L.A. Melim, D.E. Northup, M.N. Spilde, B. Jones, P.J. Boston, and R.J. Bixby – Reticulated filaments in cave pool speleothems: microbe or mineral? *Journal of Cave and Karst Studies,* v. 70, no. 3, p. 135–141.

RETICULATED FILAMENTS IN CAVE POOL SPELEOTHEMS: MICROBE OR MINERAL?

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Abstract: We report on a reticulated filament found in modern and fossil cave samples that cannot be correlated to any known microorganism or organism part. These filaments were found in moist environments in five limestone caves (four in New Mexico, U.S.A., one in Tabasco, Mexico), and a basalt lava tube in the Cape Verde Islands. Most of the filaments are fossils revealed by etching into calcitic speleothems but two are on the surface of samples. One hundred eighty individual reticulated filaments were imaged from 16 different samples using scanning electron microscopy. The filaments are up to 75 μm (average 12 μm) long, but all filaments appear broken. These reticulated filaments are elongate, commonly hollow, tubes with an open mesh reminiscent of a fish net or honeycomb. Two different cross-hatched patterns occur; 77% of filaments have hexagonal chambers aligned parallel to the filament and 23% of filaments have diamond-shaped chambers that spiral along the filament. The filaments range from 300 nm to 1000 nm in diameter, but there are two somewhat overlapping populations; one 200–400 nm in size and the other 500–700 nm. Individual chambers range from 40 to 100 nm with 30–40 nm thick walls. Similar morphologies to the cave reticulated filaments do exist in the microbial world, but all can be ruled out due to the absence of silica (diatoms), different size (diatoms, S-layers), or the presence of iron (*Leptothrix* sp.). Given the wide range of locations that contain reticulated filaments, we speculate that they are a significant cave microorganism albeit with unknown living habits.

Introduction

Microbes are well known from cave systems (Danielli and Edington, 1983; Northup and Lavoie, 2001; Barton et al., 2001; Barton and Northup, 2007). Scanning electron microscopy of the surface of speleothems commonly reveals a variety of spheroid or filamentous features, interpreted as either microorganisms or biofilms (mucous/EPS) (Jones and Motyka, 1987; Jones and Kahle, 1986; Jones, 2001; Vlasceanu et al., 2000; Baskar et al., 2006). Etching calcitic samples with weak acid sometimes reveals fossil microorganisms and/or biofilms that were entombed in the calcite (Melim et al., 2001; Boston et al., 2001). We report herein on an unusual reticulated filament found in modern and fossil cave samples that cannot be correlated to any known microorganism or organism part. Since all of our samples are from caves, we cannot comment on their possible wider distribution. We document here the morphology, distribution and context of these reticulated filaments.

Field Locations

The samples for this study (Table 1) came from caves in the Guadalupe Mountains of southeastern New Mexico, from a lava tube in the Cape Verde Islands, and a cave in Tabasco, Mexico (Fig. 1). All locations are from the aphotic zone of the cave; all but one are from speleothems that formed underwater (pool fingers) or in wet areas (cave pearls).

The Guadalupe Mountains include over 300 known caves (DuChene and Martinez, 2000) administered variously by Carlsbad Caverns National Park, the National Forest Service, and the Bureau of Land Management. Hidden Cave and Cottonwood Cave are located in the Guadalupe Ranger District of the Lincoln National Forest in southeastern New Mexico (Fig. 2). The samples from Hidden Cave are giant pool fingers (Fig. 3a), pendant features that form underwater (Davis et al., 1990; Melim et al., 2001). In Cottonwood Cave, the samples are thin pool fingers with abundant u-loops; curved connections between fingers (Fig. 3b; Davis et al., 1990). Both areas are currently dry but the features formed when the pools were full (unpublished data; Hill, 1987 Melim et al., 2001). Endless Cave is located on Bureau of Land Management property in the McKittrick Hill area of the Guadalupe Mountains (Fig. 2). The sample is a warclub (Hill and Forti, 1997) from the Warclub Room, a currently dry room. A warclub forms when the end of a stalactite is

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Table 1. List of locations containing reticulated filaments.

