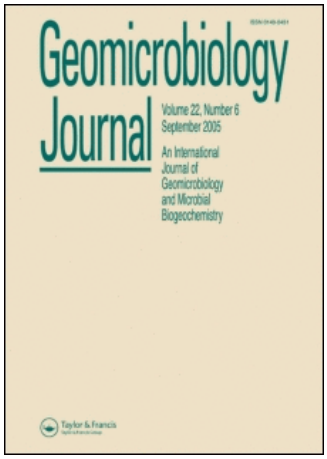

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R. H. Vreeland [a]; J. Jones [b]; A. Monson [b]; W. D. Rosenzweig [b]; T. K. Lowenstein [cd]; M. Timofeeff [cd]; C. Satterfield [cd]; B. C. Cho [cd]; J. S. Park [cde]; A. Wallace [f]; W. D. Grant [f]

[a] Ancient Biomaterials Institute, and Department of Biology, West Chester University, West Chester, PA, USA
[b] Department of Biology, West Chester University, West Chester, PA, USA
[c] Department of Geological Sciences, Binghamton University, Binghamton, New York
[d] School of Earth and Environmental Sciences, Seoul National University, Seoul, South Korea
[e] Department of Oceanography, Chonnam National University, Gwangju, Republic of Korea
[f] Department of Infection, Immunity and Inflammation, Maurice Shock Medical Sciences, Building University of Leicester, Leicester, UK, United Kingdom

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Isolation of Live Cretaceous (121–112 Million Years Old) Halophilic *Archaea* from Primary Salt Crystals

R. H. Vreeland
Ancient Biomaterials Institute, and Department of Biology, West Chester University, West Chester, PA 19383, USA

J. Jones, A. Monson, W. D. Rosenzweig
Department of Biology, West Chester University, West Chester, PA 19383, USA

T. K. Lowenstein, M. Timofeeff, C. Satterfield, B. C. Cho, and J. S. Park
Department of Geological Sciences, Binghamton University, Binghamton, New York 13902; School of Earth and Environmental Sciences, 302-19 Gwanak Campus, 151-752, Gwanak gu, Seoul National University, Seoul, South Korea

A. Wallace and W. D. Grant
Department of Infection, Immunity and Inflammation, Maurice Shock Medical Sciences Building University of Leicester, Leicester, United Kingdom LE1 9HN, UK

Recent reports have described the isolation and analysis of living microbes and/or DNA fragments from halite crystals of significant geological age. This manuscript describes the isolation of six living strains of halophilic *Archaea* from Cretaceous (121–112 MYA) halite crystals. These 6 live strains represent the oldest *Archaea* isolated to date. This manuscript also presents the first isolation of representatives from two different archaeal genera in a single event. The data presented show that the organisms that inhabited these hypersaline environments today are similar to those present during the Cretaceous age. Considering the number of ancient samples that have now yielded living microbes or DNA fragments the evidence for long-term survival of microbes (at least within halite) is becoming increasingly definitive. While there are obviously still other trapped microbes to find, it may now be time to begin investigating the implications of these ancient microbes and the mechanisms that foster long-term survival.

Keywords *Archaea*, Cretaceous, *Halobacterium*, *Natronobacterium*, long-term survival

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Address correspondence to R. H. Vreeland, Ancient Biomaterials Institute, West Chester University, West Chester, PA 19383. E-mail: rvreeland@wcupa.edu
J.S. Park's current address is Department of Oceanography, Chonnam National University, Gwangju 500-757, Republic of Korea.

INTRODUCTION

Over the last few years, several reports have described the isolation of microorganisms and/or DNA from halite crystals of significant geological age (Vreeland et al. 2000; Fish et al. 2002; Mormile et al. 2003).

The most talked about of these studies was that of Vreeland et al. (2000) who provided what has been called the best available evidence for long term survival (Parkes 2000). That same study has still been controversial due to the similarity in the sequences of the 16S rRNA genes of the ancient isolate and those of a putatively modern microbe (Graur and Pupko 2001; Nickle et al. 2002). While the molecular questions have been discussed at length, this debate is somewhat tangential since the conclusion that the bacterium was of Permian age was actually based upon the stability and age of the geological formation, the primary nature of the crystal sampled and the low level of contamination probability (1 in 10^{-9}) (Rosenzweig et al. 2000; Vreeland et al. 2000, 2006; Satterfield et al. 2005). In a separate study, Fish et al. (2002) isolated DNA from halite crystals of varying ages (11 to 425 MYA) that were first sectioned using intense lasers, analyzed for fluid inclusion chemistry, then polished and cleaned with an ultra-sonic cleaner. Crystals that were 11–16 MYA were found to contain DNA fragments from the 16S rRNA gene of at least one haloarchaeon closely related to *Halobacterium salinarum*. Crystals that were 65–425 MYA yielded bacterial DNA fragments. While this study was unsuccessful in attempts to isolate living microbes it did provide additional data indicating that halite crystals could potentially protect fragile DNA molecules for long time periods.

