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Research Paper

Discovery of Abundant Cellulose Microfibers Encased in 250 Ma Permian Halite: A Macromolecular Target in the Search for Life on Other Planets

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ABSTRACT

In this study, we utilized transmission electron microscopy to examine the contents of fluid inclusions in halite (NaCl) and solid halite crystals collected 650 m below the surface from the Late Permian Salado Formation in southeastern New Mexico (USA). The halite has been isolated from contaminating groundwater since deposition approximately 250 Ma ago. We show that abundant cellulose microfibers are present in the halite and appear remarkably intact. The cellulose is in the form of 5 nm microfibers as well as composite ropes and mats, and was identified by resistance to 0.5 *N* NaOH treatment and susceptibility to cellulase enzyme treatment. These cellulose microfibers represent the oldest native biological macromolecules to have been directly isolated, examined biochemically, and visualized (without growth or replication) to date. This discovery points to cellulose as an ideal macromolecular target in the search for life on other planets in our Solar System. Key Words: Cellulose–Permian halite–Electron microscopy. Astrobiology 8, xxx–xxx.

INTRODUCTION

Environments on Earth exist in which the labile biological constituents of cells are preserved for geologically long periods of time (*i.e.*, >10 Ma) (Vreeland *et al.*, 2000; Fish *et al.*, 2002; Jahren and Sternberg, 2003; Greenblatt *et al.*, 1999; Cano and Borucki, 1995; Schweitzer *et al.*, 2007). They have been found within a salt crystal, a fossilized bone or tree, or a drop of amber. Ancient biological materials successfully extracted from such protective environments or microenvironments provide an invaluable resource for understanding the evolutionary record over a geological time frame (Schweitzer *et al.*, 2007) that stretches at least into the hundreds of millions of years. Such molecules could also provide a unique signature of life on other planets in our Solar System. Here, we describe the discovery of cellulose macromolecules recovered from 250 Ma halite crystals and from fluid inclusions in these crystals.

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Previous studies utilizing bacterial cultivation have probed ancient halite for the presence of viable bacteria (*e.g.*, Vreeland *et al.*, 2000), and approaches using polymerase chain reaction techniques have examined salt crystals for evidence of DNA (Fish *et al.*, 2002). These powerful amplification methods produce nearly limitless modern copies of revived bacteria or ancient DNA fragments. However, these methods have limitations as well; other biological material may be undetected since it is not amplified. Further, the products of the amplification are new, not ancient; and, as a result, some information that could be mined from the ancient material itself with methods such as stable isotope analysis or analysis of the state of damage of truly ancient DNA are wiped clean in the amplification process. Thus, by directly isolating protein fragments from dinosaur bones, Schweitzer *et al.* (2007) were able to compare these proteins with modern counterparts and explore their state of preservation. In this paper, we have taken a similar approach of directly isolating ancient biological material and have used transmission electron microscopy (TEM) to obtain a broad view of the biological contents of the sample at a resolution capable of detecting, for example, large macromolecules or bacteria without any amplification steps. By avoiding amplification, we eliminate the attendant possibility of also amplifying modern contaminants. Concerns of contamination can be further minimized by limiting the TEM processing steps and including blank controls. Here, we show that cellulose microfibers, which appear remarkably intact, are a major biological component of the halite from the Permian Salado Formation. This study is predicated on a detailed geologic analysis of the state of preservation of the ancient halite deposits of the Salado Formation.

PRESERVATION CONDITIONS IN THE PERMIAN SALADO FORMATION

Halite deposits provide a protected environment in which biological material may be preserved for significant geological times. Halite crystals (as do crystals in many aqueous environments) trap small volumes (inclusions) of the parent fluid (Roedder, 1984), which provides time capsules of the ancient brine and an opportunity to unravel environmental factors such as temperature (Satterfield *et al.*, 2005b; Roberts and Spencer, 1995; Losey and Benison, 2000; Benison and Goldstein, 2000) and pH (Benison *et al.*, 1998). There is no doubt halite inclusions can trap bacteria and macromolecules (*e.g.*, Norton and Grant, 1988; Mormile *et al.*, 2003); how long bacteria may remain viable (*e.g.*, Vreeland *et al.*, 2000; Timofeeff *et al.*, 2005; Schubert *et al.*, 2005) or macromolecules may be preserved is still being determined (*e.g.*, Fish *et al.*, 2002).

The Salado Formation was deposited near the end of the Permian (253 Ma) (*e.g.*, Renne *et al.*, 2001; Mundil *et al.*, 2004), based on radiometric data from some minerals within the formation and from the ages of the underlying and overlying formations. The Salado Formation in New Mexico is a well-characterized 600 m thick sequence of halite layers, covered by approximately 300 m of overburden rock (Lowenstein, 1988; Holt and Powers, 1990a; Powers *et al.*, 2001) in the area of the sampling site (Fig. 1). Background information on the Salado Formation in the region where our samples were collected has been developed on the basis of analyses of thousands of oil and gas exploration drillholes, potash mining activities and coring, and more recent detailed mapping of large-diameter (~6 m) shafts through the upper half of the formation (Holt and Powers, 1984, 1986, 1990a).

Lowenstein (1988) examined characteristics of the meter-scale depositional cycles of the upper Salado Formation, following earlier studies (*e.g.*, Schaller and Henderson, 1932). Holt and Powers (1990a, 1990b) mapped hundreds of repetitions of these cycles through the upper Salado Formation. The cycles include features that show common evidence of initial flooding and increasing desiccation upwards, consistent with broader climatic cycles.

During the initial stage of a depositional cycle, the halite pan flooded and subaqueous environments dominated. The basal lithologies may be sulfate overlain by halite or halite beds without sulfate, depending on the salinity of the water flooding the halite pan. The lower halite of the cycle was deposited in thin beds (10–20 cm thick) with some so-called chevron or cornet halite; bands of tiny fluid inclusions oriented parallel to the crystal faces indicate phases of growth in a subaqueous environment. The inclusion bands are considered to indicate crystals that have not been recrystallized since deposition.

