

## What's Up Down There? Microbial Diversity in Caves

Microorganisms in caves survive under nutrient-poor conditions and are metabolically versatile and unexpectedly diverse

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### Summary

\*Caves provide relatively accessible sites in which individual species and microbial communities grow to levels approaching  $10^6$  cells/gram of rock under near-starvation conditions.

\*Cave-dwelling oligotrophic microbial species are phylogenetically diverse, with lineages across the breadth of the *Bacteria*.

\*Bacterial communities in caves acquire energy by several means, including by breaking down aromatic compounds, fixing gases, and oxidizing reduced metals within rocks.

\*By interacting with minerals, microbial species play an important role in reshaping the mineral environment of caves, and may help to form features such as stalactites and stalagmites.

In studying metabolic versatility, microbial ecologists often focus on those microbes that grow in extremes of temperature, osmolarity, pressure, and pH; however, these physical settings often contain microbes within high-energy systems such as sulfidic hot springs or photosynthetically driven biofilm communities. The vast majority of microbial species are not situated within such energetically favorable settings. Rather, they eke out existences under extremes of near-starvation or oligotrophy, defined as having less than 2 mg of total organic carbon (TOC) per liter. With diverse oligotrophic communities found in oceans, sediments, and within the subsurface, researchers are attempting to understand how various microbial species manage to live under such nutrient-poor conditions. Amid this heightened interest, access to such oligotrophic environments remains challenging, and doing research at such sites often requires very costly equipment, including ocean-going vessels or subsurface drilling equipment.

In the past decade, cave microbiology has emerged as a growing interdisciplinary field involving the efforts of microbiologists, geologists, and chemists to address challenging questions regarding microbial metabolism and biogeochemistry. The research also is helping land managers to recognize the importance of microbial species in cave ecosystems, further protecting cave environments. Complementing these formal efforts, many amateur cave explorers continue to play a valuable role by helping to move

research equipment into environments that are dangerous not only because they are dark but also because of their many other hazards, including loose and unstable rocks, slippery surfaces, and deep pits.

### **Subterranean Oligotrophy**

Unlike other nutrient-deprived environments where one might study oligotrophic survival, caves are readily accessible. Although appropriate training is a vital prerequisite, conducting microbial research in caves typically requires no more complicated equipment than a helmet, three sources of light, and caving ropes. Caves, which are devoid of sunlight and geologically isolated from surface-energy inputs, have only limited amounts of energy entering them. For instance, nutrient resources in caves rarely reach as high as 0.5 mg of TOC per l. Many caves are ancient, having remained static without diurnal or seasonal variations since they were formed millions of years ago. Despite the extreme oligotrophy of caves, however, they are by no means barren or lifeless. We routinely find microorganisms in amounts approaching  $10^6$  cells/gram of rock material. Such extremes of oligotrophy and their relative accessibility make caves an ideal terrestrial environment in which to study microbial adaptation to starvation.

Caves form in numerous geologic settings, including amid lava, glacial ice, volcanic tufa, mud, marble, and even boulder piles (talus). More often, caves form through the dissolution of limestone ( $\text{CaCO}_3$ ) rock by weakly acidic rainwater, forming in the subsurface and, thus, sometimes remaining difficult to identify. Fortunately, dedicated cave explorers (or cavers—the term “spelunker” has negative connotations) spend their free time locating caves through various geologic clues.

Within the United States, for example, there are more than 50,000 identified cave systems, with 7,000 in Tennessee alone. The United States also has five caves exceeding 100 miles in length, including the largest known cave, the 360-mile-long Mammoth Cave in Kentucky. Once identified, cavers record the extent of cave systems with detailed maps and attempt to protect them from vandalism, pollution, and mining. Because of these efforts, many cave systems are conserved in near-pristine conditions, making them all the more valuable when cavers bring them to the attention of geologists, biologists and microbiologists.

Until the early 1990s, little was published about cave-dwelling microorganisms, with investigators believing that most microbial species in caves were transported there by air currents, human activity, or insects. Earlier studies also tended to rely on cultivation techniques to identify the metabolically active species in such environments. However, culturing techniques have inherent limitations and are particularly problematic with respect to oligotrophs.