The most recent study to examine survival in halite studied crystals taken from Death Valley CA which were 0.097 million years old (MYA) (Mormile et al. 2003). Mormile et al. (2003) successfully isolated a representative culture of *Halobacterium salinarum* directly from brine inclusions in primary crystals. These authors also demonstrated that contamination was not an issue since their control crystals consistently produced no growth (Mormile et al. 2003).

Due to the high impact attached to claims of the isolation of living organisms from ancient materials, efforts aimed at duplication become highly desirable. Given the facts that detectable microbial populations are not homogeneous within ancient formations (Vreeland et al. 1998), and with the limited number of properly equipped dedicated laboratories, reproducing the work of others in this field is difficult. The survival of life held in stasis for significant geological periods, has implications for evolution, macromolecule preservation, suspended animation, energetics during starvation, panspermia and a host of other issues. This manuscript presents additional evidence confirming the survival of non-spore forming halophilic Archaea for significant periods of time. The organisms described herein arose from crystals that were 121–112 MYA.

METHODS

Samples used for this study were taken from the Aptian aged salts of the Sergipe basin of Brazil (Timofeeff et al. 2006). Each crystal was examined and photographed at facilities other than those to be used for microbiological or molecular extraction. During this examination, any portions of the crystals that appeared to be compromised in any manner were removed leaving only fragments that were unequivocally primary.

Geological Background

The Sergipe salts formed during the early rifting phase of the South Atlantic Ocean (Evans 1978). The Sergipe salts are Aptian in age (121.0–112.2 MYA), and have been interpreted as marine transgressive sediments of the proto Atlantic Ocean deposited in a tectonic setting similar to the modern Red Sea (Wardlaw 1972; Evans 1978). The Sergipe basin occurs on the northeastern continental margin of Brazil, and extends offshore, with a total aerial extent of $\sim$20,000 km^2 (Silva 1983). The Aptian evaporites occur in the Ibura Member of the Muribeca Formation, an evaporite sequence up to 800 m thick (Figure 1) (Wardlaw 1972; Silva 1983; Oliver 1997).

Preservation of primary sedimentary features in some Ibura Member evaporites suggests little post-depositional alteration and mineralogical changes. Primary features identified include laminated halite and fluid inclusion-banded chevron halite (Oliver, 1997). Chevron halite consists of bottom grown halite crusts in which individual crystals contain alternating bands rich (cloudy) and poor (clear) in fluid inclusions (Figure 2) (Lowenstein and Hardie 1985). These inclusions typically make up about 1% of the mass of chevron halite crystals (Roedder and Bassett 1981). This type of primary, subaqueous-grown halite can be distinguished petrographically from diagenetic halite cement and recrystallized halite. The lowermost part of the Ibura Member from the GTP-8 core was studied by Oliver (1997) and Timofeeff et al. (2006). Fluid inclusions from a layer of primary chevron halite, at a depth of 768 m in the GTP-8 core, were analyzed in this study (Figure 1). Crystals for microbiological analysis were removed from cores and examined via a petrographic microscope. Each crystal fragment was placed into clean, unused sample vials and transported to the microbiological laboratory.

Isolation of Live Microbes

Halite crystals were sampled in a biological safety level three (BSL-3) clean room. Prior to use the entire room (including all horizontal surfaces) was cleaned with anti-bacterial cleaners, then sprayed with freshly made Cidex Plus liquid sterilizing agent. All cleaning was performed using a two mop/two bucket system. All personnel involved in the sampling were gowned in clean, bleach washed coveralls, sterile latex gloves, hair caps and boots. Those working directly with crystals also wore sterile, close-front surgical gowns over their coveralls, caps, boots as well as sterile latex gloves. The class IIB laminar flow hood, where the crystals would be sampled, was first treated for 2 hours under a germicidal UV light, followed by spraying and wiped using freshly mixed liquid sterilizing agent (Cidex Plus). Each crystal was sterilized inside the hood using previously published sterilizing procedures (Rosenzweig et al. 2000).

