

GEOMICROBIOLOGY IN CAVE ENVIRONMENTS: PAST, CURRENT AND FUTURE PERSPECTIVES

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Abstract: The Karst Waters Institute *Breakthroughs in Karst Geomicrobiology and Redox Geochemistry* conference in 1994 was a watershed event in the history of cave geomicrobiology studies within the US. Since that time, studies of cave geomicrobiology have accelerated in number, complexity of techniques used, and depth of the results obtained. The field has moved from being sparse and largely descriptive in nature, to rich in experimental studies yielding fresh insights into the nature of microbe-mineral interactions in caves. To provide insight into the changing nature of cave geomicrobiology we have divided our review into research occurring before and after the Breakthroughs conference, and concentrated on secondary cave deposits: sulfur (sulfidic systems), iron and manganese (ferromanganese, a.k.a. corrosion residue deposits), nitrate (a.k.a. saltpeter), and carbonate compounds (speleothems and moonmilk deposits). The debate concerning the origin of saltpeter remains unresolved; progress has been made on identifying the roles of bacteria in sulfur cave ecosystems, including cavern enlargement through biogenic sulfuric acid; new evidence provides a model for the action of bacteria in forming some moonmilk deposits; combined geochemical and molecular phylogenetic studies suggest that some ferromanganese deposits are biogenic, the result of redox reactions; and evidence is accumulating that points to an active role for microorganisms in carbonate precipitation in speleothems.

INTRODUCTION

Life on Earth has been microscopic for much of its 3.7 billion year history (Schopf and Walter, 1983). Nonetheless, the metabolic activity of these organisms has left its mark on every conceivable planetary structure, from isotopic fractionation of ore deposits in the deep subsurface to the oxygenation of the atmosphere (Newman and Banfield, 2002; Schopf and Walter, 1983). Such metabolic activities continue to be critically important in the maintenance of the biosphere, where microorganisms sustain higher forms of life through primary production, nitrogen fixation and organic carbon mineralization. Despite the planetary evolution of our bio- and geospheres, historically researchers tended to ignore microbial activity in geological environments due to an inability to explain many geochemical reactions through purely inorganic chemistries and the inability to culture microorganisms from these sites (Amann et al., 1995). Eventually these limitations were removed with the development of molecular-scale geochemistry, while molecular biology allowed investigators to examine such environments without the need for cultivation (Banfield and Nealson, 1997; Hugenholz et al., 1998; Newman and Banfield, 2002; Pace, 1997).

Such techniques, and their resultant findings, also facilitated the interactions of microbiologists and geologists to understand the natural history of life processes and biogenic changes identified under geologic conditions (Banfield and Nealson, 1997). This scientific revolution at the boundary of geology and biology, which became known as geomicrobiology, extended into all arenas of

geology and revealed processes occurring under previously unrecognized physical and chemical conditions (Newman and Banfield, 2002). Historically, as investigators began to examine cave environments in closer detail, they identified unusual structures that hinted at the important role that microbial species might play in these systems (Cunningham et al., 1995; Hess, 1900; Høeg, 1946).

In creating a comprehensive review of the advances in cave geomicrobiology, we have built upon the earlier reviews (Northup and Lavoie, 2001; Northup et al., 1997) and have chosen to use the Karst Waters Institute *Breakthroughs in Karst Geomicrobiology and Redox Geochemistry* conference in 1994 (hereafter referred to as the Breakthroughs conference; Sasowsky and Palmer, 1994) as a watershed event in the history of such studies within the US. This conference brought together an international group of scientists to present their microbiological research, allowing ideas to be discussed and debated between karst and non-karst researchers. These cross-disciplinary interactions sparked a greater recognition and quickening of cave geomicrobiology within the US. Indeed, a search of the literature indexed in *Scisearch* (1977–present), *BIOSIS* (1969–present), and *Zoological Record* (1978–present), using search keywords representative of the secondary

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minerals, microorganisms, and caves, returned 165 articles of which 134 were published after 1994. To highlight the rapid evolution of cave and karst geomicrobiology, we will review studies on the secondary deposits identified in caves carried out in the years preceding the Breakthroughs conference and then examine the representative studies that followed. The latter clearly demonstrates the impact that mainstream geomicrobiological techniques have had on cave geomicrobiology and how subterranean processes in caves have led new investigators to enter the field.

CAVE GEOMICROBIOLOGY BEFORE 1994

Initial work on cave microbiology prior to the 1990s tended to concentrate on descriptive studies, with many investigators noting the presence of microorganisms in cave secondary mineral environments. Generally, such observations were dismissed as the result of transport into the system through air movement or vectors (animal or human) (Cunningham et al., 1995; Northup et al., 1994; Palmer, 1991). Researchers suggested that due to geologic isolation from allochthonous surface energy input, microbial species would be limited to the relatively few able to eek out an existence in this extremely starved environment (Palmer, 1991). Nonetheless, certain geochemical processes were difficult to explain by purely inorganic processes.

NITRATES

Cave nitrate (a.k.a. nitrocalcite or calcium nitrate) is the saltpeter commonly found in dry cave sediments and historically was an important component of gunpowder manufacture (Faust, 1949). As early as 1900, Hess questioned the origin of such deposits and proposed a seeping ground-water hypothesis in which bacterial decomposition of organic matter above the cave released nitrate ions that were transported via ground water. Subsequently, evaporation of water in dry passages would result in a buildup of nitrate in the saltpeter earth (Hess, 1900). Hill (1981), Hill et al. (1983), and Pace (1971) proposed modifications on this seeping ground-water mechanism, suggesting that organic-rich ammonia or ammonium ions were carried in from surface soils. Other suggested sources of nitrates in caves included bat guano (Hill, 1987); ammonium-urea from amberat (cave rat feces and urine) (Moore and Sullivan, 1978); bacterial nitrogen fixation (Faust, 1949, 1968; Lewis, 1992); fertilizers and sewage; volcanic rocks; and forest litter (Hess, 1900; Hill, 1981; Moore, 1994). Studies in Mammoth Cave demonstrated the presence of nitrifying bacteria, specifically *Nitrobacter* spp., in densities 100 times higher than surface soils, although no consensus was reached on a biogenic source for these nitrates (Fliermans and Schmidt, 1977).

SULFUR

Early studies of sulfur in caves concentrated on descriptive studies of microorganisms in caves with sulfide

inputs. Principi (1931) first proposed sulfuric acid-driven speleogenesis, and suggested that a small Italian cave was created by the interaction of sulfidic waters with limestone (noted in Vlasceanu et al., 2000, who also reviews early non-cave and karst sulfuric acid corrosion). Morehouse (1968) first described cave dissolution by sulfuric acid in the English-language literature based on his studies in Level Crevice Cave, Iowa. Of particular interest in establishing a microbial role in sulfuric acid-driven speleogenesis was the first documentation of isotopically light sulfur and gypsum deposits. These lighter isotopes are preferentially used by cellular enzymes; and thus, such fractionation usually indicates biological activity. Hill (1987) provided the first $\delta^{34}\text{S}$ values for a range of geological environments, including sulfur isotope analyses on sulfur and gypsum deposits from several Guadalupe caves. The comparison of observed data with theoretical values led her to conclude that biological fractionation had occurred in the pathways leading up to the cave deposits. In a later publication Hill (1994) concludes that biogenic fractionation comes from the initial reduction of sulfate to hydrogen sulfide; that the cave elemental sulfur deposits are not biogenic, while the gypsum deposits are. Since these early papers, other studies have implicated sulfuric acid in the formation of numerous caves (Davis, 1980; Egemeier, 1981; Galdenzi, 1990; Hill 1987, 1990; Jagnow, 1979; Korshunov and Semikolennyh, 1994); however, in these inactive cave systems, the cause and effect of microbial metabolisms on speleogenesis remained elusive.