The evidence for extant microbial processes in caves became more compelling with the discovery of other mineral deposits that were difficult to explain through purely geologic or inorganic processes, such as U-loops and pool fingers; these calcified structures more

closely resemble microbial biofilms than inorganic mineral deposits (Fig. 1). Subsequent research suggested that pool fingers are indeed biogenic in origin, arising from important geomicrobial interactions that underpin energy conservation and life in caves under near-starvation conditions.



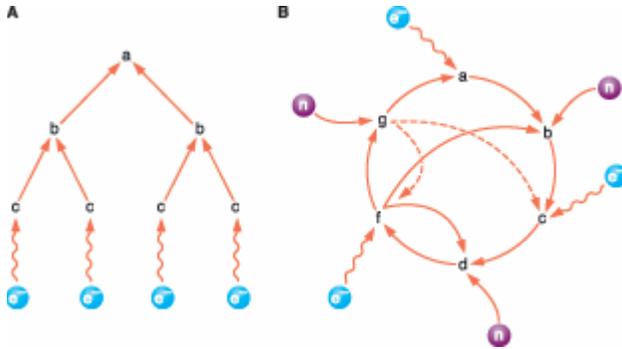
**Figure 1. Pool fingers in a paleo-pool within Carlsbad Caverns, Carlsbad Cavern National Park, New Mexico. These subaqueous, finger-like structures were difficult to explain by inorganic processes and proved to be microbial in origin. Photograph courtesy of Kenneth Ingham, 2006.**

### **Microbial Communities Adapt to Near-Starvation Conditions**

Our research is geared toward understanding the metabolic processes that allow both microbial communities and individual microbial species to survive under energetically unfavorable and nutrient-limited conditions. Our first studies entailed examining a site deep within a cave with no obvious signs of energy input from dripping or seeping water. Although we expected to find only oligotrophic species with limited diversity, to our surprise, our initial phylogenetic screening with 16S rRNA gene sequences identified a diverse community with lineages across the breadth of the Bacteria. By comparing the closest cultivated representative with phylotypes from the cave, we learned that the bacterial community acquires energy by several means, including by breaking down surface-derived complex aromatic compounds; fixing volatile organics, carbon dioxide, and nitrogen from the atmosphere; and also by oxidizing reduced metals within the cave rocks. Thus, rather than being lifeless environments, caves supply a blend of barely perceptible energy sources to maintain a diverse ecosystem at a subsistence level.

After repeating these phylogenetic studies in other caves, the same profile emerged, with species numbers estimated in the many hundreds. Finding such broad diversity under near-starvation conditions is a paradox under the general rules of ecology and

competitive exclusion, which holds that two species will not coexist when they both need a limited resource. However, high diversity under extreme starvation conditions was observed by G. Evelyn Hutchinson of Yale University in the 1960s, who described it as the “paradox of the plankton.”



**Figure 2. Hypothetical models for comparative energy and nutrient flow in high and low energetic microbial systems: A) This pyramid represents the classical view of energy flow; energy (e-) flows into the community and is fixed by primary producers (c). These primary producers are in turn consumed by species in higher trophic levels (b), until the top consumer is reached (a). B) Our proposed microbial community energetics in cave systems. Some species (a, c and f) are better adapted for energy (e-) acquisition from the available sources, with the release of secondary metabolic products to support other species (b, d and g). Mutualism is driven by the ability of these species to fix other essential nutrients (n) from the environment, which are recycled to the primary energy fixing species.**

We suspect that, because limited but chemically complex nutrients enter the cave system, very few microbial species are capable of encoding all the necessary uptake and catabolic reactions to support growth. To overcome this limitation, selfish competition for resources is replaced by cooperative and mutualistic associations, such as have been seen in biofilm communities (Fig. 2).

Such mutualism may also be explained by a phenomenon observed in chemostat cultures when nutrient sources become limiting, slowing catabolism and allowing more complete oxidation of substrates. As limited nutrient concentrations persist, respiration rates drop, while metabolic products such as acetate accumulate. These partially oxidized products create a niche that could support additional microbial species, increasing the likelihood of further cooperative resource-sharing.