Due to the need for high levels of contamination control, extensive contamination checks were conducted throughout all experiments. These controls included: (1) autoclaving of all steam stable equipment, media and chemicals for at least 60 minutes; (2) pre-incubation of all sterilized media for at least 14 days prior to use, all medium batches were also prepared in groups of 50 flasks with the rule that all were discarded without use if even a single flask was contaminated; (3) sampling of all wash brines after treatment of six crystals, once again any contamination event resulted in discarding the 6 samples even if any of these already produced positive growth; 4) during incubation the wash brines were incubated along with samples and needed to remain negative for at least 14 days after the last positive crystal from that set of 6 samples; (5) presence of open control plates of solid media scattered inside the laminar flow hood during the entire sampling operation. In this case, any contamination resulted in the rejection of all samples taken during that session. Finally, all media used for contamination checks were selected at random from the flasks being used for actual isolation.

The individual halite crystals were surface sterilized using the 5-minute NaOH, 5-minute HCl procedure of Rosenzweig et al. (2000) in groups of 6 using sterile 24-well tissue culture plates. At the end of the surface sterilization, the crystals were placed individually (one per flask) into small 50 ml screw cap Erlenmeyer flasks containing 25 ml of CAS medium (Vreeland et al. 1984) that had been autoclaved at 121°C for 60 minutes and incubated before use to check for contamination. All media used in these experiments contained 20% NaCl so the crystals were allowed to dissolve slowly after they were placed in the medium. The inoculated medium was allowed to stand undisturbed for at

