO 100×/1.4 OIL) facilitated study of prokaryotes in fluid inclusions. Prokaryotes were documented with an AxioCam MRm B&W camera and AxioVision software (Figs 3D, 4C and 5F).

Surface sterilization and crystal dissolution

Preparation for surface sterilization included autoclaving all glassware, tweezers, rinses and pipette tips for 30 min (121°C, ~17 psi). Growth media (see details below) were made in 500 ml batches and then allocated to 50 ml glass Kimax flasks with screw caps (25 ml of medium per flask) or 16 × 125 mm Fisherbrand glass test tubes with screw caps (7 ml of medium per tube) and autoclaved. All surface-sterilization procedures were completed in a Class IIA laminar flow hood with an HEPA filter (Baker, SterilGARD III). The laminar flow hood was disinfected with germicidal UV light for at least 1 h before use and all hood surfaces were wiped down with 95% ethanol. Halite crystals were sterilized following procedures adapted from Rosenzweig and colleagues (2000). In brief, the sterilant (10 M NaOH) and NaCl saturated brine washes (~5 ml) were poured into separate sterile beakers. Halite crystals were immersed in 10 M NaOH for 5 min followed by two consecutive washes in sterile NaCl saturated brine. The pH of the second rinse remained neutral. Crystals were transferred between beakers using sterile tweezers.

Following sterilization, halite crystals were aseptically transferred into growth medium where they passively dissolved. One millilitre of the second NaCl wash (experimental control) was inoculated using a sterile pipette into a separate test tube or flask containing the same medium type as the paired crystal sample. The test tube or flask with the dissolved crystal and the experimental control were incubated together, under the same conditions (37°C, indirect sunlight) (Isotemp Incubator, Fisher Scientific). Test tubes and flasks were incubated upright and manually shaken several times per week. The lack of growth in control samples suggests that cultured prokaryotes did not result from laboratory or environmental contaminants on crystal surfaces.

Two media types (CAB and PGB) at four NaCl concentrations (CAB – 0.7, 1.4 and 3.4 M; PGB – 4.3 M) were used (Vreeland *et al*., 1984) (Table 2). The pH was adjusted to 8.0–8.2 for CAB medium and 7.3–7.5 for PGB medium. The inorganic salt chemistry was designed to approximate the major ion chemistry of modern brines from Death Valley (Li *et al*., 1997). Inoculated media were incubated for up to 28 months or until growth was observed (indicated by turbidity or colour change), although all growth occurred in 92 days or less (Table 3). Cultures showing signs of growth were isolated on plates (1.5–2.0% agar) that matched the liquid media chemistries.

The sterilization protocol was tested to show that the isolates cultured from the Death Valley core could not have survived on crystal surfaces when submerged in 10 M NaOH for 5 min. Halite crystals (Diamond Crystal Solar Salt Extra Coarse, Cargill Incorporated, Minneapolis, MN) were sterilized by heating at 500°C for 72 h. Once at room temperature, each crystal was streaked across the surface of an agar plate containing colonies of Death Valley isolates DV427, DV462A, DV582A-1 and DV582B-3. A total of 64 halite crystals (16 crystals streaked for each of the four isolated microorganisms) were then surface sterilized in 10 M NaOH and rinsed twice in sterile NaCl-saturated brine following protocols described above. The crystals and 1 ml of the second rinse were then added to separate test tubes containing PGB medium. Cultures were incubated for 168 days at 37°C.

DNA extraction

DNA was prepared from isolates by aseptically transferring approximately two 'loopfuls' of cells into a sterile 1.7 ml microcentrifuge tube containing 170 μl of Tris EDTA (pH 8.0) Triton-X (1%) lysing buffer to solubilize cell walls. The cells were mixed by vortexing and then heated in a water bath at 95°C for 5 min to lyse the cells. An equal volume of chloroform (170 μl) was added to the sample to denature proteins and remove lipids. The sample was inverted gently five times and then centrifuged at 12 000 *g* for 5 min (Eppendorf AG MiniSpin, Hamburg, Germany). The upper aqueous phase (containing the DNA) was then transferred to a sterile 1.7 ml microcentrifuge tube.