CARBONATES

An early morphological study of limestone types by Shoji and Folk (1964) first indicated the possible role that microorganisms might play in carbonate deposition, revealing inclusions within rock that were later shown to be microbial in origin (Folk and Chafetz, 1980). At the same time, geologists were recognizing a microbial component to carbonate precipitation in stromatolites from the fossil record (Logan et al., 1964), which provide evidence of some of the earliest life on Earth (Schopf and Walter, 1983). While studies on stromatolites suggested that microbial activity was limited to the trapping of calcite crystals within an algal film, subsequent work demonstrated that changes in the microenvironment through photosynthetic activity induced this precipitation of calcite (Walter, 1976). While much early work concentrated on saltwater environments with photosynthetically-driven calcite precipitation, Chafetz and Folk (1984) began to examine calcite precipitation in freshwater, travertine deposits. These investigators were among the first to recognize that the high temperatures and sulfide chemistry of these environments limited algal growth and photosynthesis. As a result, they were able to demonstrate that as much as 90% of the deposited travertine in these springs was bacterially precipitated (Chafetz and Folk, 1984). These investigators went on to demonstrate that local changes in geochemistry altered the

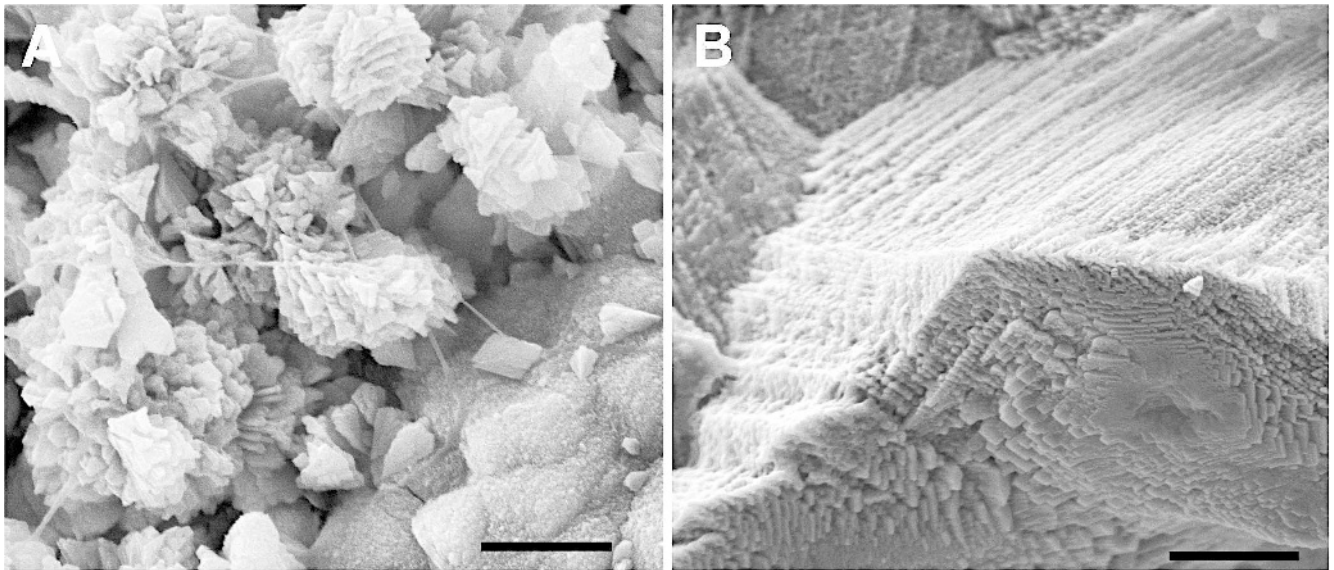


Figure 1. Scanning electron micrographs of calcite crystals formed on Boquet B-4 media (Boquet et al., 1973) by bacteria isolated from a cave environment. **A,** Calcite crystals forming on a bacterial colony; filaments of individual bacterial cells are visible (scale bar 5 μm). **B,** Individual calcite crystal formed on a bacterial colony, confirmed by energy dispersive spectroscopy (EDX; scale bar 10 μm).

crystal structure of such deposits, allowing the nature of such biogenic deposits to be identified within the geologic record (Chafetz, 1986; Chafetz and Folk, 1984; Love and Chafetz, 1988).

At the time of the discoveries by Chafetz and Folk (1984), the possibility that microorganisms were involved in calcium carbonate (CaCO_3) precipitation was not new. Indeed, the earliest indication of such deposition was made by Wollny in 1897 (as referenced in Hall and Miller, 1905). Later investigators went on to conclude that such precipitation was caused by biological surfaces that could coordinate ions and facilitate biologically controlled mineralization (Chafetz and Buczynski, 1992). Nonetheless, it was not until the experiments of Boquet et al. (1973) who grew soil bacteria on agar plates that contained calcium, but lacked any source of carbonate, that the importance of bacterially controlled mineralization (BCM) was realized (Fig. 1). Interestingly, the work of Boquet et al. suggested that the ability to precipitate calcite was a common occurrence among soil bacteria and is conserved across multiple evolutionary domains. Buczynski and Chafetz (1991) were able to confirm the importance of BCM by showing that only metabolically active bacteria could precipitate CaCO_3 , and that the subsequent mineral structures (calcite versus aragonite) were dependent on the viscosity of the medium on which they were grown. Such results emphasized the importance of the terrestrial environment on calcite precipitation (Buczynski and Chafetz, 1991; Chafetz and Buczynski, 1992).

With the stimulus of such work being carried out in carbonate precipitation, it is not surprising that the myriad of speleothems found in caves prompted early studies to

examine the potential role of microorganisms in the formation of these deposits. In the 1960s, Thraillkill (1964) was the first to suggest a link between the origin of cave popcorn and microorganisms, while Went (1969) suggested that fungi played an important role in stalactite growth (Fig. 2), although no biogenic component was identified by Folk and Assereto (1976). In 1983, Danielli and Edington demonstrated that bacterial species isolated from secondary cave deposits displayed a greater capacity to precipitate CaCO_3 than surface species (Danielli and Edington, 1983). Such activity led the authors to suggest a metabolic link between using an organic calcium salt for energy and excreting calcium ions as a waste product, which would result in precipitation when the calcium exceeded the solubility threshold (Danielli and Edington, 1983). Additional support for the role of microorganisms in speleothem formation was primarily circumstantial and consisted of a number of investigators finding micro-fossils within carbonate speleothems (e.g., Cox et al., 1989; Jones and Motyka, 1987; Polyak and Cokendolpher, 1992), until the identification of pool fingers by Davis et al. (1990). These subaqueous pool fingers demonstrated a truly biogenic structure, with parabolic u-loops connecting pendant fingers, the formation of which was difficult to describe using solely abiotic processes (Fig. 3). The associated webulites seen with these pool fingers (Davis, 2000) also appeared biogenic in origin, more closely resembling microbial biofilm structures than mineral precipitates.