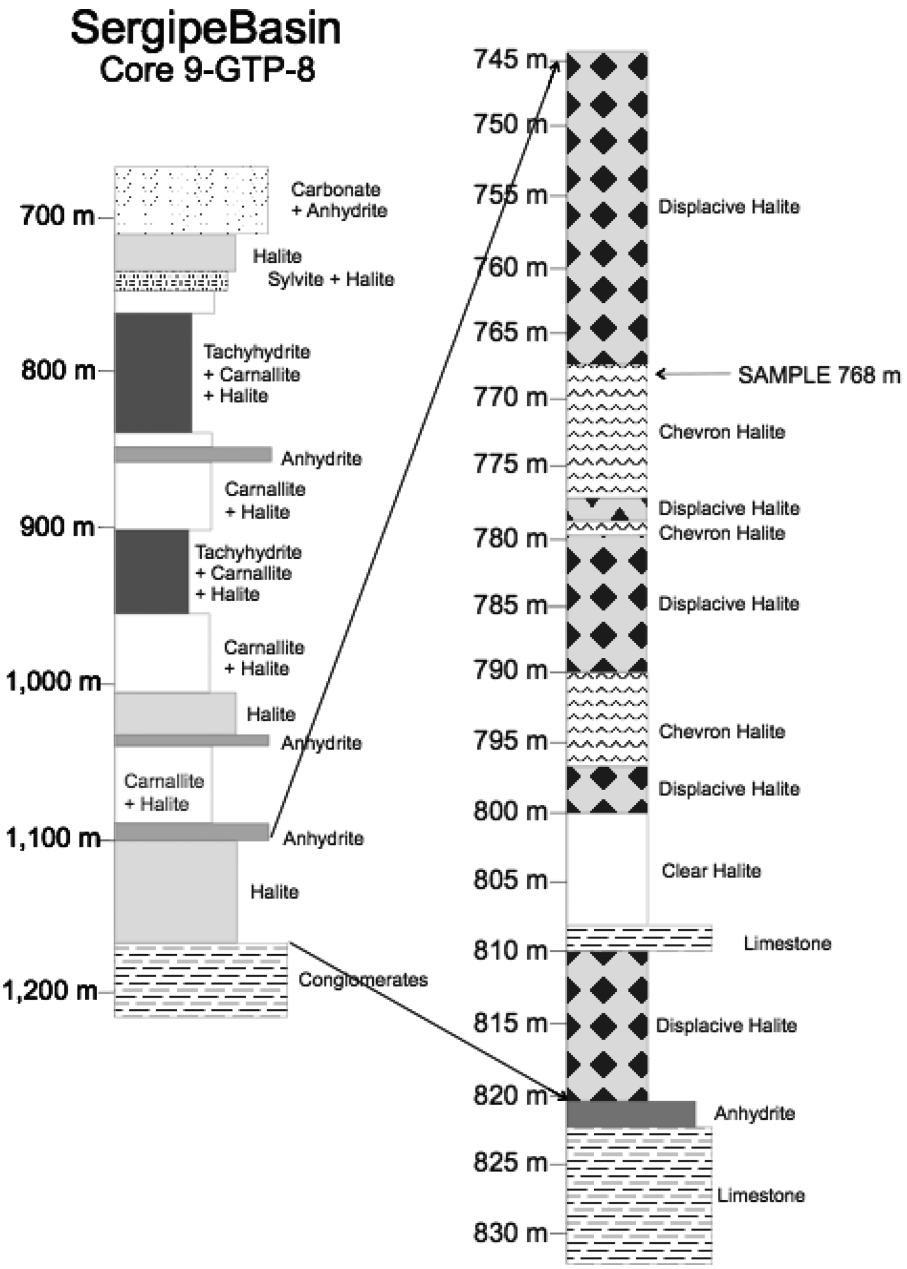

FIG. 1. Complete stratigraphic section of the Ibura Member from a borehole core through the Muribeca Formation is shown on the left (modified from Figure 4 of Wardlaw 1972). The lowermost section of the Ibura Member, core GTP-8 is shown on the right. The first cycle of the Ibura Member (833–766 m) contains laminated halite, chevron halite and displacive halite (Oliver 1997 PhD thesis; Universidade Federal Fluminense). Chevron halites from the 768 m layer, which contained large numbers of fluid inclusions were analyzed in this study. This figure has been adapted from actual core logs from 2 cores and the stratigraphic layers have been drawn to correspond.

least 2 hours after the crystals dissolved. The media were then placed into an incubated shaker at 30°C. The flasks were checked weekly for several weeks.

Lipid Analysis

Lipid analyses were performed according to the method of Torreblanca et al. (1986) as recommended for the identification of haloarchaea (Oren et al. 1997). All cultures were also grouped into the Archaea due to the presence of intact phosphatidyl glycerol ether moieties following acid methanolysis of whole cell lipid extracts (Ross et al. 1981).

Sequencing of 16S rRNA

DNA was extracted directly from a pelleted cell mass using the Genomic Prep Cell and Tissue DNA Isolation Kit (Pharmacia Biotech, Amersham,UK) following the manufacturer's protocol. To confirm genomic DNA was present the extracted samples were electrophoresed on 1% agarose gels. The PCR protocol