Cave	Location	Speleothem	Collector	Number of Imaged Filaments
Hidden Cave	Guadalupe Mountains, New Mexico, USA	Pool fingers	Authors	92
Cottonwood Cave	Guadalupe Mountains, New Mexico, USA	Pool fingers and U-loops	Authors	65
Endless Cave	Guadalupe Mountains, New Mexico, USA	Warclub	Authors	9
Carlsbad Cavern	Guadalupe Mountains, New Mexico, USA	Webulite	Authors	5
Cueva de Las Canicas	Tabasco, Mexico	Cave pearls	Arturo C. Conde	2
Fogo Island, Basalt Lava Tube	Cape Verde Islands	Coating on basalt lava flow	Peter Roe	11

submerged by a rising pool level and is then coated in pool spar. Our sample is a surface piece of this pool spar. Two more samples came from Carlsbad Cavern in Carlsbad Caverns National Park (Fig. 2). One sample is a thin pool finger from an active pool, whereas the other is webulite from a dry pool in Lower Cave, a portion of the cave generally closed to the public. Webulite is a thin draping calcite web that connects adjacent pool fingers (Davis et al., 1990, Queen and Melim, 2006).

The Cape Verde sample is from a small, unnamed lava tube on Fogo Island. The sample came from a purple and white crust on the wall of the basalt lava tube (Peter Roe, personal communication). The Mexican sample is a cave pearl collected from Cueva de Las Canicas, a cave in Tabasco famous for containing millions of cave pearls (Pisarowicz and Snow, 2003).

METHODS

All samples were collected under permit from the appropriate agency (U.S. National Park Service, U.S. Forest Service, and Bureau of Land Management) or landowner permission (Sr. Arturo Cano, Cueva de Las Cañicas) and were selected from those broken by past visitors to avoid disturbance of intact speleothems. Thin sections were cut from all samples from Hidden Cave, Cottonwood Cave, and Carlsbad Cavern and examined for possible microbial fabrics in a standard petrographic microscope. The cave pearl was cut in half, ground flat, and then etched. For SEM analysis, samples were either etched with 5% hydrochloric acid for 10–15 seconds before rinsing in distilled water, drying and mounting on SEM stubs (Hidden Cave, Cottonwood Cave, Carlsbad Cavern

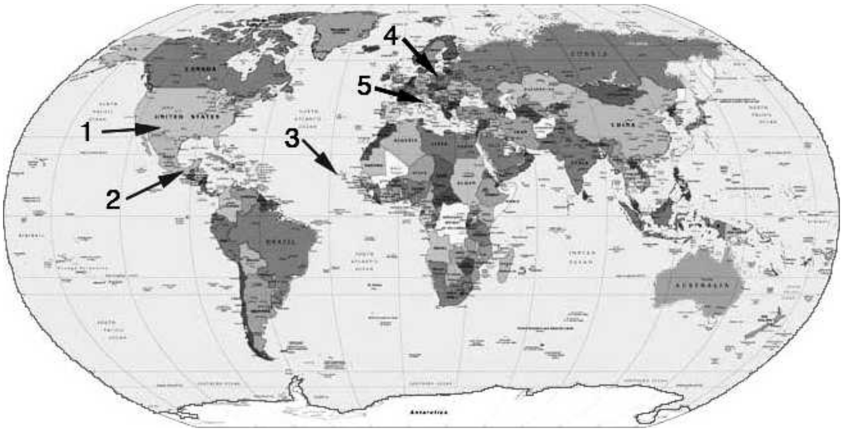

Figure 1. World map of locations where reticulated filaments have been found by this study: 1. Guadalupe Mountains, New Mexico, U.S., 2. Tabasco, Mexico, 3. Cape Verde Islands. Also shown are locations for two examples found by other workers: 4. Poland, Gradzinski (2003); 5. Frasassi Caves, Italy, Macalady (pers. comm.).