Another carbonate deposit that has long attracted microbiologists is moonmilk, also known as mondmilch and a variety of other names (Bernasconi, 1981; Reinbacher, 1994). Moonmilk, which has differing structural

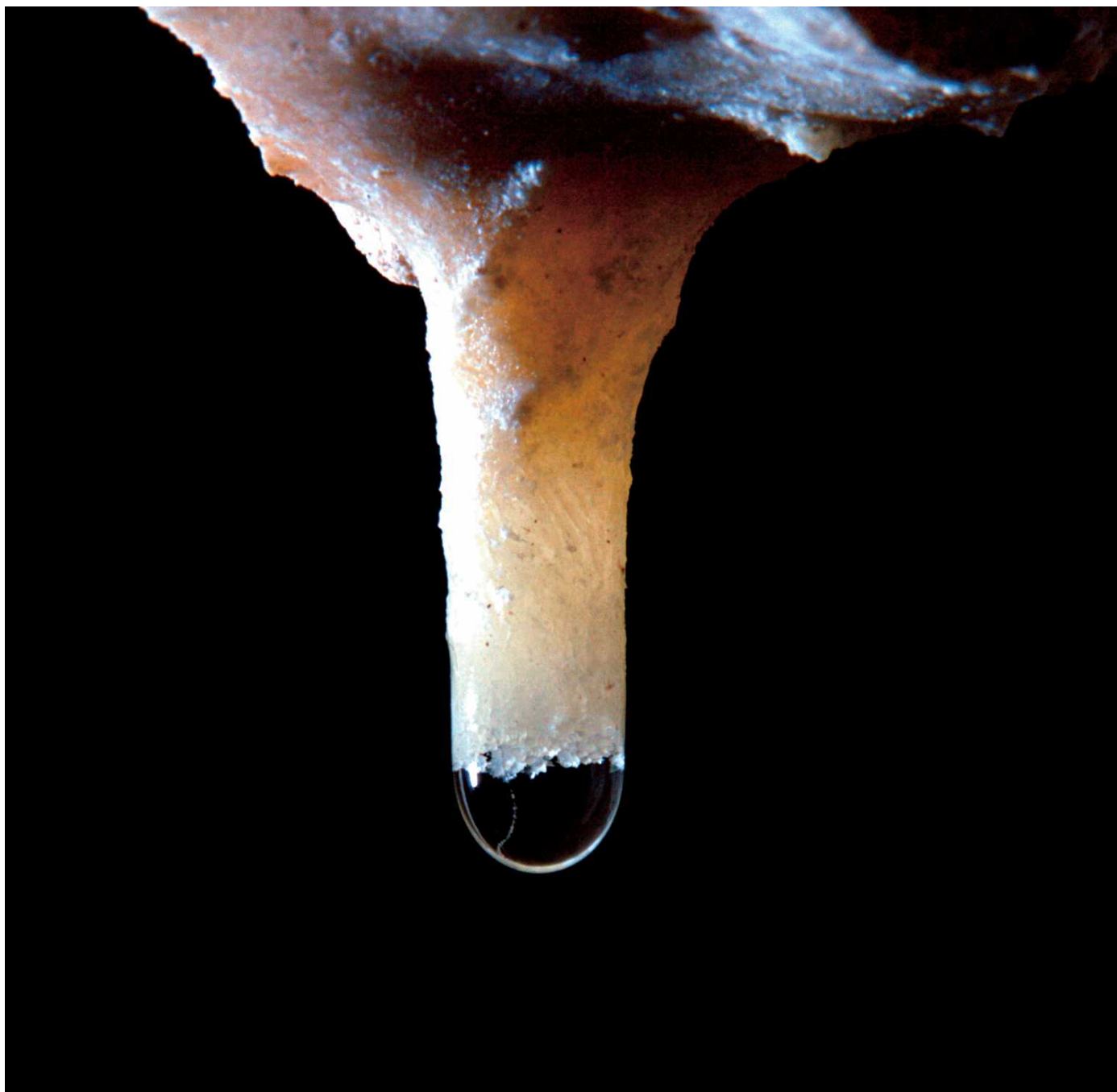


Figure 2. Circumstantial evidence, such as this fungal mycelium emerging from the end of a calcite soda straw and its associated calcite crystals, prompted early investigators to postulate on a role for microbial species in the deposition of speleothems.

forms, from a soft, granular paste to a loosely aggregated powder, can be composed of calcite, aragonite or hydromagnesite crystals, depending upon the structure of the cave in which it is deposited (Hill and Forti, 1997). Høeg (1946), in one of the earliest papers on microbial-mineral interactions, suggested that the metabolic activity of microorganisms was the cause of moonmilk deposition, an idea later supported by Davies and Moore (1957). In

support of this hypothesis was the work of Went (1969), who demonstrated the ability of fungal hyphae to act as attachment and nucleation sites for CaCO_3 precipitation. Nonetheless, with the formation of soft deposited forms of moonmilk on much harder bedrock, other investigators suggested that corrosion rather than deposition mechanisms were responsible. Early studies by Caumartin and Renault (1958) and Caumartin (1963) suggested that



Figure 3. Small, double u-loops connecting the pendant-like pool-fingers in Hidden Cave, New Mexico. These small structures were difficult to explain using abiotic processes. It is now known that such structures are biogenic in origin.

moonmilk could be the result of microbial metabolic products that corroded underlying bedrock. Despite numerous hypotheses, at the time of the Breakthroughs conference, no clear picture emerged of whether these intriguing deposits are biogenic or abiotic in origin.

OTHER MINERALS

Of the other secondary mineral species observed in caves, an abundance of circumstantial evidence for iron biomineralization exists: Caldwell and Caldwell (1980), Caumartin (1963), Crabtree (1962), Dyson and James (1981), Jones (1991), Jones and Motyka (1987), Klimchouk (1994), Luiszer (1992), Maltsev (1997). One of the only experimental studies was that of Peck (1986), who recovered the iron-oxidizing species *Gallionella ferruginea* and *Leptothrix* sp. from cave pools, sumps and moist Fe/Mn structures in Level Crevice Cave near Dubuque, Iowa. This study established that sterile controls showed no iron precipitation, while live inoculations of subterranean bacterial species precipitated iron hydroxides (*Gallionella ferruginea* cultures) and iron-impregnated sheaths (*Leptothrix* sp). Such iron-encrusted filaments were also identified in the rusticles of Lechuguilla Cave, wherein Davis et al. (1990) used scanning electron microscopy (SEM) to show the filamentous bacterial shapes associated with these interesting formations.