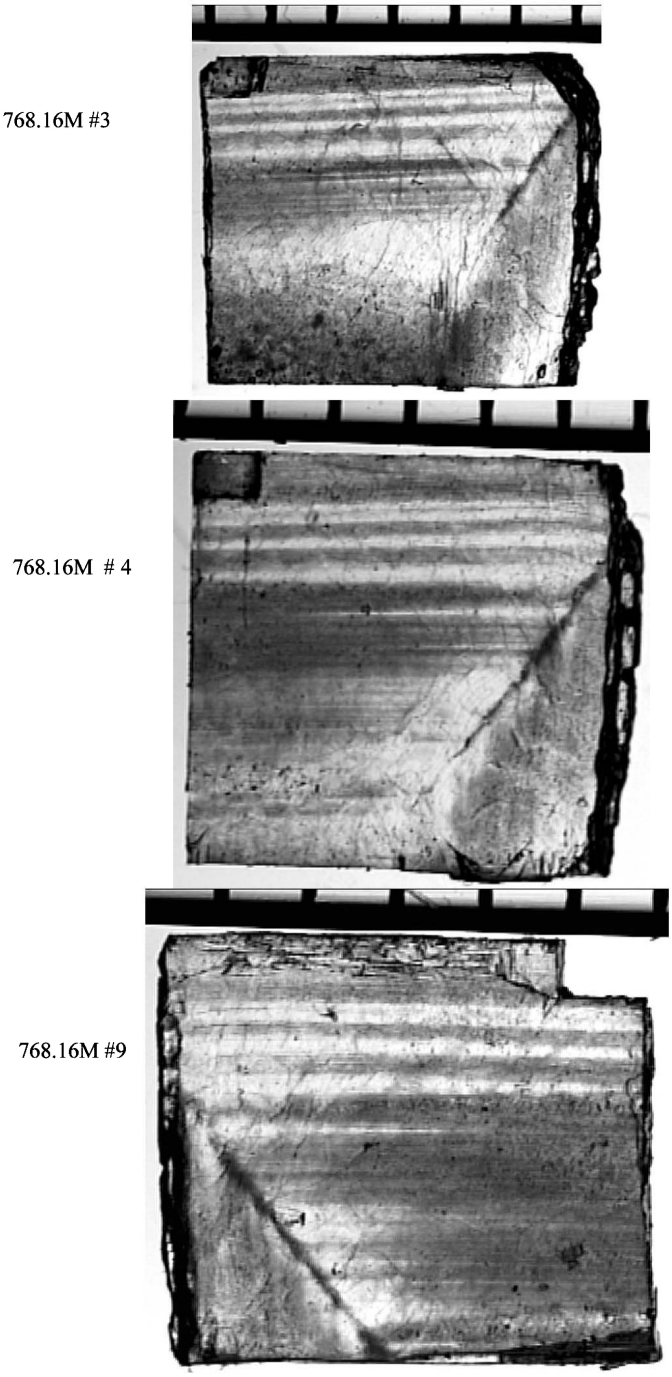

FIG. 2. Photographs of 3 of the 4 Cretaceous (121–112 MYA) chevron crystals with abundant fluid inclusion banding that contained living halophilic Archaea. (A) crystal that contained Archaeal strains 2-24-2, 2-24-3. (B) crystal that contained 2-24-5. (C) crystal that contained 2-24-7. The crystal that yielded 2-24-6 is not shown. Scale bars equal 1 mm center to center.

for the amplification of 16SrRNA genes has been described previously (Grant et al. 2004). Primers used were:

27Fa (archaeal forward) (5′-TC(CT)GGTTGATCCTG(CG)CG G-3′)

1492R (universal reverse) (5′-ACGG(ATC)TACCTTGTTACG ACTT-3′)

The PCR products were then purified using the QIAquick PCR Purification Kit (Qiagen, Crawley, UK). PCR products were checked for the presence of 16S rRNA genes using 1% agarose gel electrophoresis and the products were sequenced using the same primers.

The BLASTN program at NCBI and the FASTA program of the GCG Wisconsin package were used to identify archaeal sequences closest to those of the isolates. Sequences were aligned using the Clustal X program, version 1.81 <http://inn-prot.weizmann.ac.il/software/ClustalX.html>. The alignment was then transferred into the Genedoc program (http//www.psc.edu/biomed/genedoc) for final manual editing. The phylogenetic tree was constructed from the alignments using the TREECON for Windows package (Van der Peer and De Wachter 1994). Distance estimations were calculated using the substitution rate calibration of Jukes and Cantor (1969). Tree topology was inferred by the neighbor-joining method. A 1000 replicate bootstrap analysis was performed to estimate the reliance of the clusters (Felsenstein 1985).

RESULTS

Isolation of Halopilic Archaea

A total of 56 crystals from the Sergipe basin core were used for enrichment of live microbes. The Brazilian crystals all arose from the stratigraphic layer at 768 m. Six live, extremely halophilic microorganisms were isolated from the Cretaceous halite crystals. Four of the 56 Cretaceous crystals 6 yielded viable organisms. Three of the organisms were enriched from individual crystals, while the other 3 isolates came from a single crystal. These isolations met all non-contamination criteria. The remaining 52 enrichments never developed any signs of contamination.

Each of the Sergipe Basin isolates was purified via several successive single colony isolations from the initial enrichment cultures and assigned permanent collection tracking numbers 2-24-2, 2-24-3 and 2-24-5 thru 2-24-8. Three of the 4 crystals that contained viable organisms are shown in Figure 2. The crystal shown in Figure 2A contained 2-24-2, 2-24-3 and 2-24-8; the crystal shown in Figure 2B contained 2-24-5 and that shown in Figure 2C produced 2-24-7. The purified isolates were subjected to lipid and molecular analyses for preliminary characterization.

The lipid analyses (Figure 3A) clearly show that all of the Sergipe Basin isolates (designated culture collection numbers 2-24-2, 2-24-3 and 2-24-5 thru 2-24-8) possessed the typical glycerol di-ether moieties present in all members of the Archaea (Ross et al. 1981). They did not possess any fatty acid methyl esters. Consequently, all of the isolates can be designated as members of the Domain *Archaea*. A comparison of the diagnostic glycolipids (Fig. 3B) showed that strain (2-24-8) had a lipid pattern identical to members of the genus *Natronobacterium*