During the later stage of the depositional cy-

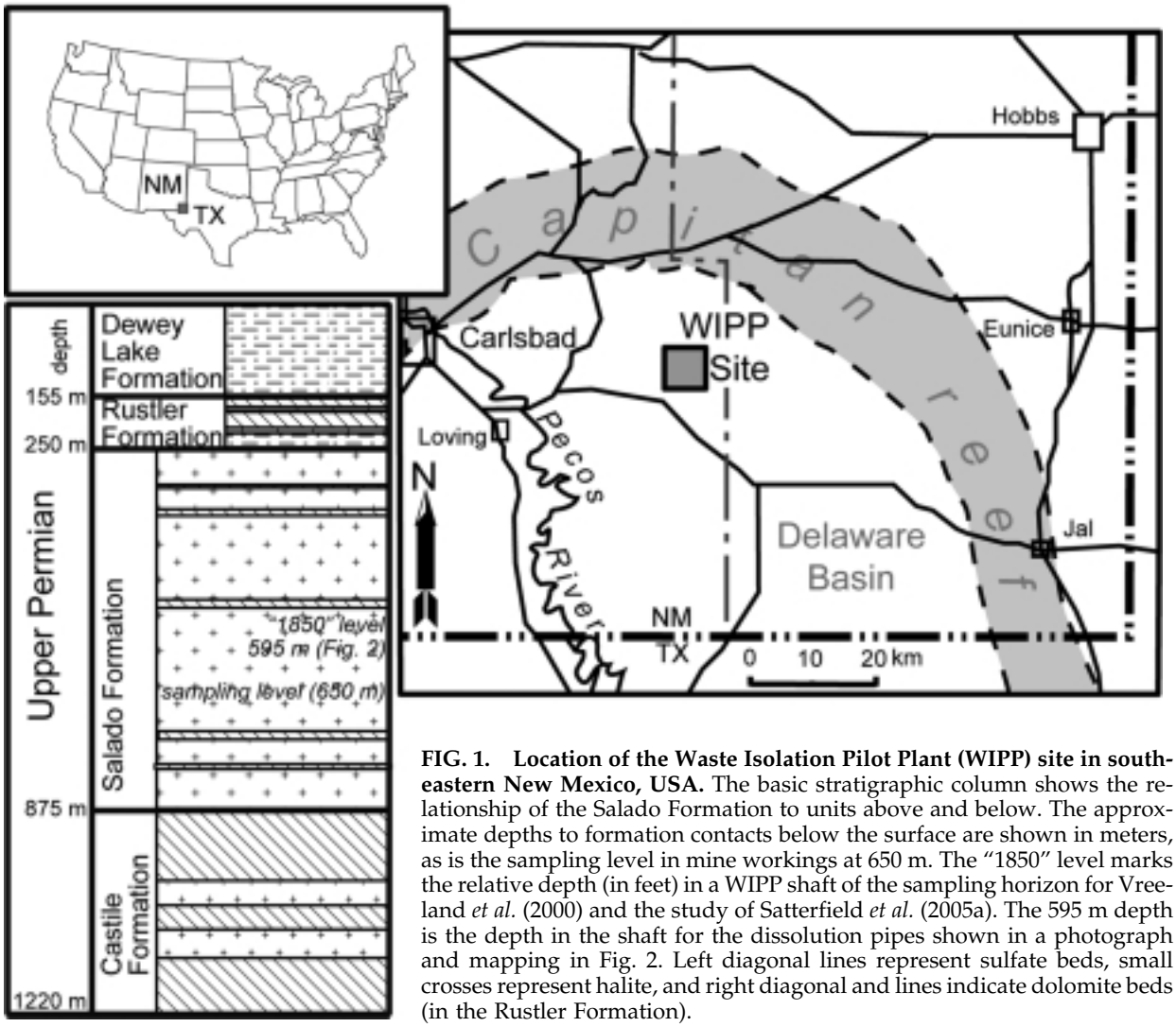

FIG. 1. Location of the Waste Isolation Pilot Plant (WIPP) site in southeastern New Mexico, USA. The basic stratigraphic column shows the relationship of the Salado Formation to units above and below. The approximate depths to formation contacts below the surface are shown in meters, as is the sampling level in mine workings at 650 m. The "1850" level marks the relative depth (in feet) in a WIPP shaft of the sampling horizon for Vreeland *et al.* (2000) and the study of Satterfield *et al.* (2005a). The 595 m depth is the depth in the shaft for the dissolution pipes shown in a photograph and mapping in Fig. 2. Left diagonal lines represent sulfate beds, small crosses represent halite, and right diagonal and lines indicate dolomite beds (in the Rustler Formation).

cle, flooding waned and subaerial exposure dominated. Fine siliciclastics accumulated on the surfaces as wind-blown dust and as sediment from intermittent runoff from the area surrounding the halite pan. Desiccation cracks developed at the exposed surface, and teepee structures developed within the polygonal areas between cracks. Siliciclastics were much less soluble than halite, and most major exposure surfaces were draped by concentrated siliciclastics. Teepee structures were truncated by exposure surfaces in many cycles, and siliciclastics filled or outlined desiccation cracks. Well-developed exposure surfaces are characteristic of the uppermost part of a depositional cycle. They represent significant periods of time in the overall development of the deposit. Extensive re-flooding of the Salado brine pan ended the desiccating-upwards part of the depositional cycle and initiated a new cycle.