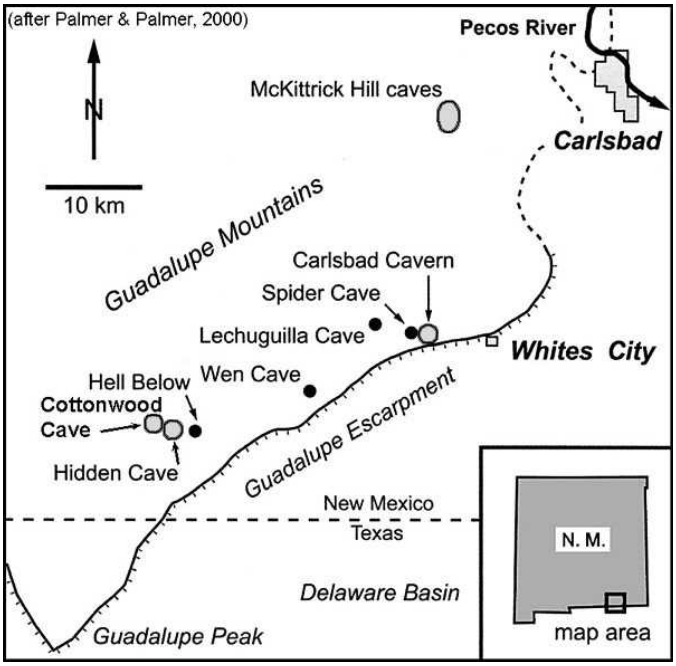

Figure 2. Locations of major caves in the Guadalupe Mountains. We have found reticulated filaments in samples from Hidden Cave, Carlsbad Cavern, Cottonwood Cave, and Endless Cave. (Map, after Palmer and Palmer, 2000.)

and Cueva de Las Canicas) or mounted without etching (Cape Verde lava tube and Endless Cave).

All samples were examined with either a JEOL 5800LV scanning electron microscope at the University of New Mexico (UNM) or a JSM 6301FXV field emission scanning electron microscope at the University of Alberta. The UNM samples were coated with approximately 200 angstroms of gold-palladium alloy in an evaporative coater. The thin film of gold-palladium provides a conductive layer that is relatively free of artifacts and allows light element x-rays of carbon to pass with only moderate attenuation. The University of Alberta samples were coated in gold alone, which resulted in minor artifacts at higher magnification. The UNM SEM is equipped with an Oxford Isis 300 Energy Dispersive X-ray (EDX) analyzer. This modern EDX system utilizes a thin polymer-film window, which allows the analysis of low-energy x-rays of light elements such as boron and carbon ($Z > 5$). The SEM was operated at 15 kV accelerating voltage, the beam current was 10 picoamps as measured in a Faraday cup. This gives a beam diameter of less than 50 nm. However, the beam samples a greater volume. With these operating conditions, the EDX provides a qualitative estimate of elements present in the upper 2–3 µm of a calcitic sample.

Three samples were analyzed for total organic carbon using a Carlo Erba elemental analyzer from samples dissolved in 6 N hydrochloric acid to remove carbonate.

Occurrence of Reticulated Filaments

Smooth, commonly hollow, filaments are locally abundant in our cave samples and have been interpreted as fossil microbes (Melim et al., 2001; Boston et al., 2001). We have also found a less common reticulated filament that is similar in size to the smooth filaments but has a reticulated form (Fig. 4). Over 180 individual examples of these unusual reticulated filaments were found in 16 different cave samples (out of 22 samples examined in this study). Most (87%) of these filaments are in pool fingers from Hidden and Cottonwood Caves (Figs. 2 and 3), but this at least partly reflects the extended time we have spent with these samples. In addition, Gradzinski (2000, 2003) imaged an example from Polish cave pearls and J. Macalady has found them in the Frasassi caves in Italy (Macalady, personal communication).

Filaments are preferentially found in dense micritic calcite, but can also occur in clear spar (Melim et al., 2001). All but two of our samples come from cave pool biothems,

Figure 3. (a) Giant pool fingers in Hidden Cave, NM. (b) Small pool fingers coating an earlier stalactite in Cottonwood Cave, NM (Spanish moss of Hill, 1987).