Such niche dynamics are possible only at the microscopic level, explaining why ecologists who focused on macroscopic systems failed to recognize this potential driver of diversity. Indeed, such metabolic activity could help to resolve the paradox of the plankton. To address this hypothesis, we are further characterizing the energetics of cave systems and the metabolic properties of the microbial species making a living within them.

## Cave System Energetics

Because caves are geologically stable structures, it is possible to identify, measure, and model how nutrients and other energy sources enter them. Based on our phylogenetic profiles of cave-dwelling microorganisms, we believe that there are three major routes for energy to enter these subterranean systems and support microbial growth. Energy sources and nutrients can enter (i) as atmospheric gases such as nitrogen, CO<sub>2</sub>, and organic molecules, including methyl halides and aromatic hydrocarbons; (ii) as soil-derived aromatic and polyaromatic compounds percolating into the system with surface water; and (iii) as reduced metal ions, such as Mn(II) and Fe(II) within the rock itself.

Other microbial species that we find in caves can mobilize inorganic phosphate, oxidize methane and hydrogen, and derive energy by hydrolyzing proteins, lipids, and other macromolecules that are released by other members within the microbial community, allowing recycling of those macromolecules. Having identified the major sources of nutrients and energy within caves, we began to explore their roles in supporting microbial communities.

Caves breathe: as high and low pressure weather systems move over a cave's entrance, air enters or exits the cave to equilibrate. In larger cave systems, the volume of air exchange is so large that air speeds as high as 80 miles per hour have been measured. Therefore, except under rare exceptions, there is no lack of atmospheric gases within caves. Nitrogen is a limiting nutrient in caves and, despite high-energy requirements for nitrogen fixation, we identify a large portion of nitrogen fixing and nitrifying species within our cave microbial population profiles. To determine whether atmospheric organic molecules are present for microbes to use, we developed a portable (under the limitations of cave travel) atmospheric condenser for analyzing the cave atmosphere using gas chromatography/mass spectroscopy (GC/MS). Such analyses indicate that cave condensates contain various small aromatic compounds that microbes likely can use as carbon and energy sources. We plan to measure microbial uptake of these compounds using radiolabeled tracer molecules.

Caves also contain rainwater that filters through the soil, picking up dissolved organic carbon before percolating through the rock and into the cave. The TOC of this water entering the cave can range from 0.500–0.020 mg/l and contains many small aromatic and polyaromatic carbon molecules. By comparing these molecules with extracts from the soil above a cave, it is possible to confirm soil-derived sources of these organics.

Geologists have mapped the flow of percolating groundwater into many caves, greatly aiding us in understanding this energy movement. For example, in Carlsbad Cavern in New Mexico, a limited number of cracks in the bedrock do not provide an easy flow path for water entering this cave, forcing water to percolate through the pore-space between the minerals of the rock itself. Such percolation slows the entry of organics into the system, with some nutrients likely taking more than 50 years to travel 300 m from the surface into the cave.

Meanwhile, other investigators study how reduced metal ions in the bedrock of cave systems provide energy for microbial growth. As microbial species access these energy sources, the structure of the host rock breaks down while metal oxides accumulate, producing a powdery surface that can extend many centimeters into the rock. This rock powder sometimes contains significant numbers of both iron- and manganese- oxidizing species, including large numbers of archaea. We see a direct correlation between the amount of energy entering a system and the preferential use of such energy sources by microbial species. By combining petrology, X-ray diffraction, and scanning electron microscopy with energy dispersive spectroscopy, we see that as sample sites become more oligotrophic, there is an increase in the mineral transformation of the host rock, presumably as microorganisms both mobilize and use mineral energy sources.

### **Metabolic Activities of Culturable Cave-Dwelling Microbial Species**

To verify our phylogenetic profiles, we are isolating, culturing, and further analyzing species from cave environments. Our initial efforts were cumbersome, using the same techniques that work for isolating species from nutrient- rich and soil environments. However, by adjusting nutrient media to mirror chemical conditions in the caves, we could cultivate a higher diversity of bacterial species.