Several early studies also proposed microbial participation in the formation of cave manganese deposits: Broughton (1971), Cílek and Fábry (1989), Crabtree (1962), Hill (1982), Jones (1992), Laverty and Crabtree (1978), Moore and Sullivan (1978), Peck (1986) and White (1976). A range of manganese forms are found, such as coatings on walls or speleothems (Gascoine 1982; Hill, 1982; Kashima, 1983; Moore and Sullivan 1978; Rogers and Williams, 1982), soft deposits in clastic deposits (Cílek

and Fábry, 1989), and consolidated crusts (Hill, 1982; Jones, 1992; Moore, 1981; Peck, 1986). Moore (1981) found manganese-oxidizing bacteria such as *Leptothrix* in a stream in Matts Black Cave, West Virginia, and attributed the formation of birnessite in this cave to the precipitation of manganese around sheaths of bacteria. The presence of rods, sheets, strands, and smooth spheroid morphologies in the fossil remains of manganese precipitates in stalactites, karst breccia and root calcrete crusts in Grand Cayman caves led Jones (1992) to conclude that some of these manganese precipitates were biogenic; however, as with much of the pre-1994 cave geomicrobiology literature, many of these studies provide only descriptive, circumstantial evidence. The degree to which the phylogenetically diverse group of microorganisms known to oxidize reduced manganese can promote such oxidation, passively or enzymatically, is debated. Microorganisms can increase the rate of manganese oxidation by up to five orders of magnitude (Tebo et al., 1997) and the large accumulations of manganese oxides that occasionally occur in caves represent potentially microbial mediated production.

Literature on other biogenically mediated mineral structures from caves is limited; studies of silicate speleothems and clay mineral forms have been conducted mainly in Japan and Venezuela. Early studies of microorganisms associated with opal speleothems demonstrated the presence of microbial morphologies in the speleothems (Kunicka-Goldfinger, 1982; Urbani, 1976, 1977). *Meolosira*, a siliceous algal diatom, were found in twilight zone coralloids in Togawa Sakaidanipdo Cave, Japan (Kashima, 1986; Kashima, et al., 1989). Little early work exists on clay-containing speleothems such as vermiculations, although Anelli and Graniti (1967) hypothesized that the halo surrounding vermiculations is caused by acids and other organic substances secreted by fungi.

CAVE GEOMICROBIOLOGY AFTER 1994

At the beginning of the 1990s, new molecular techniques increased the number of environments that could be successfully studied by microbiologists (Pace, 1997). Such techniques allowed researchers to examine the complex chemical interactions of microbial physiology with redox active minerals, in what had previously been considered abiotic, geological environments (Banfield and Nealson, 1997; Newman and Banfield, 2002). The bringing together of cave geologists and biologists at the Breakthroughs conference mirrored the evolution of the science of geomicrobiology; biologists brought alternative principles (respiration across redox gradients) and unique techniques (DNA purification and molecular phylogenetics) to their geologic peers (Newman and Banfield, 2002). Geologists likewise exposed biologists to the principles of mineralogy and novel techniques (x-ray powder diffractometry and energy dispersive spectroscopy) (Banfield and Nealson, 1997). The introduction of new tools and techniques

provided opportunities to pose novel questions in cave environments. While this initial euphoria was not without its drawbacks [the *de facto* hypothesis for many biologists is that everything is biogenic, with the converse being true for geologists (Barton et al., 2001)], the initial discoveries brought cave geomicrobiology to a new found audience. This work also showcased the significance of cave microbiology to speleologists and allowed them to recognize potentially important geomicrobial structures during exploration (Davis, 2000; Davis et al., 1990). The melding of such activities has taken the value of such research beyond the interest of speleologists and into the broader scientific realm (Newman and Banfield, 2002).

NITRATES

George Moore's 1994 title, "When will we have an accepted explanation for cave nitrate deposits?" captures the essence of a debate that has spanned more than a century. Despite the significance of cave nitrates in the early history of cave microbiology, there has been little work that has advanced our understanding beyond the hypotheses reviewed in the earlier section of this paper. The stable isotope work of Jameson et al. (1994) did demonstrate that saltpeter is enriched in the lighter isotope of nitrogen, supporting the hypothesis that microbial activity is involved in the formation of cave nitrates. Microbiologists have continued to debate the degree to which bacteria, such as *Nitrosomonas* and *Nitrobacter*, facilitate the creation of the cave saltpeter deposits and the origin of the nitrogen. Nonetheless, to date, no consensus exists to explain the formation of these minerals in caves, likely a reflection of the loss of any commercial value for such deposits with the advent of industrial chemistry. Even so, nitrogen is a limiting nutrient for microbial growth in all environments and a clear understanding of how such deposits form would lead to a greater understanding of how microbial growth can be supported in subterranean environments (Newman and Banfield, 2002).

SULFUR

The intense exploration of Lechuguilla Cave and the discoveries of massive sulfur related deposits provided substantial support for the theory of sulfuric acid driven speleogenesis (Spirakis and Cunningham, 1992; Cunningham et al., 1993, 1994) [an expanded history of this idea in the Guadalupe Mountains is traced in Jagnow et al., 2000]. Nonetheless, the metabolic role of microorganisms in producing such sulfuric acid was based almost entirely on the study of inactive cave systems in which cavern enlargement through active microbial processes was no longer occurring. The rare exception were initial studies in Parker Cave (Kentucky), which suggested that sulfur and gypsum deposited on artificial substrates in Sulphur River resulted from extensive bacterial sulfide-oxidizing activity (Angert et al., 1998; Olson and Thompson, 1988; Thompson and Olson, 1988). Other investigators also began

examining microbial sulfur cycling in active cave systems, leading to a greater understanding of the role of bacteria in cave dissolution. Our discussion of this work will be brief as readers are referred to the current review of sulfuric acid speleogenesis by Engel (this issue).

In the underground aquifer of the Bahamas and Yucatan Peninsula, the hydrology results in the formation of anchialine caves. These caves contain an upper freshwater lens, a brackish mixing zone (halocline), and underlying seawater that intrudes from the coast (Bottrell et al., 1991; Moore et al., 1992) and creates a stratified water column within the cave system, based on chemical, temperature and density gradients (Moore et al., 1992; Pohlman et al., 1997; Stoessell et al., 1993). Within the halocline itself, stratified zones of SO_4^{2-} , NO_3^- , NO_2^- , and pH have been observed, suggesting the presence of an active microbial ecosystem (Pohlman et al., 1997; Socki et al., 2001; Stoessell et al., 1993). Work by Socki et al. (2001) has shown that $\delta^{34}\text{S}$ values for the sulfide in these systems are isotopically light, as much as -63.2‰ , suggesting that the H_2S comes from bacterial cycling, and not from degradation of plant material entering through the cenote, supporting microbially driven sulfuric-acid production (Marcella et al., 1994; Martin and Brigmon, 1994). The importance of such subterranean sulfur-cycling is emphasized by the discovery by Sarbu et al. (1994) of a sulfur-based cave ecosystem in Movile Cave, Romania, where a macroscopic-ecosystem is supported by chemoautotrophic bacterial communities (Sarbu et al., 1996). The basis of this ecosystem was a staggering level of dissolved H_2S in the water, approaching $1300\ \mu\text{M}$, although the majority of microbial mats in this system developed on the surface of pools in isolated air pockets, rather than in a stratified water column (Sarbu et al., 1994, 1996). The discovery of such a diverse subterranean ecosystem driven by chemoautotrophic microorganisms was a significant advance in our understanding of biological diversity. Other cave systems have similarly contributed to our understanding of biologically mediated sulfuric acid speleogenesis, including Cueva de Villa Luz, Mexico (Hose et al., 2000), Frasassi Cave, Italy (Galdenzi and Menichetti 1995; Vlasceanu et al., 2000), Cupp Coutunn Cave System, Turkmenia (Maltsev 1997), Lower Kane Cave, Wyoming (Engel et al., 2004), and Cesspool Cave, Virginia (Engel et al., 2001). These systems demonstrate many of the subaerial microbial activities thought to have occurred within Lechuguilla Cave, confirming a broader biogenic component in the speleogenesis of sulfuric acid caves (Davis, 2000).