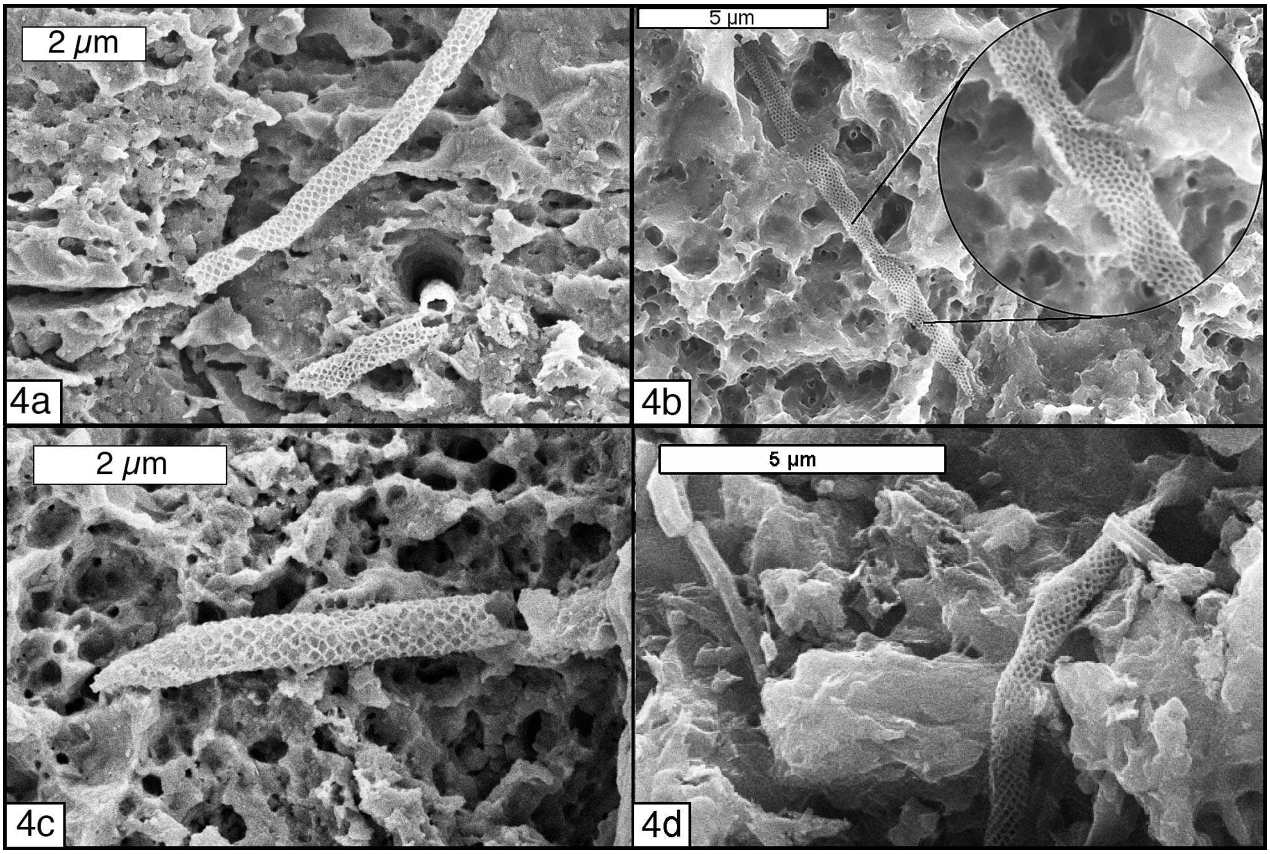

Figure 4. Scanning electron micrographs of reticulated filaments. (a) Hollow reticulated filament with diamond-shaped chambers that form a spiral. Hidden Cave pool finger, etched sample. (b) Reticulated filament that is hollow and torn open, with chambers that align along length of filament. Hidden Cave pool finger, etched sample. (c) Reticulated filament that is solid with diamond-shaped chambers that spiral. Hidden Cave pool finger, etched sample. (d) Reticulated filament from recently living sample (not etched). Note similarity to etched samples, particularly (a). Cape Verde lava tube.

features with external morphology suggestive of microbial involvement (Cunningham et al., 1995; Queen and Melim, 2006). This, however, could be an artifact of our study, as we have not closely examined many other cave pool precipitates, other speleothems or similar features in non-cave environments. Other speleothems or other environments may contain similar filaments, but etching of samples and meticulous, very high magnification SEM work is required to find them.