During the latter stages of a depositional cycle, when subaerial exposure dominated, the brine table dropped below the surface. Shearman (1970) noted crystal-size (cm scale) dissolution pipes cemented with clear halite at the surface of modern evaporite deposits; he interpreted the dissolution as a response to a drop in the location of the brine table. Lowenstein and Hardie (1985) recognized such features in their examination of the textural distinctions among halite deposits of differing origins. Powers and Hassinger (1985) reported similar, but much larger, features from the Salado Formation and speculated about their origin. Holt and Powers (1990a, 1990b) found the same features spectacularly displayed (Fig. 2) in a large-diameter shaft at the Waste Isolation Pilot Plant (WIPP). Detailed mapping vertically through hundreds of depositional cycles clearly revealed that multiple meter-plus–scale dissolu-

tion pipes developed downward from individual exposure surfaces. As the brine table rose, the pipes filled with coarse, clear halite cement, deposited via a mechanism similar to that proposed by Shearman (1970) for the much smaller dissolution pipes he examined. The dissolution pipes, with their Permian-age crystals and common, larger fluid inclusions (see also Satterfield *et al.*, 2005a), were targeted especially for possible recovery of bacteria (Vreeland *et al.*, 2000; Powers *et al.*, 2001). Coarse halite crystals from such dissolution pipes were also sampled for this study. These sedimentologic features and simple stratigraphic relationships are the fundamental bases for interpreting these coarse halites as a part of the depositional system and as a suitable sampling material rather than as some later diagenetic cement caused by the passage of water through the formation at some unknown later time.

The halite has been isolated from contaminating groundwater since shortly after its deposition, as indicated by uninterrupted bedding above and below the sampled zone (Holt and

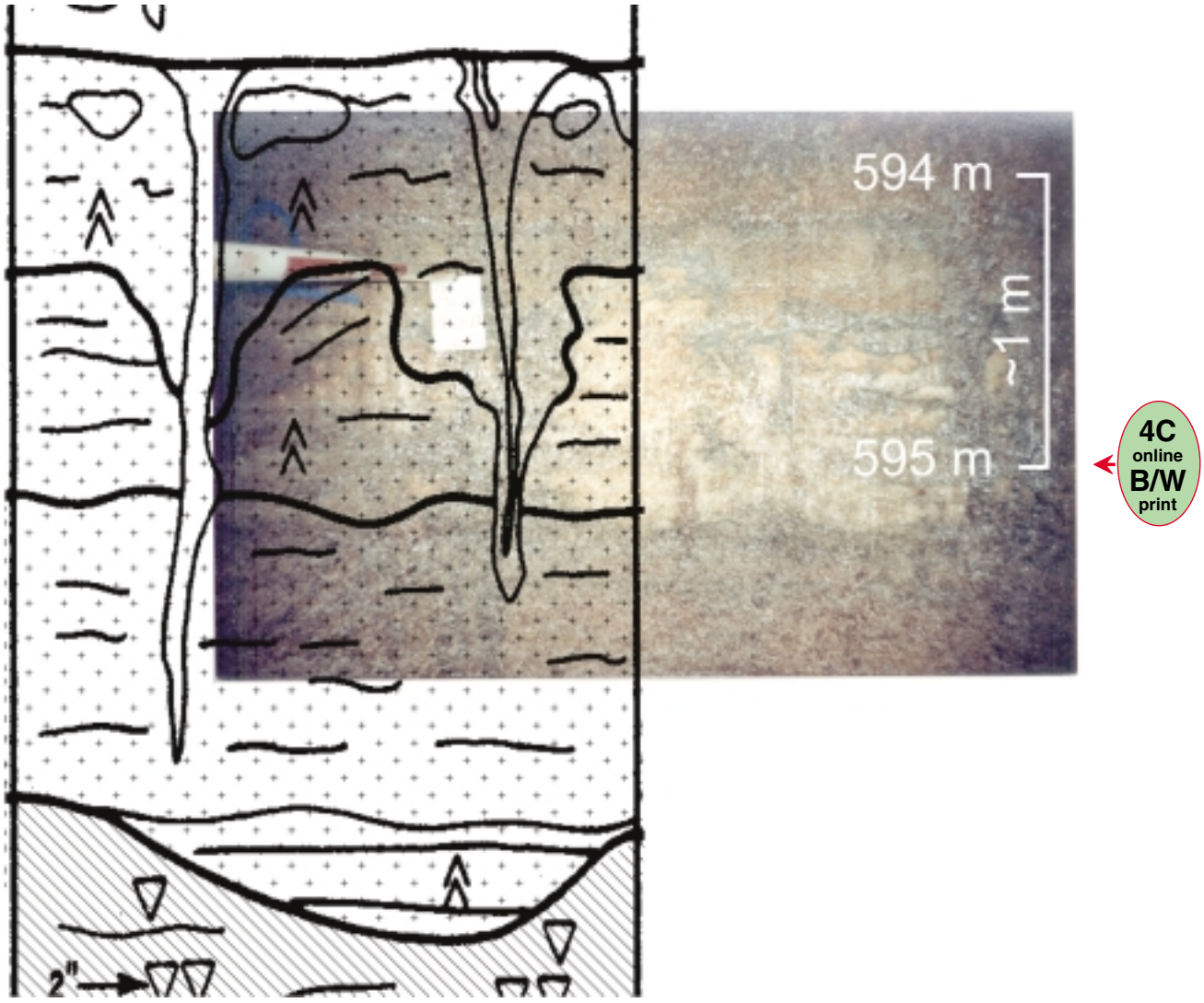

FIG. 2. Photograph of well-displayed m scale dissolution pipes. The photograph shows m scale dissolution pipes from ~595 m below the surface in a shaft at the WIPP, with superimposed general map of 1 feature (from Holt and Powers, 1990a). Other pipes are displayed right of the graphic overlay, originating mainly from the exposure surface at about 594.4 m. Detailed mapping also showed some pipes from a higher exposure surface (~593.5 m) followed by the earlier, lower trend. Bedding between the dissolution pipes included some halite crystals, with intense banding of fine fluid inclusions, which are called "chevron" halite. Note that this figure illustrates dissolution pipes and is not a sampling location for this study.