In order to better understand the microbial metabolic processes that lead to cavern enlargement, Engel et al. (2004) demonstrated localized dissolution of carbonates by *Epsilonproteobacteria* in Lower Kane Cave, Wyoming. The bacteria locally produced sulfuric acid that dissolved the host rock, leaving behind obvious solution pockets where the microorganisms attached to the surface of the mineral

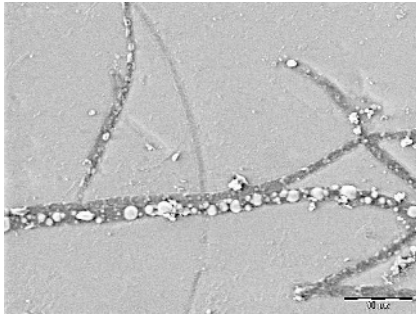


Figure 4. Unpreserved calcite chip and filamentous microbial cells (some with intracellular sulfur globules) examined with environmental scanning electron microscopy; calcite chip was exposed to the microbial mat for three months, resulting in an etching of the surface by the microbial activity. Scale bar 10 μm .

(Fig. 4). Surprisingly, these investigators demonstrated that dissolved H_2S in the cave spring was quickly consumed by sulfide-oxidizing bacteria before it could generate inorganic sulfuric acid, in direct contradiction to the classic sulfuric acid speleogenesis model of Palmer (1991; Engel et al., 2004). In contrast, other caves such as Cueva de Villa Luz have aggressive subaerial microbial activity resulting in the presence of microbial biofilm communities (snottites; Fig. 5) with observable sulfuric acid production (Hose et al., 2000). Such communities are actively producing gypsum precipitates that slough off the walls to enlarge the cave and rillenkarren that were taken as evidence of past microbial activity within Lechuguilla Cave (Davis, 2000). In order to identify the microbial activity responsible for the formation of caves on the scale of Carlsbad Caverns and Lechuguilla Cave, Barton and Luiszer (2005) recently proposed a metabolic model wherein sulfite and sulfuric acid could be produced in such systems in the absence of significant oxygenated water input. This theory also suggested that subaerial dissolution in the presence of oxygen would result in localized pockets of aggressive dissolution, as has been seen in both Lechuguilla and Villa Luz cave systems (Hose et al., 2000; Davis, 2000). Whatever the microbial mechanisms in place, there remain important questions regarding observable differences in speleogenesis and cavern enlargement on the molecular and geological scales (Engel et al., 2004; Klimchouk et al., 2000) that promise to be an exciting and innovative area in cave geomicrobiology as we work toward a comprehensive model of sulfuric acid speleogenesis.

CARBONATES

In the past decade, there has been a rapid expansion in our understanding of carbonate biogeochemistry and the deposition of CaCO_3 in reactions that range from the molecular to environmental scale (Banfield and Nealson, 1997; Banfield et al., 2005; Mozely and Davis, 2005;



Figure 5. Microbial biofilms, such as these snottites from Cueva de Villa Luz, produce water droplets with a pH of 0 to 2. Such biofilms play important roles in secondary dissolution processes within sulfidic cave systems.

Neuweiler et al., 2000; Newman, 2001; Woods et al., 1999). Together, these investigations are piecing together a more complete understanding of the role that biological processes, whether direct or indirect, play in the formation of CaCO_3 deposits within the geologic record (Banfield and Nealson, 1997; Bosak and Newman, 2003; Neuweiler et al., 2000; Woods et al., 1999). Likewise, the importance of speleothems as terrestrial travertine deposits has led to greater research in these environments, increasing our understanding of the potential role that microorganisms play in the structure and formation of such cave deposits (Cacchio et al., 2004; Frisia et al., 2002; Galy et al., 2002; Melim et al., 2001; Saiz-Jimenez, 1999; Sanchez-Moral et al., 2003; Tooth and Fairchild, 2003). Together this work indicates that, as has been observed in hot spring travertines, the local geochemistry, temperature, rate of CO_2 off-gassing and precipitation, and microbial activity all play critical roles in carbonate deposition and structure (Fouke, et al., 2000; Frisia et al., 2002; Sanchez-Moral et al., 2003).

There has been a greater recognition of the different roles that biologically induced and biologically controlled precipitation play in CaCO_3 biomineralization (Bosak and Newman, 2005; Braissant et al., 2005); biologically induced precipitation (BIM) refers to the effect that organismal

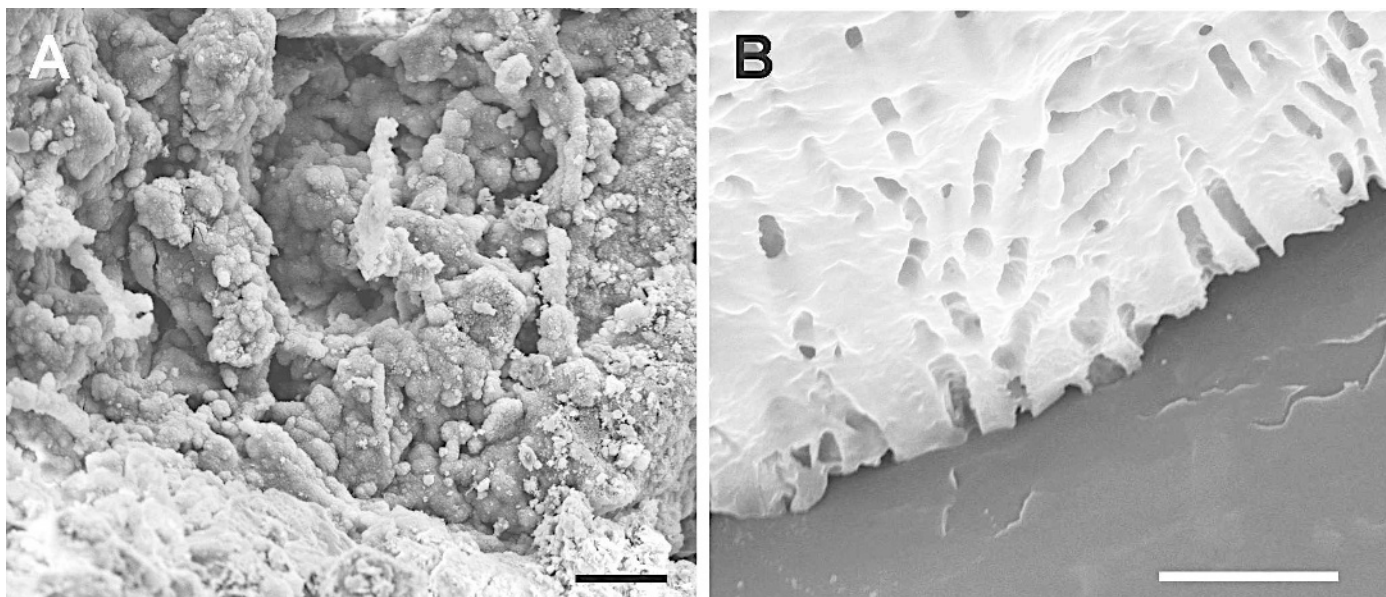


Figure 6. Microorganisms growing on the wall of a cave in Kentucky have become encased in a calcite matrix (A), as confirmed by EDX analysis (scale bar 20 μm). Viable bacterial cells were isolated from this area and shown to also become encased in calcite during growth on B-4 media, leaving behind cell-shaped cavities when washed from the precipitated calcite (B; scale bar 5 μm).

metabolic activities and by-products have on the local physiochemical environment, favoring conditions that promote precipitation (Frankel and Bazylinski, 2003). Biologically controlled precipitation (BCM), also called organic matrix-mediated mineralization or boundary-organized mineralization, refers to the role that cellular substrates play in promoting coordination and growth of biominerals (Bazylinski and Frankel, 2003; Simkiss and Wilbur, 1989).