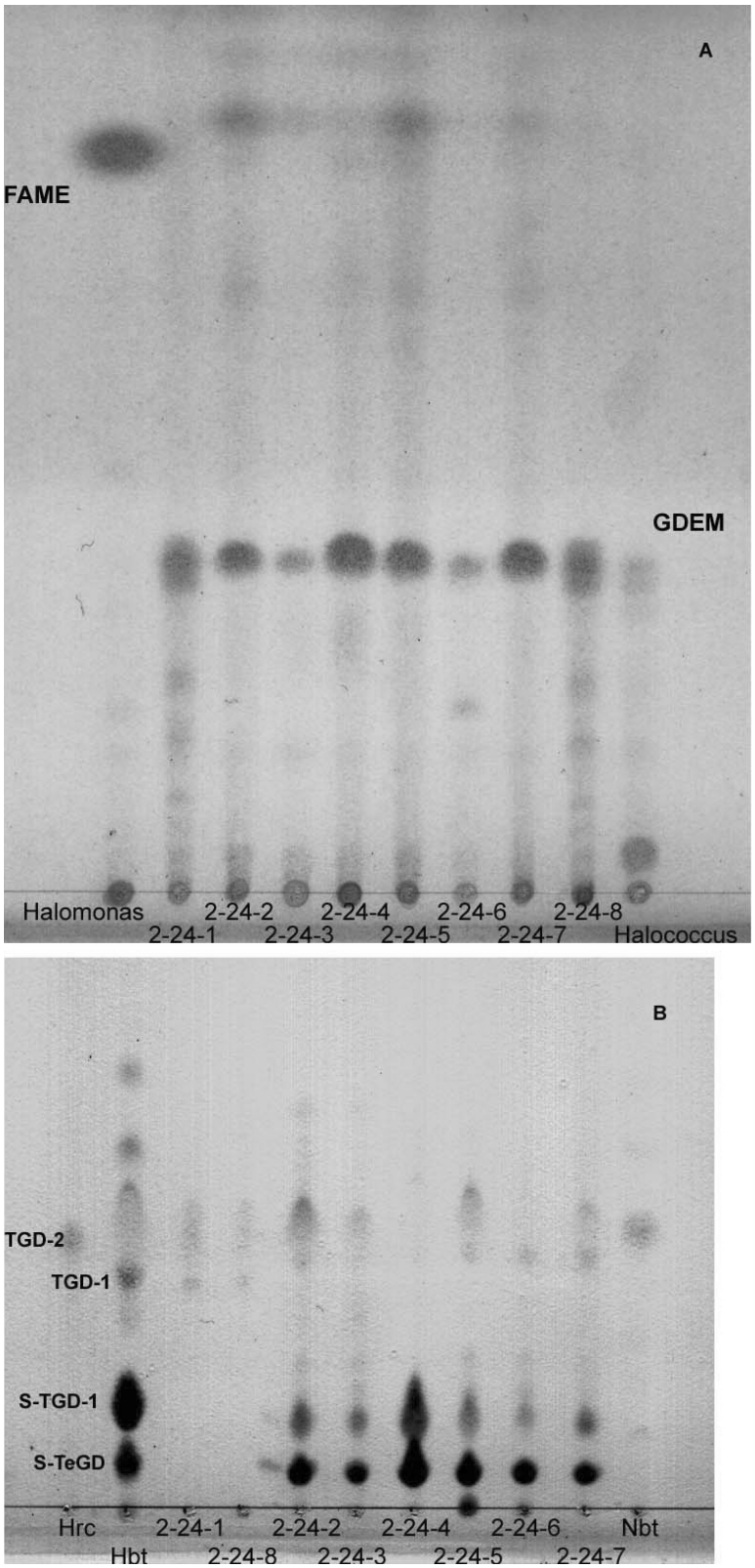

FIG. 3. Lipid patterns of the 6 Cretaceous haloarchaeal isolates obtained in this study (strains 2-24-1 and 2-24-4 were isolated from 31,000-year-old Death Valley Salts and will be described elsewhere. (A) Glycerol diether patterns (GDEM). FAME: fatty-acid methyl esters; (B) Glycolipid patterns. TGD-1: Tri-glycosyl-diether #1; TGD-2: Triglycosyl-diether #2; S-TeGD: Sulfated Tetra-glycosyl-diether; Hrc: *Haloarcula*, Hbt: *Halobacterium*, Nbt: *Natronobacterium*.