Powers, 1990a; Powers *et al.*, 2001), ultra-low permeability of the halite (Beauheim and Roberts, 2002), cm scale chemical inhomogeneity (Stein and Krumhansl, 1988), and a marine origin for similar halite (Satterfield *et al.*, 2005a). Beds at variable scales within the Salado Formation are traceable laterally over tens of km in underground mine workings to more than one hundred km, based on distinctive geophysical log properties. Interruptions in bedding, such as desiccation cracks, are restricted to single deposition cycles. The deepest of the dissolution pipes mapped by Holt and Powers (1990b) approach 10 m and may penetrate underlying depositional cycles. Nevertheless, they are clearly related to a distinctive exposure surface that is overlain by another depositional cycle. Casas and Lowenstein (1989) showed that permeability within halite beds is strongly occluded by halite cements that formed early in the diagenetic history—by 45 m burial. The permeability of the Salado Formation is at the extreme low end of any *in situ* tested material (Beauheim and Roberts, 2002); if permeability in such rocks follows Darcy's law, movement of fluids should be limited to the order of 1 m over the period since deposition. In addition, extensive testing of *in situ* fluids at the horizon sampled for this study shows that fluids have not homogenized at the scale of ~10 cm (Stein and Krumhansl, 1988) over this period of time. Satterfield *et al.* (2005a) additionally investigated fluid inclusions in halite from the dissolution pipe sampled by Vreeland *et al.* (2000) and found that the chemistry was consistent with crystallization from marine-derived brines, not as halite recrystallized somehow by infiltrating rainwater, as suggested by Hazen and Roedder (2001) following O'Neill *et al.* (1986). These combined studies are very strong indicators of stability of the evaporite beds and isolation from infiltrating fluids (Powers *et al.*, 2001).

The scale of the dissolution pipes and their relationship to bedding in the Salado Formation, as well as the hydrological and geochemical evidence, are not interpretable on the basis of petrographic and core-scale evidence, which are more common. Early study of the coarse, clear halite from the Salado Formation prior to its having been mapped in detail (especially by Holt and Powers, 1990a, 1990b) did not account for such stratigraphic relationships, as such analysis predated much of the sedimentologic data, and proceeded without an understanding of the hydraulic system within evaporites. Thus, O'Neill *et al.* (1986) started with the assumption that such coarse, clear halite must be "recrystallized" at some post-depositional period and ended with the interpretation that meteoric water isotopic signatures from fluid inclusions in the Salado Formation halite indicated continuing infiltration of water through the evaporites. Hazen and Roedder (2001) continued such arguments in commenting on the report of Vreeland *et al.* (2000) "that, in the absence of primary growth features in the specific halite crystals studies, the age of those crystals and their fluids must remain in doubt." As pointed out by Powers *et al.* (2001), the sedimentologic data regarding these dissolution pipes and the coarse, clear halite cements lead instead to an interpretation that is consistent with the data and requires processes neither at odds with the stratigraphic, hydrologic, and geochemical data nor in violation of basic hydraulic conditions; the isotopic data indicate that halite cements in some dissolution pipes trapped brines that formed as Permian rainwater fell on the exposed salt pan surface and filled these spaces, which were created while the brine table was lowered. They are Permian, they are not recrystallized, and they are part of the depositional system.

Here, we have combined TEM with biochemical treatments to show that cellulose microfibers are a major biological component of the halite. The cellulose microfibers appear remarkably intact. This may be due to many factors, including a high initial load, the great stability of the cellulose microfibers, and, finally, at the depth of sampling, the protection of halite from most cosmic sources of ionizing radiation; furthermore, halite contains low levels of naturally occurring radioactive isotopes. In nature, cellulose is synthesized by simple cells such as cyanobacteria, and it is also produced by multicellular eukaryotic organisms in particular plants. The ability of cellulose to remain in a native form over hundreds of millions of years in the halite, and the fact that cellulose is synthesized by even primitive cells, points to cellulose microfibers as a significant marker for life. Finding this macromolecule in halite also contributes to the growing evidence that evaporites are remarkable in preserving biological materials over long geological periods and present an excellent medium to investigate life on planets such as Mars (Benison, 2006), if halite is present.

MATERIALS AND METHODS

Sterile methods

"TEM grade" water prepared each day was used to make all solutions. House steam-distilled water was redistilled using a 2 L boiling flask, a 4-foot vertical vacuum silvered column and a 1 L collecting flask. The flasks were cleaned with chromic acid before each use. Prepared this way, the water is both highly purified and sterile, as it is reduced to vapor and then recondensed in a closed system. Ethanol was also redistilled in this way and was free of any contaminants as detected by TEM. All pipettes, tips, tubes, and other tools used to extract liquid from fluid inclusions in halite and to recover Salado Formation brine were sterilized by autoclaving for 30 min. Sterile latex gloves were used for handling halite crystals. Steel drills (25 mil) were treated briefly with 12 *N* HCl before and after each use. The solid chemicals used were of highest laboratory grade powder. Once the solutions were prepared and autoclaved, they were kept frozen at −20°C. We have found that this additionally ensures that no bacterial growth can occur in the autoclaved concentrated buffer solutions.

Plastic snap cap Eppendorf vials used to hold the liquid inclusions were autoclaved and then rinsed with sterile TEM grade water prior to use. All steps at the University of North Carolina, Chapel Hill (UNC) that involved handling of the crystals were done with the use of sterile latex gloves. Disposable pipette tips were sterilized, and the Gilson Pipetman pipettors were disassembled and carefully washed between uses. Fiber-plugged tips were avoided due to possible contamination.

Sample collection and isolation of liquid inclusions

Halite crystals were collected from several locations in the WIPP at roughly the 650 m level with a 7.5 cm diameter coring drill. Synsedimentary dissolution pipes with large halite crystals were identified along mined walls. Samples were successively taken from the core hole to depths of ~15 cm. There was no attempt to sterilize sampling equipment and surfaces underground, as the sterility of the sampling process is very difficult to verify and the specimen selection is the critical step. Samples were collected and bagged for transport to the laboratory. All remaining work was done at UNC.

