DATA REPOSITORY 2009266

This is supplemental material for the paper, "How do prokaryotes survive in fluid inclusions in halite for 30,000 years?" by Schubert et al., submitted to *Geology*, containing methods (including microscopy, culturing, DNA sequencing, and calculations of carbon availability) and tables (DR1, DR2, and DR3), followed by the appropriate references.

Methods

Microscopy. Large format thin sections (5 x 7.5 x 0.1 cm) prepared from the Death Valley core were studied microscopically with a Leica Wild M3Z stereomicroscope. Prokaryotes were identified in fluid inclusions *in situ* using a Zeiss compound microscope (AXIO Imager.A1) at 1000x magnification with an oil immersion objective (PLAN APO 100x/1.4 OIL). Autofluorescent microparticles were studied using an HBO 100 mercury lamp and Chroma Technology Corp. filters [DAPI: exciter D350/50 nm, emitter D460/50 nm, beamsplitter 400dclp; Green Fluorescent Protein (GFP): exciter HQ450/50 nm, emitter HQ510/50 nm, beamsplitter Q480lp; and TRITC: exciter HQ545/30 nm, emitter HQ620/60 nm, beamsplitter Q570lp]. Prokaryotes were photographed with an AxioCam MRm B&W camera and AxioVision software (Version 4.5).

The distribution of prokaryotes in the Death Valley core was determined microscopically along random transects of thin sections from 38 stratigraphic layers using the 100x oil immersion objective. The percentage of fluid inclusions containing prokaryotes was calculated for each layer. A total of 6823 fluid inclusions were examined for prokaryotes.

Culturing. Single crystals were cut from freshly broken interior sections of the Death Valley core (6 cm diameter) using a single-edged razor. Fresh, inner sections of core reduced the potential for contamination. Selected halite crystals had numerous primary, single-phase (liquid) inclusions and no conspicuous fractures.

Preparation for surface sterilization included autoclaving all glassware, tweezers, rinses, and pipette tips for 30 minutes (121 ^{o}C, ~17 psi). Growth media (see below) were made in 500 ml batches and then transferred to 50 ml glass Kimax flasks with screw caps (25 ml of medium per flask) or 16 x 125 mm Fisherbrand glass test tubes with screw caps (7 ml of medium per tube) and autoclaved. All surface sterilization processes were performed 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 one hour before use and all hood surfaces were wiped down with ethanol. Halite crystals were sterilized following procedures adapted from (Rosenzweig et al., 2000). In brief, the sterilant (10 M NaOH) and NaCl saturated brine washes (~5 ml) were placed into sterile beakers. Halite crystals were immersed in 10 M NaOH for five minutes followed by two consecutive washes in sterile sodium chloride saturated brine. The pH of the second rinse remained neutral. Crystals were transferred between beakers using sterile tweezers and then transferred into growth medium. Crystals slowly dissolved in the medium. One milliliter of the second NaCl wash was inoculated using a sterile pipette into a separate test tube or flask containing the same medium type as the associated crystal sample. The test tube or flask with the dissolved crystal and the experimental control were incubated together, under the same conditions (37 ^{o}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

any controls suggests that cultured prokaryotes did not result from laboratory or environmental contaminants on crystal surfaces.

Two media types (CAB – Casamino Acid Binghamton; PGB – Pyruvate Glycerol Binghamton) at four NaCl concentrations (CAB – 0.7, 1.4, and 3.4 M; PGB – 4.3 M) were used (Vreeland et al., 1984) (Table DR1). The pH was adjusted to 8.0-8.2 for CAB medium and 7.3-7.5 for PGB medium. Inorganic salt chemistry of all media was designed to approximate the modern major ion brine chemistry of Death Valley (Li et al., 1997). Inoculated media were incubated for up to 28 months or until growth was observed (indicated by turbidity or color change). Cultures showing signs of growth were isolated on plates (1.5-2.0% agar) of the same medium chemistry in which they grew.

The sterilization protocol was tested to show that the cultured strains isolated from Death Valley halite could not survive on crystal surfaces when submerged in 10 M NaOH. Halite crystals (Diamond Crystal Solar Salt Extra Coarse, Cargill Incorporated, Minneapolis, MN) were sterilized by heating at 500 ^{o}C for 72 hours. Once at room temperature, each crystal was streaked across the surface of an agar plate containing colonies of prokaryotes isolated from the Death Valley salt core (DV427, DV462A, DV582A-1, and DV582B-3). A total of 64 halite crystals (16 crystals streaked for each of the 4 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 test tubes containing the medium from which the organism originally grew. Cultures were incubated for 168 days at 37 ^{o}C.

Two strains, DV582A-1 and DV582B-3, were cultured in GB medium (Table DR1) to test their ability to grow in medium containing glycerol as the only carbon

source. The cultures were incubated at 37 ^{o}C under aerobic conditions. Growth was observed for both strains. These results indicate that halophilic *Archaea* isolated from the Death Valley core can utilize glycerol as a carbon source.