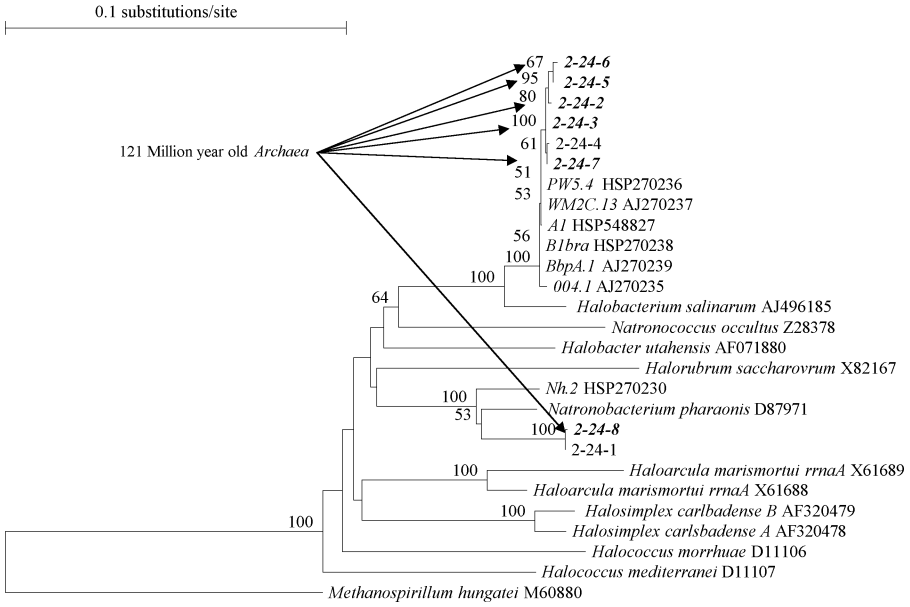

FIG. 4. Phylogenetic tree comparing 16S rRNA sequences of the live Cretaceous haloarchaea with other isolates and type strains. Bootstrap values (> 50%) from distance estimations (1,000 replicates) are only shown at the nodes. The following strains and accession numbers represent sequences amplified live isolates from 20 MYA salts by Fish et al. (2002): PW5.4 (HSP 270236); WM2C.13 (AJ 270237); A1 (HSP 548827: Hbt noricense); BiBra (HSP 2702238); BbpA.1 (AJ270239) and 0004.1 (AJ 270235). The other strains referenced by accession numbers in this figure represent known cultures isolated from modern surface habitats.

(Tindall et al. 1984; Oren et al 1997; Grant et al. 2001). The remaining five organisms possessed the glycolipid patterns characteristically possessed by members of the *Halobacterium* (Figure 3B). These data were also supported by physiological characterizations which showed that *Natronobacterium* strain (2-24-8) grew best in alkaline media while the other strains preferred more neutral conditions (data not shown).

Molecular analysis of these organisms agreed precisely with the chemical and physiological data. Isolate (2-24-8) was associated with the *Natronobacterium* clade while all of the others grouped with *Halobacterium salinarum* (Figure 4). The raw sequence similarity between the strains from the crystals and those from culture collections was also quite high. Isolate 2-24-8 was 96% similar to *Natronobacterium pharaonis* whereas 2-24-2, 2-24-3 and 2-24-5 thru 2-24-7 proved to be >99% similar to members of the *Halobacterium salinarum*—associated clade containing strain PW5.4 and other isolates from Permo-Triassic salt deposits (McGenity et al. 1998, 2000). The 16S rRNA gene fragments isolated from 11-16-million-year-old crystals described by Fish et al (2002) are also associated with this clade. The strains isolated from these primary salt crystals are members of 2 familiar haloarchaeal genera, *Natronobacterium* and *Halobacterium*. The 16S rRNA gene sequences from the Cretaceous crystals have been deposited in GenBank under accession numbers AJ878078, AJ878079, AJ878081-AJ878084.

DISCUSSION

The findings reported here have significance beyond the identification of the strains or their presence within these primary halite crystals. Based upon their presence inside surface sterilized 121 MYA primary salt crystals the six strains described here represent the oldest living *Archaea* ever isolated. This particular conclusion is not based on the biological characterization presented here. The age of these isolates must be inferred from the primary nature of the crystals from which they were enriched; from the geologically determined age of the Sergipe Basin and from the lack of any sign of contamination in any of the contamination checks or the 52 crystals sampled in the same experiment and which proved to be negative.

This most significant finding is supported by data showing that the crystals in which the strains were found, are primary materials, from well dated, un-deformed geological strata (Lowenstein et al. 2005; Timofeeff et al. 2006). These isolates also provide at least some information relative to the possible survival frequency within these formations. Research on modern samples has shown that nearly 100% of the crystals contain live microbes upon initial formation. These isolations have shown that less than 8% (4/56) contain live cells after 120 MYA. These data are consistent with data from numerous other formations of varying ages which also show a decreasing rate of survival over time.