In the laboratory, each core was broken apart, and crystals 2 to 10 cm in the long dimension containing liquid inclusions were selected. Crystals were examined under a dissecting microscope, and inclusions adjacent to a fracture plane were excluded. They were not examined to determine whether daughter crystals or visible artifacts were observable. Inclusions were of variable size, commonly roughly cubic, and not arranged as chevron bands.

Crystals were soaked in saturated NaOH for 3 min, rinsed in saturated NaCl, soaked in 12 *N* HCl for 3 min, rinsed again with saturated NaCl and sterile distilled TEM grade water (Rosenzweig *et al.*, 2000) to remove any biological material that might have contaminated the surface of the crystals or entered any fractures. The final water wash with TEM grade water was extensive to the degree that at least 1 mm of the surface of the crystal was removed, a step which by itself should have removed any surface contamination. Drilling was performed with a 25 mil drill bit and a Cameron Micro Drill Press (Sonora, CA) attached to a Variac transformer. Crystals were viewed under a dissecting microscope while a hole was drilled from the crystal surface to the inclusion (Fig. 3). Liquid inclusions were withdrawn using a fine glass capillary (freshly drawn under gas flame) attached to a Hamilton syringe. Samples were stored in sterile 250 μl Eppendorf snap cap tubes.

Collection of material from solid halite

Halite crystals were also collected in which large fluid inclusions were absent, though very small inclusions may have been present. Multiple crystals, pretreated as described above, were grouped into pools of approximately 50 g each and were slowly dissolved in TEM grade water. The sterilized crystals were placed in 500 ml small-neck glass flasks that had been washed with chromic acid followed by TEM grade water. TEM grade water was added to the crystals and the mixture stirred with a sterilized magnetic stir bar in a cold room with the flask capped until the halite had fully dissolved. The solution was placed in new polycarbonate ultracentrifuge tubes and centrifuged in a Beckman SW27 rotor at 22,000 rpm for 18 h at 14°C. Prior to centrifugation, the SW27 rotor tubes were carefully washed and rinsed with TEM grade water. The pellet was resuspended in 3 ml of 10 m*M* Tris-

FIG. 3. Primary halite crystals with inclusions. Primary halite crystals were surface-sterilized and washed with highly purified water (see Methods and Materials). (**a**) Low power microscope (~5× objective) view into a halite crystal that contains numerous small inclusions and 1 larger inclusion on the right (arrow). The smallest inclusions are less than 1 microliter in size. (**b**) A ~10× view into a halite crystal that contains 3 larger inclusions into which holes (dark arrows) were drilled to remove the inclusion liquid, which ranged from a few microliters (lower right) to ~40 microliters for the large inclusion on the left. The surfaces were not polished, which is why cleavage lines (white arrow) that intersect the surface of the thin section are visible.

HCl pH 7.5, 0.1 m*M* EDTA (4°C) (TE) prepared in TEM grade water as described above. Large mineral particles were allowed to settle at 1× g for 30 min and discarded. Aliquots (20 μl) of this material were incubated with 50 μg/ml cellulase (106 units/μg) (Worthington Biochemical Corp, Lakewood, NJ) for 2 h at 37°C in 50 m*M* acetic acid. The sample was centrifuged in a microfuge, and the pellet was resuspended and prepared for TEM. For NaOH/$NaBO_4$ treatment, aliquots (20 μl) of the 3 ml suspension above were suspended in 20 μl of 0.5 *M* NaOH, 50 m*M* $NaBO_4$, incubated for 30 min at 65°C, re-pelleted, and prepared for TEM.

TEM sample preparation

Liquid inclusions were pooled, if necessary, to obtain a 20 μl volume. To collect any fast-sedimenting biological material, the samples were centrifuged in an Eppendorf microcentrifuge with a swinging bucket rotor at 10,000 rpm for 30 min. TEM grade water (20 μl) was placed on top of the pelleted material for 10 min to dissolve any salts, followed by centrifugation again for 30 min. This washing step was repeated twice. The sample was centrifuged again and the pellet suspended in 4 μl of 50% ethanol in water. Two μl of the suspension were placed on each of two 200-mesh copper grids (Ted Pella) covered by a thin carbon foil. Carbon-covered copper mesh grids were prepared as described in Griffith and Christiansen (1978). The sample was allowed to air dry and then was rotary shadowcast with Pt:Pd (80%:20%) at 1×10^{-6} Torr. Samples were examined in a FEI Tecnai 12 TEM at 40 kV. Micrographs were collected on 3.25×4 inch sheet film and with a Gatan Ultrascan 4000 CCD camera. Length measurements were made with Gatan Digital Micrograph software. Sheet films were scanned with an Imacon 848 film scanner (Hasselblad USA Inc.), and Adobe Photoshop software was used to arrange panels for publication and invert the contrast (common for metal shadowcast sample TEM images) of the final figure.

RESULTS

Locations (in a shaft at the WIPP) at the exact site sampled by Vreeland *et al.* (2000) were not available for additional sampling. However, the background knowledge of the geological features (coarse halite cements in dissolution pipes) for the Vreeland *et al.* (2000) investigation and available data (Holt and Powers, 1984, 1986, 1990a, 1990b; Powers and Hassinger, 1985) aided in the selection of sampling sites within the WIPP underground area. Features similar in origin, and similarly constrained by stratigraphy, hydrology, and geochemistry, were selected to provide more extensive samples as well as samples from 2 successive depositional cycles.

Primary halite crystals were collected at a depth of ~650 m (Materials and Methods). In the laboratory, the crystals were soaked in saturated NaOH followed by 12 *N* HCl (Vreeland *et al.*, 2000), and this was followed by abrading at least 1 mm of the surface with highly purified water. This stringent treatment will destroy and wash away any biological material which might have adhered to the surface of the crystals or entered via fractures. With a micro drill press and glass capillary, liquid ranging from 1 to 80 μl was recovered in a sterile manner from more than 80 individual different inclusions taken from a half-dozen different coring sites in the WIPP. The largest inclusion encountered yielded 80 microliters of liquid, but most were in the size range of 1–20 microliters. As shown in Fig. 3, some crystals exhibited large numbers of tiny inclusions, likely a fraction of a microliter in volume.