DNA Sequencing. DNA was amplified using the polymerase chain reaction (PCR). First, a ~1446 bp section of the 16S rRNA gene was amplified using primers to target *Archaea* (arch16sfs and rp1a) (Table DR2). Reaction conditions for each sample were 11.2 µl water, 1.6 µl $MgCl_2$ (25 mM), 2.0 µl 10X buffer, 4.0 µl dNTP (1 mM each), 1.2 µl of each primer (25 µM), 0.08 µl Taq polymerase (5U/µl), and 0.4 µl of DNA template for a final volume of 21.68 µl. Samples were amplified in a MJ Research PTC-200 thermal cycler. A touchdown cycling program was used for the primer set arch16sfs/rp1a involving 8 cycles of 94.2 ^{o}C for 20 s, 58.0 ^{o}C for 15 s (-0.5 ^{o}C per cycle), 72.0 ^{o}C for 80 s, followed by 26 cycles of 94.2 ^{o}C for 20 s, 54.5 ^{o}C for 15 s, 72.0 ^{o}C for 80 s, with a final extension of 3 min at 72.0 ^{o}C. The PCR products were separated by agarose gel electrophoresis and reamplified using internal primers (Table DR2).

16S rRNA genes from one culture (17.8 m; 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 was extracted, amplified, and sequenced.

PCR products were sequenced on both strands using an Applied Biosystems model 310 automated sequencer with protocols recommended by the manufacturer. Sequences were manually assembled and edited to make an 1118-1406 bp contiguous 16S rRNA sequence. Sequences were compared with the GenBank database using a BLAST search (Altschul et al., 1990). Partial 16S rRNA gene sequences from the

Death Valley core were deposited in GenBank under accession numbers FJ492047-FJ492052.

Carbon availability. We calculated the maximum time over which one miniaturized prokaryote cell could metabolize all the carbon provided by the glycerol from one *Dunaliella* cell. The calculation used one *Dunaliella* cell (10 μm diameter; 5.5 M glycerol), one prokaryote cell [10 fg of carbon (Whitman et al., 1998)], and an average metabolic rate of 10^{-7} hr^{-1} needed for a cell to survive and repair macromolecular damage (Price and Sowers, 2004). The calculated time of 12 million years indicates that prokaryote cells in fluid inclusions in halite from the Death Valley core are not carbon limited if *Dunaliella* co-exist in the same inclusions.

TABLE DR1. MEDIA RECIPES USED TO CULTURE DEATH VALLEY PROKARYOTES [RECIPES MODIFIED FROM VREELAND ET AL. (1984)].

Component	CAB*	PGB†	GB§
	(gram liter^{-1})		
Casamino acids	7.5	0.0	0.0
Yeast extract	1.0	0.0	0.0
Protease peptone	5.0	0.0	0.0
Sodium citrate	3.0	0.0	0.0
K_2HPO_4	0.5	0.5	0.5
$MgSO_4 \cdot 7H_2O$	2.0	2.0	2.0
KCl	2.0	4.0	4.0
Glycerol	0.0	2.5	5.0
Sodium pyruvate	0.0	2.5	0.0
$(NH_4)_2SO_4$	0.0	1.0	1.0
NaCl	40, 80, or 200	250	250

*Casamino Acids Binghamton (pH adjusted to 8.0-8.2).
†Pyruvate Glycerol Binghamton (pH adjusted to 7.4).
§Glycerol Binghamton (pH adjusted to 7.4).

TABLE DR2. SUMMARY OF GROWTH CONDITIONS FOR *ARCHAEA* CULTURED FROM THE DEATH VALLEY CORE.

Organism	Medium	pH	NaCl (M)
DV427	PGB	7.4	4.3
DV472A	PGB	7.4	4.3
DV582A-1	PGB	7.4	4.3
DV582B-3	PGB	7.4	4.3
DV582c2	PGB	7.4	4.3
DV582c4	PGB	7.4	4.3
Natronobacterium sp. 2-24-1*	CAS*[†]	ND	3.4*
Halobacterium sp. 2-24-4*	CAS*[†]	ND	3.4*
Halobacterium salinarum strain BBH 001[§]	HM[#]	7.5[#]	3.4[#]

*Reported in Vreeland et al. (2007).
[†]Reported in Vreeland et al. (1984).
[§]Reported in Mormile et al. (2003).
[#]Reported in Norton and Grant (1988).
PGB = Pyruvate Glycerol Binghamton.
CAS = Casamino Acids.
HM = Halophile Medium.
ND = No Data.

TABLE DR3. OLIGONUCLEOTIDE PRIMERS USED IN THIS STUDY.

Primer	5' → 3' nucleotide sequence	Position of primer*	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

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

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