The isolation of a halophilic neutrophile and a halophilic alkaliphile from crystals sterilized with concentrated acid and base provides strong evidence that the organisms were contained within the stable confines of the crystal protected from the harsh conditions. The presence of these two *Archaea* in the same culture is also not altogether surprising. While the pH optimum, lipid patterns and rRNA showed that strain 2-24-8 is clearly a *Natronobacterium* it has a wide pH tolerance and is able to grow

from pH 7 to 10. Since the pH of the enrichment medium was 7.5–8.0 this organism could have easily survived and grown in the enrichments. Therefore, the organisms must have been present at the time the crystals formed. As such, these strains represent the state of microbial evolution during the Cretaceous period.

The isolation of living organisms from ancient samples obviously has the great potential impact on our overall knowledge of ancient life. Such isolations invariably raise questions about the mechanisms of survival within these materials. Several previous studies using both ancient halite (Vreeland et al. 2000) and amber (Cano and Borucki 1995; Greenblatt et al. 2004) isolated spore forming microbes that could be thought to survive such long-term suspension as cryptobiotic spores. Such is not the case in this instance since halophilic Archaea do not form such protective structures. How then might non-sporing organisms such as those isolated here survive? At the biological level this is a difficult question to address with currently available data. As is obvious from Figures 1 and 2 this particular halite region and the crystals sampled represent very stable formations. So stable that individual crystals have quite clearly maintained their most delicate primary features.

In addition, numerous chemical analyses have demonstrated that these crystals retain the chemistry of their original source waters (Lowenstein and Hardie 1985; Satterfield et al. 2005). Timoffeef et al. (2006) have used other primary crystals from this site to demonstrate distinct differences between the chemistries of the Cretaceous and modern oceans. Since the inclusion brines are also of very high ionic strength many damaging compounds [i.e., oxygen free radicals or even beta particles (Nicastro et al. 2002)] are rapidly absorbed by the large number of oppositely charged ions in the solutions leading again to a highly protective stable environment for the trapped microbes. In pure distilled water ionized particles such as beta and alpha radiation diffuse away at some distance from their original flight path thereby creating a slightly larger cone of damage. However in solutions already containing large amounts of dissolved ions the opposite charges serve to attract these forms of ionized radiation, holding them to a smaller flight path while effectively transferring some of the energy away from the radiation. This then serves to decrease but not necessarily eliminate the damage cone since a particle generated (or flying directly at) a sensitive bond could still impact he bond. High levels of dissolved ions do however significantly lessen the probability of such an impact.

At the taxonomic level the isolation of several strains that key out so precisely with currently recognized genera is a fascinating aspect indicating that these organisms have obviously populated hypersaline environments on the Earth's surface for many years. In fact, this close taxonomic relationship also points directly to the long term stability of these so-called extreme environments. From an evolutionary viewpoint such close relationships over long periods could only occur within conditions that do not impart intense selection pressures upon already adapted species, making those genetic systems extremely stable since radical changes would be undesirable. This lack of selection pressure can also be gleaned from the fact that these living isolates possess the same 16S rDNA genes found by others in both modern hypersaline areas, 0.1 MYA (Mormile et al 2002) and 20 MYA materials (Fish et al. 2002). The isolation of these microbes is also significant from the fact that these living organisms possess 16s rDNA genes that precisely match fragments isolated from another formation by a completely separate research group (Fish et al. 2002).

Numerous laboratories studying many different materials of varying ages have consistently isolated halophilic *Archaea, Bacteria*, and DNA fragments (Cano and Borucki 1995; Stan-Lotter et al. 1999, 2002; McGenity et al. 2000; Vreeland et al. 2000; Fish et al. 2002; Mormile et al. 2003; Greenblatt et al. 2004). Particularly compelling is the fact that several independent laboratories have now reported detecting representatives of the same *Halobacterium salinarum*-associated haloarchaeal clade (Stan-Lotter et al. 1999, 2002, this study; McGenity et al. 2000). Based upon these numerous findings we suggest that the evidence for such survival, including the results presented here, is becoming overwhelming.

Recently, Stan-Lotter et al. (2002) described the new taxon *Halobacterium noricense* to include microbes isolated from Austrian salt rocks. These latter authors used much larger salt rocks than those shown here and still isolated the same microbes. While there are obviously more ancient environments to be investigated (and undoubtedly more microbes to be found) it is time to focus attention on other important questions. These include the mechanisms by which microbes accomplish such survival, the careful comparison of the geochemical environments that foster survival with those that do not, examination of the evolutionary implications for microbes isolated for such long periods of time and the impact of these finding on astrobiology.

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