Microorganisms can promote CaCO_3 precipitation through processes of BCM by altering the saturation index (SI) of the solution, or by removing kinetic inhibitors of crystallization, such as magnesium, sulfate or phosphate ions (Bosak and Newman, 2005, and references therein). While such BCM activities have been shown to play an important role in calcite crystal morphology (Bosak and Newman, 2005; Braissant et al., 2005; D'Souza et al., 1999; Orme et al., 2001; Schultze-Lam et al., 1992), it has been more difficult to assess a role for BIM in CaCO_3 deposition and the role of microorganisms in speleothem development. Castanier et al. (1999) have suggested that bacterial autotrophic processes cause CO_2 depletion surrounding the cell, favoring the precipitation of calcium ions as CaCO_3 . Other investigators have proposed that heterotrophic processes of nitrogen fixation and release play a critical role in raising the pH of the local environment, again favoring the precipitation of carbonates (Cacchio et al., 2004; Hammes and Verstraete, 2002). In cyanobacterial species, fixation of CO_2 increases the concentration of bicarbonate ions, which may be excreted into the extracellular medium, causing CaCO_3 precipitation if

calcium ions are present (Badger and Price 2003; Hammes and Verstraete 2002). While such photosynthetic reactions are not possible in cave environments, the carbonic anhydrases responsible for CO_2 uptake are common among bacterial species (Merlin et al., 2003). McConaughy and Welan (1997) also suggested that bacterial calcification may generate energy for nutrient uptake in starved environments and may explain the ubiquitous nature of calcite precipitation originally observed by Boquet et al. (1973).

Intellectually a problem arises in that as microorganisms carry out calcite precipitation, such activity invariably leads to entombment within the growing crystal and death (Barton et al., 2001) (Fig. 6). This makes understanding an evolutionary advantage for such activity difficult to assess. It is known that bicarbonate ions can serve as a buffer, which allows microorganisms to carry out cellular processes that would otherwise lead to acidic conditions; if bicarbonate ions were serving this function under such conditions, Ca^{2+} would quickly accumulate to toxic levels. Nonetheless, evidence is starting to emerge that bacteria, like their eukaryotic counterparts, have Ca^{2+} antiporter protein pumps that selectively detoxify calcium from the cell by pumping it into the extracellular medium (Cai and Lytton, 2004). Indeed, a study by Anderson et al. (1992) demonstrated that under toxic calcium concentrations, *Pseudomonas fluorescens* actively precipitated calcite.

This work is further supported by Cacchio et al. (2004), who demonstrated a selective enrichment for microbial species capable of carrying out CaCO_3 deposition from speleothems within Cervo Cave, Italy. These investigators

demonstrated that bacterial species isolated from different speleothems displayed an unusually high rate of carbonate precipitation, when compared with organisms from non-karst environments; however, these investigators relied on cultivation techniques that favored microorganisms capable of surviving the transition from the starved, oligotrophic conditions of cave environments to the eutrophic conditions of a nutrient plate. Such cultivation strategies selectively identify microbial species that are known to display the metabolic phenotypes consistent with carbonate precipitation, including *Pseudomonas* and *Bacillus* species (Boquet et al., 1973; Cacchio et al., 2003, 2004; Koch, 1997). Nonetheless, these investigators did demonstrate using oxygen and carbon isotopic fractionation that three distinct processes appeared to be involved in bacterial calcite precipitation (Cacchio et al., 2004). Whatever the metabolic process responsible for calcite deposition (and the work of Cacchio et al., (2004) suggests they may be numerous) it is important to remember that a balance must exist between biological and inorganic processes in such precipitation (Fouke et al., 2000; Palmer, 1996).

Regardless of the metabolic activity directly responsible for CaCO_3 deposition within caves, investigators have continued to examine such deposits for the presence of microfossils, petrographic fabrics that are indicative of microbially mediated precipitation and isotopic fractionation of the carbonate (Melim et al., 2001; Boston et al., 2001). Using SEM, Melim et al. (2001) examined the layered calcite pool fingers, first identified by Davis (2000) as potentially biogenic in origin. These investigators found an abundance of fossil filaments within micritic layers, but not in the inter-layered clear calcite spar. They also found a small shift in the carbon isotope composition of the micritic versus clear calcite layers. Together, this association suggested that microorganisms were involved in the deposition of this formation (Melim et al., 2001). Such work was recently supported by Baskar et al. (2006), who demonstrated microcrystalline deposition of calcite within stalactites that appeared to be mediated by microbial processes. A study by Contos et al. (2001) also demonstrated the presence of subaqueous calcite precipitates associated with microbial biofilms in Weebubbie Cave, Australia. These deposits formed in waters well below the saturation index of calcite and demonstrated a unique structure, which could only be replicated *in vitro* with the addition of organic acids (Orme et al., 2001). Such results led the investigators to conclude that the surface of the *Gammaproteobacteria* species found within the bacterial filaments of the cave (Holmes, et al., 2001) played a crucial role in calcite deposition (Contos et al., 2001).

The most convincing evidence of microbial involvement in speleothem formation comes from the formation of moonmilk (Cañaveras et al., 2006). While moonmilk was one of the earliest calcite cave deposits to be associated with microbial activity (Høeg, 1946), its needle-fiber structure is delicate and easily altered by the constructive

or destructive processes of diagenesis (Jones, 2001). Thus it has remained difficult to determine the role that microorganisms play in the structural formation of moonmilk. By using a combination of cultivation, molecular phylogenetics and petrographic analyses, Cañaveras et al. (1999, 2006) demonstrated that moonmilk does not contain fungal filaments, but rather numerous filamentous *Proteobacteria* species that demonstrate a calcite precipitation phenotype. Morphological evidence suggested that moonmilk forms through the microbial colonization of rock surfaces, followed by calcite deposition along bacterial surfaces, microstructural breakdown, and accumulation of collapsed fibers (Cañaveras et al., 2006). As this process repeats through seasonal oscillations, moonmilk deposits become thicker, forming the significant deposits observed in numerous caves (Cañaveras et al., 2006). These investigators also identified the presence of *Crenarchaeota*, members of the Archaea, in these moonmilk deposits; their role in moonmilk formation remains unclear (Gonzalez et al., 2006). Together, such work represents one of the most complete pictures of the physiological and geochemical relationships of biogenic deposit formation within cave environments.