It was necessary to lower the salt to ~0.1 *M* or less to allow any biological material in the liquid to adhere to the TEM support. This was accomplished by centrifuging aliquots of the inclusion liquid in a microfuge followed by washing the pellet with water and finally suspending the pelleted material in 50% ethanol and placing it, in its entirety, on the TEM grid, followed by air drying and metal coating in a vacuum for contrast enhancement (Materials and Methods). This procedure was developed to provide the least number of steps between collection of the liquid and drying onto the TEM support, which thus greatly minimizes chances for laboratory contamination. Fast-sedimenting high–molecular weight biological material and mineral particles were collected and visualized by this approach. The supernatant from the centrifugation was found to contain only a small amount of biological material, which will be described elsewhere.

Examination of the samples by TEM at low magnification revealed that in one half of the examples from either individual inclusions or pools of several inclusions, the supporting surface was covered with long, thin fibers arranged into ropes, sheets, and mats (Fig. 4a, 4b, 4c). Individual fibers frequently spanned 10 microns or more, and the diameter of the thinnest fibers (Fig. 4a arrows, 4b, 4c) was 3–5 nm (after correction for metal coating). The thinner fibers, which appeared stiff and brittle with sharp ends, frequently coiled about each other, forming higher-order ropes and tangles (Fig. 4a, 4b). The most common thicker fiber measured 16 nm (n = 50 measurements) (Fig. 4b arrows), but fibers of 10 nm, 20 nm, and greater diameter were also present. Often, the 5 nm microfibers were laid out in flat sheets (Fig. 4c). The other 50% of the inclusions or inclusion pools were apparently barren

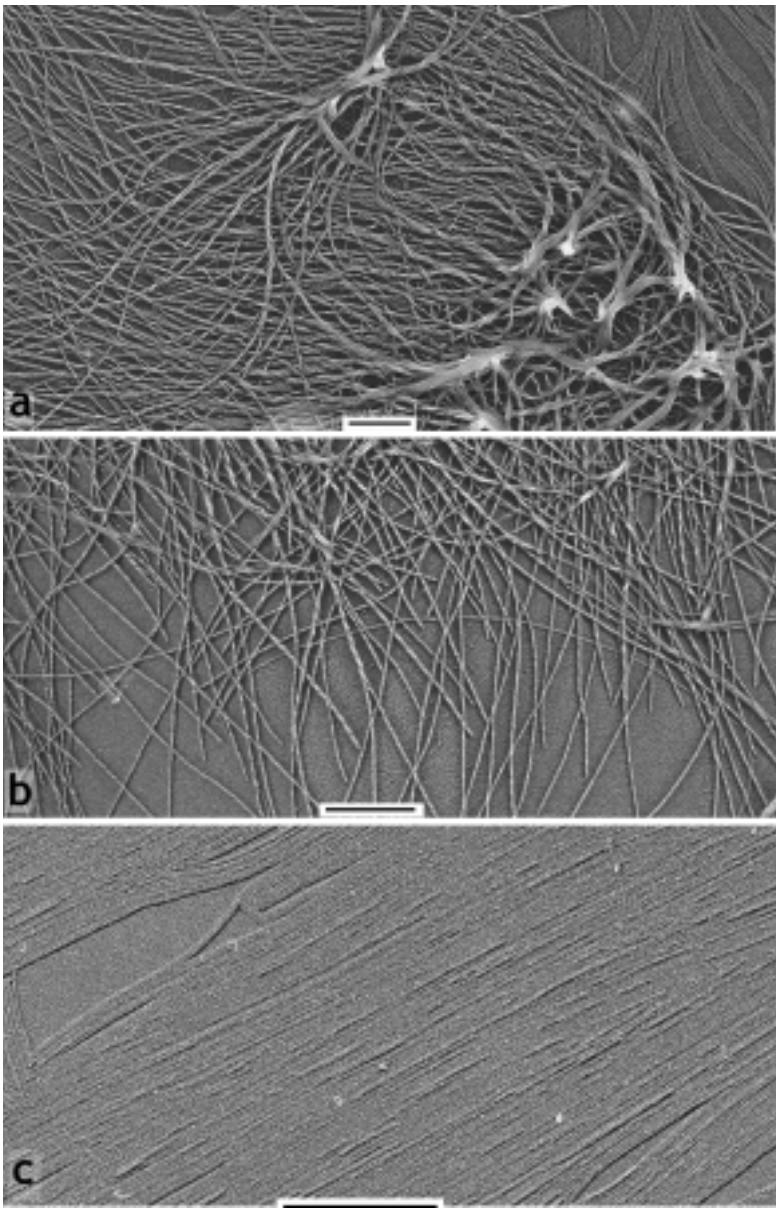

FIG. 4. TEM visualization of Permian-age cellulose fibers. (**a**) A sample of inclusion brine was collected by centrifugation, deposited directly on the TEM support, air dried and rotary shadowcast with Pt:Pd (Materials and Methods). (**b**) Cellulose fibers were collected by centrifugation from solid halite and prepared as in (a). The width of the thinnest fibers (arrows in a) is 5 nm, and the most abundant thicker fiber (arrows in b) is 16 nm. (**c**) A flat sheet of 5 nm microfibers deposited on the TEM support. Bars equal 500 nm. Shown in reverse contrast.

of this material, which reflects the variable nature of the formation of each inclusion. In every experiment, parallel samples were prepared with only the buffers and reagents, and in no cases were such fibers observed. Further, examination of the highly purified / sterile "TEM grade" water was carried out as an additional blank control and has never shown fibrous material or other macromolecules. In 30 years of TEM work in this (Griffith) laboratory, no fibers such as these have been encountered, which argues against contamination from the preparative steps.