Our phylogenetic profiles suggested that the caves contain a broad diversity among members of the *Alphaproteobacteria*, *Betaproteobacteria*, *Gammaproteobacteria*, *Firmicutes*, and *Actinobacteria*. As we refined our growth media, we were reassured to see a similar distribution in our isolates.

The diversity of microbes within our collection from caves is impressive. For instance, we grew more than 400 cultivars from Lechuguilla Cave, N.M., of which (to date) 83 have been identified and represented 27 different genera. Of these 83, 40 appear to represent previously uncultivated species (based on 16S rDNA gene sequence fragments displaying 89–97% identity to known species), and we are working toward identifying the metabolic properties of many of these novel species. Early indications suggest phenotypes include an ability to fix nitrogen, metabolize complex aromatic compounds, turn over lipids, and scavenge scarce nutrients.

If the microbial species in caves must cooperate to extract available poor nutrient and energy sources, then examining single colonies is counterproductive to gaining an accurate understanding of how such communities function. Our reliance on this approach may explain how, despite the broad distribution of cultivars we have isolated, there is rarely a match between the species observed through cultivation and those identified by molecular phylogenetic techniques.

To overcome this limitation, we are isolating and culturing microbial consortia from such environments, adding nutrients and energy sources that we find within the caves. In one such consortium, we identified a group of bacterial species in distilled water that could not be grown individually. Gas chromatography and mass spectrometry (GC/MS)

analysis indicated that plasticizers leaching from Petri plates were supporting the growth of these species. Those plasticizers resemble the aromatic hydrocarbons that are carried into these caves from water that passes through soil.