Reticulated filaments have an open cross-hatched pattern reminiscent of a fish net or a honeycomb (Fig. 4). Preservation of filaments varies substantially between individuals. The best preserved are hollow tubes that are partly collapsed (Fig. 4a). Others are torn open (Fig. 4b) or completely filled with calcite (Fig. 4c). Recently living filaments are hollow tubes (Fig. 4d). The filaments are up to 75 µm (average 12 µm) long. The measured length, however, is largely an artifact of preservation as virtually all of the specimens are torn, broken or have their ends buried in the matrix. Filament diameter is 300–1000 nm (average 590 nm) but this overall range encompasses two overlapping populations; one with an average diameter of 200–400 nm and the other with an average diameter of 500–700 nm. Both populations are found in the same samples (Fig. 5).

The filaments are characterized by two different styles of cross-hatched patterns, both with individual chambers 40 to 100 nm long and walls between chambers that are 30–40 nm wide. The larger diameter filaments typically have larger chambers, but not always. The more common form (77% of imaged filaments) has approximately hexagonal chambers that align in rows parallel to the length of the filament (Figs. 4b, 5). The less common form (23%) has more diamond-shaped chambers that spiral along the filament (Fig. 4c). The cross-hatch pattern does not correlate to filament size.

Composition of Filaments

In etched samples, the reticulated filaments occur within the sample and are partially revealed by the removal of the surrounding calcite (Fig. 4). Preliminary data show 1–2% organic carbon in the sample containing the most filaments (from Cottonwood Cave). EDX analysis of individual

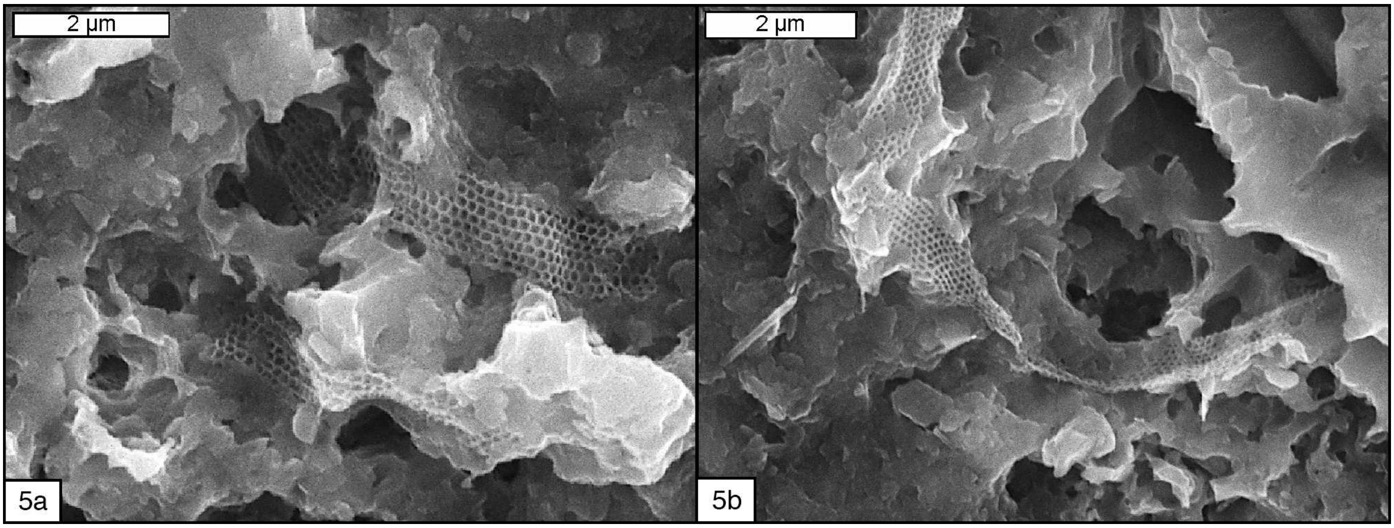

Figure 5. Scanning electron micrographs showing the two different sizes of reticulated filaments with parallel hexagonal chambers. Note the scale is identical in these images. (a) Larger filament. (b) Smaller filament. Hidden Cave pool finger.