Sequencing

Purified DNA was used as template DNA for PCR amplification of a ~1446 bp section of the 16S rRNA gene using primers arch16sfs and rp1a (Table 4). Reaction conditions for each sample were 11.2 μl of water, 1.6 μl of $MgCl_2$ (25 mM),

2.0 μl of 10× buffer, 4.0 μl of dNTP (1 mM each), 1.2 μl of each primer (25 μM), 0.08 μl of *Taq* polymerase (5 U μl^{-1}) and 0.4 μl of DNA template for a final volume of 21.68 μl. The samples were amplified in a MJ Research PTC-200 thermal cycler. A touchdown cycling programme was used involving eight cycles of 94.2°C for 20 s, 58.0°C for 15 s (–0.5°C per cycle), 72.0°C for 80 s, followed by 26 cycles of 94.2°C for 20 s, 54.5°C for 15 s, 72.0°C for 80 s, with a final extension of 3 min at 72.0°C. The PCR products were separated by agarose gel electrophoresis and re-amplified using internal primers (Table 4).

Polymerase chain reaction products were sequenced on both strands using an Applied Biosystems model 310 automated sequencer with protocols recommended by the manufacturer. The resulting sequences were manually assembled and edited to make a 1118–1406 bp contiguous 16S rRNA sequence. The sequences were compared with the GenBank database using BLAST searches (Altschul *et al.*, 1990). The partial 16S rRNA gene sequences from the Death Valley salt core were deposited in GenBank under Accession No. FJ492047–FJ492052.

Cloning

The 16S rRNA genes of one culture from 17.8 m depth (34 000 years old), amplified by PCR, were cloned into *Escherichia coli* using a TOPO TA Cloning Kit from Invitrogen Life Technologies (Carlsbad, CA) following the manufacturers' protocols. DNA from two clones (DV582c2 and DV582c4) was extracted, amplified and sequenced following procedures described above.

Phylogenetic tree construction

A maximum likelihood tree was constructed from aligned 16S rRNA gene sequences using PAUP 4.0 beta for Windows (Swofford, 2003). The general time reversible model of nucleotide substitution was used (Lanave *et al.*, 1984). *Archaea* for the tree were selected from the order *Halobacteriales* (Wright, 2006). A bootstrap analysis (200 replicates) was performed to estimate support for each branch. *Methanospirillum hungatei* (M60880) was used as the outgroup taxon.

Growth conditions

DV582A-1 and DV582B-3 were studied to determine their optimal NaCl concentration, temperature and pH for growth. DV427 and DV462A were not studied due to transfer difficulties. The culture represented by DV582c2 and DV582c4 could not be studied because a single-colony isolate was not obtained from this sample. PGB medium was used to determine the optimal NaCl concentration for growth (Table 2). The NaCl concentration was varied from 2.9 to 5.0 M at temperatures of 21°C, 37°C and 45°C (Table 5). The pH tolerance for growth was determined at the optimal NaCl concentration and temperature by varying the pH of the medium from 6 to 10 (Table 6). All cultures were incubated under aerobic conditions and manually shaken several times per week. Cultures were examined for growth for up to 3 months.

Re-entrapment in fluid inclusions in halite

DV582A-1 and DV582B-3 were trapped inside halite crystals formed under laboratory conditions to confirm that these microorganisms could survive in fluid inclusions. Isolates were grown in 50 ml conical centrifuge tubes containing 25 ml of PGB medium. The cultures were centrifuged (Jouan Inc. CR3-12 with swing-out rotor, Winchester, VA) at 1750 *g* for 30 min to pellet cells at the bottom of the tube. The medium was decanted under a laminar flow hood and 25 ml of sterile, halite-saturated brine was then added to the cells to dilute the nutrient medium and increase the NaCl concentration. The pellet was re-suspended in the brine. Two millilitres of the brine was then transferred to every other well of a sterile, 24-well microtitre plate (Becton Dickinson Labware, Franklin Lakes, NJ). The brine slowly evaporated to dryness and the resulting halite crystals were cleaved, mounted to glass slides and microscopically inspected for entrapped cells. After 74 (DV582A-1) or 474 (DV582B-3) days, the crystals were surface-sterilized and dissolved into sterile PGB medium. One millilitre of the final NaCl saturated rinse was inoculated into PGB medium as a surface-sterilization control.

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