While a significant amount of work has been geared toward understanding microbial involvement in carbonate constructive processes, there is an increasing interest in the destructive, erosional processes of microbial activity (Jones, 2001). Even while microbial surfaces may bind Ca^{2+} ions, increasing the likelihood of calcite crystallization (Bosak and Newman, 2005), certain biological molecules have a sufficiently high affinity for these ions that they actually promote dissolution (Perry et al., 2004; Friis et al., 2003). Such structures include exopolysaccharide (a major component of biofilms), siderophores and other secreted chelators, and even the bacterial cell wall (Perry et al., 2004; Friis et al., 2003). Through their metabolic processes, bacteria also secrete a number of organic acids, which actively dissolve carbonates. Conversely, a number of microbial structures, including lipids and phospholipids, actually inhibit dissolution, while soil derived humic acids, which form a significant portion of the organic carbon found within cave drip waters (Saiz-Jimenez and Hermosin, 1999), also inhibit calcite dissolution. It is likely that the governing factors controlling microbial involvement in calcite constructive or destructive development will involve a balance between the local conditions, the geochemistry and physiochemistry of the local environment, and the microbial metabolic processes that predominate under such conditions (Pohl and Schneider, 2002; Vlasceanu et al., 2000).

OTHER MINERALS

Two decades ago, Peck (1986) described the presence of microbial species in manganese and iron oxides within caves and proposed the possibility of chemolithotrophic primary producers in these systems. Unfortunately most

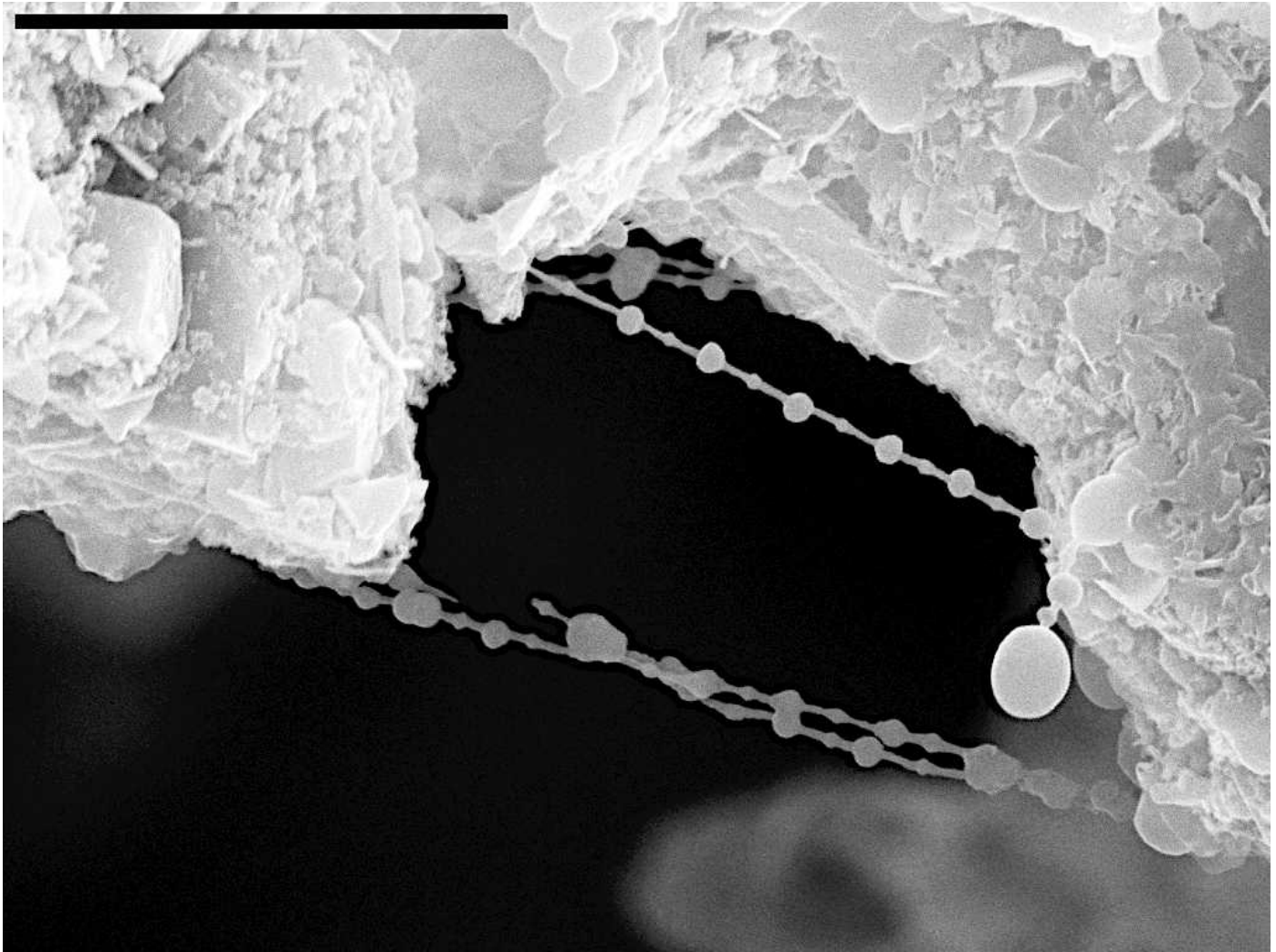


Figure 7. Unusual beads-on-a-string morphologies of manganese-oxidizing bacterial species are seen throughout several of the ferromanganese deposits from Snowing Passage in Lechuguilla Cave. EDX analyses show that the mineral matrix is manganese oxide. (Scale bar 5 μm).

biologists did not recognize the significance of this observation. Such indifference mirrors the general lack of interest by microbiologists (primarily due to the funding climate at the time), who tended to concentrate on medically important pathogens. Events in the exploration of Lechuguilla Cave, first explored in the same year as Peck's study, would provide the impetus to explore microbe-mineral interactions on manganese and iron environments in caves; one of the deposits that generated the greatest interest were the colorful deposits on walls and ceilings (Cunningham, 1991). These iron and manganese oxide-rich layers were initially believed to be the insoluble residue from the attack of corrosive air on the carbonate bedrock and were called corrosion residues (Queen, 1994; Cunningham, 1991). These corrosion residues, now referred to as ferromanganese deposits, occur in a range of colors and are diverse in composition with variable amounts of clay and Al-oxide minerals; all are rich in

Mn- and Fe-oxides (Spilde et al., 2005). The work of Cunningham was the first to recognize an association between microbial species and ferromanganese deposits within Lechuguilla Cave (Cunningham et al., 1995). Inspired by Cunningham, the team of Boston, Northup, Spilde and others established the presence of a diverse community of microorganisms, some of whom were related to known manganese- and iron-oxidizing bacteria and others who appear to be previously unknown (Northup et al., 2003) (Fig. 7), and documented the presence of metabolically active bacteria in the punk rock underlying the ferromanganese deposits (Spilde et al., 2005). Their geochemical studies documented a four-fold enrichment of reduced manganese between the bedrock and ferromanganese deposits. Spilde et al. (2005) also demonstrated that some of the mineral species identified in these deposits can be reproduced *in vitro* by microbial species inoculated from these environments and fed a chemical diet of the reduced

metal ions present within the rock of the cave, while killed controls did not produce the crystalline forms. While this does not conclusively demonstrate a cause-and-effect, it takes a significant step toward understanding the microbial activities responsible for the formation of such deposits.