filaments commonly shows calcium, oxygen, and more carbon than the surrounding calcite (Fig. 6). Since the depth of analysis (2–3 µm) is greater than the thickness of the filament, the results are a mixture of the surrounding calcite and the filament. We speculate that the extra carbon found is either a coating on the filament, protecting it from the acid etch (Melim et al., 2001), or the filaments are simply composed of a carbon-rich (hence organic) material and the etch has removed the surrounding calcite. Since the filaments from surface samples (not etched, Fig. 4d) and the embedded samples (etched out, Fig. 4a–c) have identical textures, it seems more likely that the filaments are preserved organic material and not carbon-coated calcitized filaments.

Possible origins

The morphology and high carbon content of these filaments suggests that they are biogenic. An extensive survey of known microorganisms and associated structures, and consultation with colleagues who work with a number of different microbial forms, however, shows nothing similar to the reticulated filaments in the same size range. Although the general filament shape is a common morphology, reticulated chambers are not. We are uncertain as to the origin of the reticulated chambers, as known microbes lack any structure of comparable size. Larger chambers are common; for example, some diatoms (Bacillariophyceae) have similar complex structures in their cell walls (areolae), but they are far larger in scale and very different in aspect ratio. Filamentous diatoms such as those in the genus *Aulacoseira* have similar cell morphology with regular patterns of pores termed areolae, but these filaments are significantly larger in diameter (3–30 µm

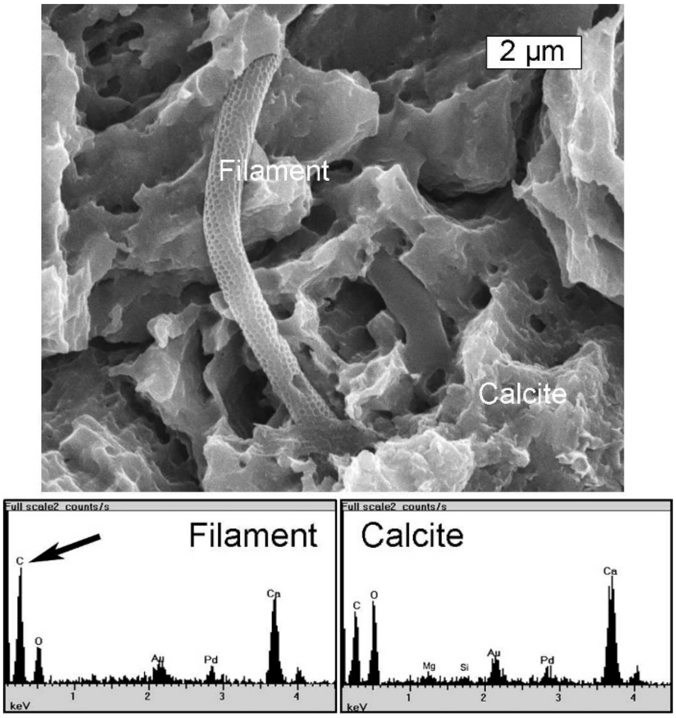

Figure 6. Scanning electron micrograph of reticulated filament and two EDX analyses; one centered on the filament and one centered on the calcite next to the filament. The analyses extend 2–3 µm into the sample so both plots show calcite (Ca + C + O), trace amounts of clays (Mg + Si + O) and the gold-palladium coating medium (Au + Pd). Note that the filament contains more carbon than the calcite sample indicating that the filament itself is carbon-rich.

diameter, 2.5–24 µm mantle height; Krammer and Lange-Bertalot, 1991). In general, diatoms in valve view range from eight or ten microns to a millimeter in length and are commonly 10–20 microns in diameter. At the extreme size ranges, diatoms can be as small as one micron in diameter (rarely) and as wide as 30–40 microns in diameter (Round et al., 2000). Some diatoms, including *Aulacoseira*, produce resting cells of similar sizes to the normal cells (Round et al., 2000). Thus, there is only the most marginal overlap in size with our observed structures. More importantly, all diatoms contain biogenic silica as a major component of their cell walls (Round et al., 2000). The reticulated filaments found in the cave samples completely lack silica.