It was of interest to ask whether the fibrous material could be isolated from solid halite. Eight 50 gm pools of solid halite crystals were dissolved in water, the high–molecular weight material pelleted by centrifugation (Materials and Methods) and examined by TEM. Of the 8 pools, 5 showed large amounts of the fibrous material. Repeated preparation of aliquots of the fiber-positive pools always showed the fibers. Fibers were not observed in buffer-only samples or in repeated aliquots from the pools lacking fibers, which further argues against contamination. We estimate that, on average, a few milligrams of the fibrous material were present in the fiber-positive 3 ml extracts from the 50 g pools of halite crystals. This high abundance further argues for their presence in the halite and not as a contaminant of the preparation. We recovered more of the fibers from the

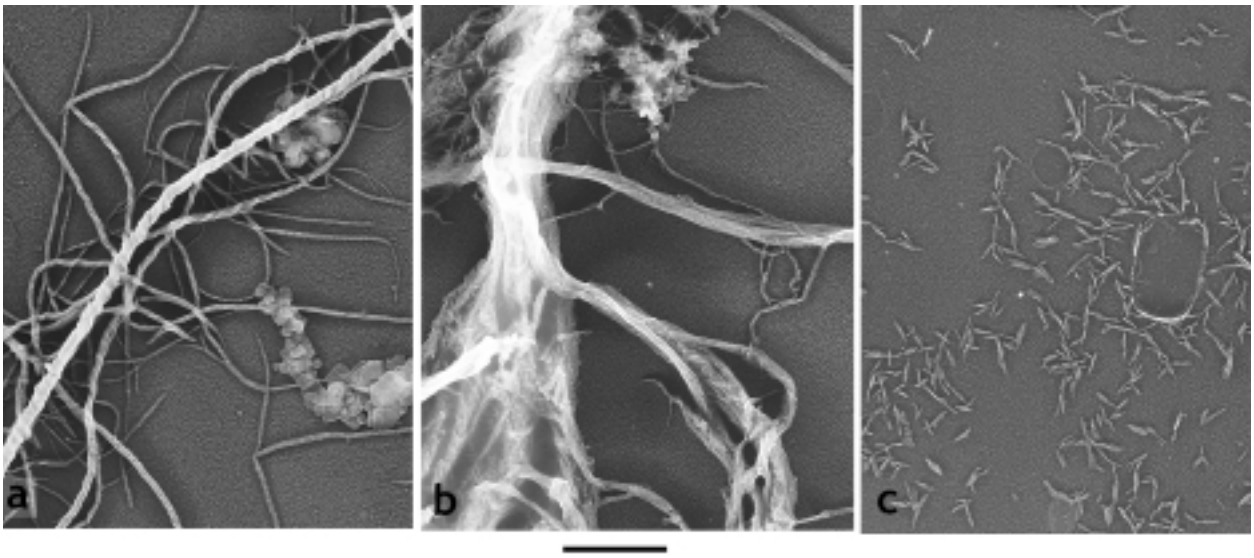

FIG. 5. TEM visualization of chemical and enzymatic treatment of ancient cellulose. (**a**) Cellulose that was collected from solid halite by centrifugation, treated with 0.5 *N* NaOH, 50 m*M* $NaBO_4$, and prepared for TEM as in Fig. 1. (**b**) Commercial cellulose (KimWipes® fragment) treated as in (a). (**c**) An aliquot of the cellulose pellet from (a) was treated for 30 min with cellulase and prepared for TEM. Bar equals 500 nm. Shown in reverse contrast.

solid halite than from fluid inclusions, but since most crystals contained some tiny inclusions, we cannot rule out their contribution to the yield.

These microfibers are similar to modern cellulose microfibers observed by others using TEM (Okuda *et al.*, 1993; Kvien *et al.*, 2005; Nobles *et al.*, 2001). Cellulose resists treatment with 0.5 *M* NaOH and 0.05 *M* $NaBO_4$ at 65°C for 30 min, while most other biological material is destroyed. Aliquots of the 3 ml extracts of solid halite were treated this way and prepared for TEM. No significant reduction in the amount of microfibers was observed (Fig. 5a). For comparison, a sonicated fragment of commercial cellulose (KimWipes® towelette) showing a similar fibrous appearance was similarly treated and was also stable under these harsh conditions (Fig. 5b). As a definitive test, aliquots of the 3 ml extracts were incubated with cellulase at 37°C for 2 h (Materials and Methods). This eliminated nearly all the fibers, leaving only short fragments (Fig. 5c). Control incubations without cellulase had no effect (not shown).

DISCUSSION

The age of the cellulose fibers described here is estimated to be approximately 250 Ma, which makes these ancient macromolecules the oldest piece of Earth's evolutionary record to have been directly isolated and visualized to date. The minimal steps involved in the TEM processing, the abundance of the cellulose in the halite and absence in all of the controls, the stringent surface abrading and sterilization used prior to sampling, and the geological evidence of isolation of the halite following its deposition all argue strongly against any modern contamination as a source of the cellulose fibers. The preservation of the cellulose fibers undoubtedly reflects the fact that the Salado Formation harbors deposits in a highly protected microenvironment that offers shelter from potentially damaging chemical and physical processes ubiquitous at Earth's surface.

Three different verifications have been presented to show that the fibrous material is cellulose: chemical, enzymatic, and ultrastructural. The fibrous material was resistant to chemical treatment at 65°C with 0.5 *N* NaOH and 50 m*M* Na-borohydride. This is diagnostic of cellulose, as this method will rapidly degrade other biological macromolecules, including chitin, DNA, and RNA. In contrast, the fibrous material was rapidly digested by cellulase enzyme at 37°C. This combination of chemical resistance and enzymatic sensitivity is highly diagnostic of cellulose microfibers. Finally, based on the TEM structural characterization alone, the only 2 candidates for these fibers would have been cellulose or possibly chitin, but the latter was eliminated by the chemical treatment and enzymatic degradation.

The cellulose microfibers were the major biological constituent of the halite, which we were able to detect using TEM. Other biological mate-

rial, including DNA, was observed but at much lesser amounts. This will be described elsewhere. Not knowing whether salt-resistant Permian-age bacteria were cellulose producers, we must presume that algae and washed-in plant debris contributed the bulk of the cellulose we detected.