Somewhat similar deposits to the ferromanganese deposits of Lechuguilla Cave are those found in Ochtiná Aragonite Cave in Slovakia. These deposits, termed ochres, contain goethite, birnessite, and asbolane (Bosák et al., 2002). Besides the co-occurrence of goethite and birnessite in these deposits and those in Lechuguilla Cave, Ochtiná ochres also contain occasional occurrences of La-Nd-bearing phosphate. Lechuguilla ferromanganese deposits also contain instances of rare earth elements associated with phosphate minerals. Bosák et al. (2002) suggested that the manganese oxides were the result of microbial precipitation in pool bottoms in a manner similar to that described by Andrejchuk and Klimchouk (2001). Chelius and Moore (2004) performed a phylogenetic analysis of the Wind Cave (South Dakota) paleofill samples that contained some manganese and iron. Interesting similarities exist between the archaeal phylogenetic trees of this study and those of Northup et al. (2003). Closest relatives for both included clone sequences from the South African gold mine study (Takai et al., 2001), which were obtained from pore water that passes over wad (manganese oxide) fill (T.C. Onstott, *pers. comm.*). What role archaeal species may play, if any, in production of manganese oxides is currently unknown.

Additional forms of poorly crystalline manganese oxides and hydroxides (pyrolusite, romanechite, todorokite, and rhodochrosite) have been described from caves (Onac, et al., 1997; Gradzinski et al., 1995; Northup et al., 2000). Irregularly shaped crusts of manganese flowstone (2–20 mm thick) are found in Jaskinia Czarna Cave (Tatra Mountains, Poland). Filaments and globular bodies are interpreted as bacterial or fungal cells that participated in the formation of the flowstones, as evidenced by their three-dimensional morphology and the amorphous character that is more common in biogenic manganese oxides. The high Mn/Fe ratio of 72.1:1 in the crusts was attributed by Gradzinski et al. (1995) to biologically mediated precipitation. A little studied type of ferromanganese oxide deposit is the black coatings of littoral Mediterranean submarine caves. Alloué and Harmelin (2001) concluded that black coatings in these caves were biosedimentary deposits that form from the interaction of slime, associated with microorganisms, and dissolved manganese from the seawater. The study includes some fascinating SEM micrographs of biofilm and microbial structures and makes the observation that the Mn/Fe ratio is negatively correlated with level of nutrients.

Kasama and Murakami (2001) attempted to ascertain the microbial contribution to iron precipitation on stalactites composed of ferrihydrite. Microscopy studies showed a variety of microbial morphologies associated with the

stalactites. Their experiments demonstrated that in comparison to inorganic processes, microorganisms enhanced precipitation rates by up to four orders of magnitude. The authors argued that exopolysaccharides and microbial surface characteristics were more important than metabolic processes in the precipitation of iron in this cave.

FUTURE PERSPECTIVES ON GEOMICROBIAL ACTIVITIES IN CAVE SYSTEMS

Since the emergence of geomicrobiology as a science, our understanding of microbial interactions with minerals has evolved beyond a preliminary appreciation of their role in carbon, sulfur and nitrogen cycling. It is now recognized that many important mineral transformations, originally considered to be inorganic in nature, can be mediated by microorganisms; from the microbial precipitation of dolomite in groundwater (Roberts et al., 2004; Warthmann et al., 2000); transformation of smectite to illite clay (Kim et al., 2004); to the production of iron, uranium and even gold deposits (Newman and Banfield, 2002). Likewise, through a more thorough understanding of geochemistry, we have expanded our knowledge of the range of habitable environments on Earth; from endolithic environments of extreme temperatures (Friedmann and Ocampo, 1976; Bell, 1993) to the deep subsurface, where hydrogen produced from volcanism, serpentinization and even radiolysis provides sufficient energy to support microbial growth (Chapelle et al., 2002; Coveney et al., 1987; Lin et al., 2005). While such work allows us a more comprehensive understanding of life on Earth, it also opens a window into the possibility of life under other geochemical conditions, such as on Mars or Europa. Due to the absence of liquid water on the surface of these planetary bodies, extant life will be restricted to the subsurface (Boston et al., 1992), making it critical to understand the processes that support microbial life in all subsurface environments.

While, as the title suggests, this review primarily addresses the interactions between microbes and minerals in cave environments, microbiology in cave environments also provides information on subterranean chemolithotrophic ecosystems (Barton et al., 2004; Chelius and Moore, 2004; Groth et al., 1999, 2001; Laiz et al., 1999, 2003; Schabereiter-Gurtner et al., 2002). Together these investigations suggest that without sunlight energy and through geologic isolation, caves are extremely starved environments where the levels of available organic carbon to support heterotrophic microbial growth are often a thousand-fold lower than starved terrestrial environments (Barton, unpublished, 2006). Examining microbial ecosystems surviving under such starved conditions suggests that they produce a myriad of energy conserving reactions; from obtaining energy from the minuscule organic material percolating into the system and fixing available nutrients from the atmosphere, to reducing the trace minerals within the rock of the cave itself (Barton et

al., 2004; Chelius and Moore, 2004; Northup et al., 2003; and Spilde et al., 2005). Recent studies have also suggested that the Archaea may play important roles in cave microbial ecosystems (Chelius and Moore, 2004; Gonzalez et al., 2006; Northup et al., 2003), although an identifiable metabolic role for these microorganisms has yet to be determined.

By understanding how microorganisms survive the extreme starvation of caves, we can understand and limit human impacts on such hypogean environments (Cigna, 1993). In doing so, such work can preserve cultural treasures, such as Paleolithic paintings in the caves of northern Spain, where tourist activity altered the cave environment and brought in heterotrophic microorganisms that threaten to damage these images (Cañaveras et al., 2001; Groth et al., 1999; Laiz et al., 2003). An understanding of such processes also facilitated the development of microbially precipitated calcite coatings, which can help to preserve historical monuments and sculptures (Hoppert et al., 2004; Rodriguez-Navarro et al., 2003). Presently it is hard to predict the similar outcomes from the increasing number of microorganisms being cultured from cave environments, although they range from such beneficial activities as bioremediation to drug discovery.

Even as we write this review, new techniques are being developed in materials science, chemistry, physics, geology and biology that will allow investigators to ask more complex questions of the interactions between microorganisms and mineral surfaces. For example, in materials science, attenuated total reflectance Fourier transformed infra-red (ATR-FTIR) spectroscopy allows real-time analysis of chemical changes on surfaces through microbial activity (Omoike et al., 2001), while atomic force microscopy allows us to examine the intra-molecular forces that allow microorganisms to acquire energy from mineral surfaces (Lower et al., 2001). Within microbiology, new techniques that allow us to probe inter-species and ecosystem interactions (Caldwell et al., 2000), and advances in genomics, metagenomics and proteomics, will allow us to ask questions of community interactions within caves that could previously only be addressed under *in vitro* conditions. All the while, caves continue to be discovered, presenting new environments to be examined. As a result, our understanding of microbial activity in such subterranean systems can only continue to grow, as present questions are addressed and new questions are posed. As we look back over the advances in cave geomicrobiology since the Breakthroughs conference, we can predict that the science of cave geomicrobiology will continue to grow in both prominence and regard within the greater scientific community.

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