Fungi, especially the filamentous, microscopic forms, were eliminated from consideration due to their larger size, generally two microns in diameter or larger. Thus, while they are often tens of microns in length, their diameter is insufficiently small to qualify as a bona fide candidate. The fungal morphologies we examined, or with which we are experienced, lack this form of reticulated patterning.

Actinomycetes, a filamentous group of bacteria, commonly occur in caves, are tens of microns in length at times and have similar diameters, but all known strains that we have investigated in the literature lack this kind of reticulated patterning. The authors have examined many cultures and environmental samples of actinomycetes using scanning electron microscopy and have never observed this morphology nor seen it in the literature

There is the possibility that the reticulate structure is a form of S-layer, a symmetrical arrangement of hexagonal protein units in the outer cell surface layer that has been observed in some Gram-negative bacteria (e.g. Schultze-Lam et al., 1992) and in *Archaea* (Messner et al., 1986). Interestingly, S-layers have a cross-hatched pattern when imaged using TEM (Phoenix et al., 2005) that is akin to the chamber patterns found in the reticulated filaments. The hexagonal units of the S-layer, however, are generally <50 nm wide (Messner et al., 1986; Schultze-Lam et al., 1992; Phoenix et al., 2005) and are therefore smaller than 70–100 nm long hexagonal units found in the reticulated filament. In the reticulate filaments, the center-to-center distance between adjacent chambers is generally two to three times larger than found in S-layer units. In addition, images of S-layers give the impression of a solid lattice layer rather than the open chambers of the reticulated filaments that we have observed in our cave samples.

In reviewing bacteria described in *Bergey's Manual of Systematic Bacteriology* (Holt, 1984–1989; Garrity, 2001–), only three bacteria were found with a similar morphology. The first of these is *Nitrosomonas*, which is pictured with "an additional cell wall layer" with units that are <20 nm (see Holt 1984–1989, p. 1824). The units in this extra cell wall layer in *Nitrosomonas* are three to four times smaller than the hexagonal units found in the cave reticulated filaments and also give the impression of a solid layer. *Nitrosomonas*' additional layer may represent an S-layer, as it is similar in morphology. The image of *Prosthecomicrobium polyspheroidum* (Garrity, 2001–) depicts cells with numerous short prosthecae that give the appearance of a corn cob, superficially resembling the reticulate structures, but the comparison breaks down on closer inspection. The third possibility is an image of *Helicobacter bilis* (Garrity, 2001–) with "tightly wound periplasmic fibers and multiple sheathed flagella." Although this organism is similar in diameter, it is only three microns in length.

In a study of putative *Leptothrix* sp. from a pool enriched in iron oxides in Carlsbad Cavern, Caldwell and Caldwell (1980) described filaments with "a hexagonal matrix over the surface of the cells." These hexagonal subunits, 0.1 microns in diameter, are more irregular in shape and are composed of iron, thus ruling out *Leptothrix* as a candidate for our filaments.

Thus, similar morphologies to the cave reticulated filaments do exist in the microbial world, but all can be ruled out due to the presence of silica (diatoms), size (diatoms, S-layers), or the presence of iron (*Leptothrix* sp.), leading us to conclude that this is a heretofore unreported morphology probably bacterial in nature.

CONCLUSIONS

Reticulated filaments are common in speleothems from moist or wet environments. These reticulated filaments are tubes of cross-hatched mesh with either hexagonal or diamond-shaped chambers. They are up to 75 µm in length (avg. 12 µm) but this size range is partly an artifact of preservation. Two overlapping populations occur, one 200–400 nm in diameter and the other 500–700 nm. EDX indicates that the filaments are composed of predominantly carbon, hence they are not mineral. We speculate that reticulated filaments are from an unknown, but possibly common, subsurface type of microorganism or group of microorganisms that prefer moist cave environments. We continue to look for more examples, particularly living examples, in the hope of culturing and/or obtaining DNA in order to more precisely identify their phylogenetic position and to understand their role in the cave ecosystem.

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