The ability of the cellulose molecules to survive this long is remarkable; however, it is not possible to estimate what fraction of the material present at the time of deposition has already been degraded and what fraction persists today. In a planned study of unsterilized samples from the WIPP mine area, Vreeland *et al.* (1998) cultured halophiles that degrade cellulose. Whether these organisms were modern surface contamination or viable Permian-age bacteria was not relevant to the study of Vreeland *et al.* (1998). Our finding of abundant cellulose in the halite argues that either the cellulose degraders were modern or, if ancient, were not metabolically active within the halite. Our findings also do not differentiate between the options of modern surface contamination of the exposed Salado Formation or a limited distribution of Permian-age cellulose degraders.

Cellulose has been previously extracted from tree fossils preserved in 45 Ma lignite beds (Jahren and Sternberg, 2003), which shows that it is capable of lasting for tens of millions of years. Multiple factors likely aided in its preservation in the Salado Formation for over 250 Ma. The relatively large amount of cellulose observed likely reflects both a high initial load relative to other biological macromolecules and the extreme stability of the glycan chains. The half-life of the β 1–4 bond in cellulose has recently been predicted to be 5 Ma in low-salt aqueous solutions and may be much longer in high salt conditions (Schroeder *et al.*, 2006). As well as excluding water, the parallel array of 36 glycan chains in a semi-crystalline state in the 5 nm cellulose microfiber may impart great physical stability as compared to other macromolecular structures. Finally, the very low level of radiation in the Salado Formation would also limit damage to the cellulose microfibers by ionizing radiation.

Ionizing radiation has been considered to limit the survival of various organic compounds over geological time, even for Mars (Kminek and Bada, 2006), in the absence of repair mechanisms in a metabolizing organism. The more particular case of the effects of ionizing radiation in evaporite environments has also been considered with various assumptions, and Kminek *et al.* (2003) predicted that "the long-term survival of spores is limited to less than 109 million years in halite fluid inclusions." Nicastro *et al.* (2002) modeled double-strand breaks in DNA due to beta decay from radioactive potassium for a population of bacterial spores in halite inclusions in the Salado Formation and concluded that the model is consistent with survival of spores since the Permian.

IMPLICATIONS FOR ASTROBIOLOGY

Bacterial cellulose is used as a substrate for bacterial biofilms and was adopted by plants and algae as a major structural component. Insect chitin is a close molecular relative of cellulose. On Earth today over 100 gigatons of cellulose are produced each year, divided equally between land and water (Cox *et al.*, 2000). This makes cellulose the single most abundant macromolecule on Earth. The six-carbon sugar glucose molecule is a fundamental energy currency for many carbon-based life forms, including some primitive bacteria. It is possible that some early bacteria developed the means of polymerizing glucose units in an alternating arrangement to create the glycan chains that condense into semi-crystalline 5 nm cellulose microfibers. Cellulose is synthesized by cyanobacteria, the ancestors of which date to at least 2.8 Ga (reviewed in Nobles *et al.*, 2001). Thus, it is tempting to speculate that cellulose fibers may have appeared in evolution several billion years ago. Cellulose fibers in modern cyanobacteria, however, are involved in gliding motility (Nobles *et al.*, 2001) rather than cell wall structure, and thus an alternative explanation is that the cellulose found in these present-day bacteria with very ancient relatives is actually a relatively modern acquisition. The structural similarity of cellulose synthases between plants and modern cyanobacteria (Nobles *et al.*, 2001) does not resolve this question. This similarity could equally point to the transfer of cellulose-synthesizing ability from ancient bacteria to early plants or the acquisition of cellulose synthesis by bacteria from early plants. In the latter case, the earliest appearance of cellulose would date to the first red and green algae and plants in the Silurian and Cambrian periods 400 to 570 Ma ago. Clearly, future studies by others more precisely pinpointing the evolution of cellulose microfibers in the broad span of 400 Ma to 2.8 Ga ago will be of great value to many investigations.

There are compelling reasons to consider cellulose as a molecular target in searches for life on

other planets in addition to its abundance. Cellulose is likely to be far more resistant to ionizing radiation than other macromolecules such as DNA. DNA survival from ionizing radiation is dominated by double-strand breaks from a single track for exposures less than a few hundred million years at potassium levels found in WIPP halite (Nicastro *et al.* 2002). In addition, accumulation of single-strand breaks for longer periods eventually results in a double-strand break. On the other hand, for ionizing radiation to cleave a 5 nm cellulose micofiber completely, the equivalent of 36 proximal glycan chain breaks would be required. This many breaks from a single track is extremely unlikely, and the accumulation of single glycan chain breaks to a point where the entire microfiber cleaves would take much, much longer than for DNA. Because the 5 nm microfiber is in a semi-dehydrated state, the desiccation encountered in space is likely to be less disruptive than for many other macromolecules. Finally, while DNA and proteins are rapidly degraded by a multitude of enzymes released when cells die, cellulases are generally not present within cells, and the macroscopic cellulose fiber bundles make it relatively difficult for cellulases to digest.

In the future, it will be important to examine older halite samples to determine whether the time line of preservation can be extended beyond the 250 Ma age of the halite examined in this study. In addition, there is considerable work to be done to further examine the distribution of cellulose in the Salado Formation with respect to different parts of the depositional cycles, not just the late stage of the cycle represented by dissolution pipes.

Evaporites similar to deposits on Earth (*e.g.*, Benison, 2006) may be found on Mars and other space bodies. The halite in these locations should become a priority for exploration, as the macromolecules inside may be protected. Cellulose appears to be highly stable, and if it is relatively resistant to harsh conditions such as those found in space, it may provide the ideal "paper trail" in the search for life on other planets.

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ABBREVIATIONS

TEM, transmission electron microscopy; UNC, University of North Carolina, Chapel Hill; WIPP, Waste Isolation Pilot Plant.

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