### Cave Geomicrobiology

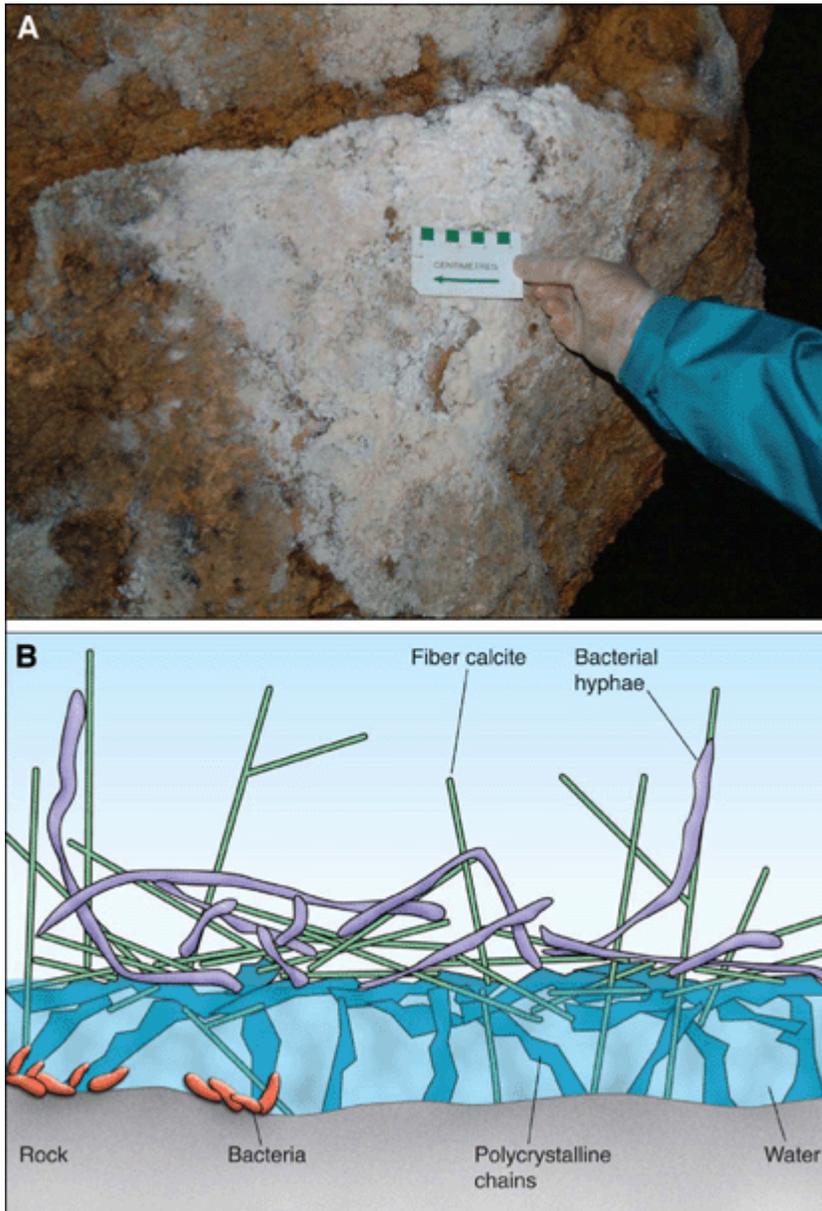


**Figure 3. A caver uses minimum-impact techniques to examine a diverse array of calcium carbonate speleothems, including stalactites, stalagmites, soda straws, and shelfstone, within Lake Castrovalva, Lechuguilla Cave, Carlsbad Caverns National Park, New Mexico. Photograph copyright David Bunnell, 2006.**

Microbial species adapt to caves by interacting with minerals there. Some of these processes reshape the mineral structure of the cave walls, floors, and ceilings—for instance, by forming speleothems such as stalactites and stalagmites. As water percolates through the soil above a cave, it becomes saturated with  $\text{CO}_2$ , creating a weak carbonic acid that reacts with the limestone rock, dissolving  $\text{CaCO}_3$ . When this water enters the atmosphere of the cave, the  $\text{CO}_2$  off-gasses and dissolved  $\text{CaCO}_3$  precipitates to produce beautiful formations (Fig. 3).

Many carbonate minerals within caves are associated with heterotrophic bacteria, including metastable carbonates, such as vaterite ( $\mu\text{-CaCO}_3$ ). Microbial species isolated from these minerals can produce similar crystals from organic calcium salts in the lab. Further, some microbial species help to form  $\text{CaCO}_3$  secondary products by boundary-organized mineralization on the surface of cells, reducing the local saturation index for precipitation. While microbial species must be metabolically active to induce  $\text{CaCO}_3$  precipitation, the role of calcium ions in this process remains controversial. Nonetheless, Sanchez-Moral and co-investigators at the Instituto de Recursos Naturales y Agrobiología, Spain, demonstrated that heterotrophic species consuming small quantities of  $\text{CO}_2$  can trigger these processes.

When microbial species induce precipitation of  $\text{CaCO}_3$ , they become trapped within that deposited mineral matrix. Although this entombment makes it possible to identify the deposited microbial cells, it does not explain how this adaptation provides any selective advantage to these species. However, carbonate plays an important role in buffering the metabolic activities of many of these species, which in culture can rapidly dissolve nearby carbonate materials, even as  $\text{CaCO}_3$  is being precipitated.



**Figure 4. (Top) A carbonate speleothem, known as moonmilk, with a cream cheese-like consistency within Altamira Cave, Spain. (Bottom) Schematic of moonmilk formation, which occurs through a combination of microbial dissolution and re-precipitation of the host rock calcium carbonate.**

The speleothem, called moonmilk (Fig. 4), forms through a series of phases, based on the topology and distribution of fiber crystals, extracellular polymeric substances (EPS), and microorganisms, according to Juan Carlos Cañaveras, Cesareo Saiz-Jimenez, and their

collaborators also at the Instituto de Recursos Naturales y Agrobiología. The process begins with microbial colonization and growth, generating a network of branching filamentous bacteria. Monocrystalline rods are then generated by calcification of microbial hyphae, which develop into a network of hyphae, fiber crystals, and EPS. In the next phase, the fibers grow until they collapse under their own weight, after which new microbial growth produces more fibers, forming a crust that isolates a water-rich, microenvironment for still further microbial and epitaxial growth (Fig. 4).

We have observed other metabolic structural interactions between microbial species and their mineral environments. For instance, we identified an unusual biofilm community floating within a 50°C sulfidic hot spring pool in a hot spring cave in Colorado. These biofilms, which form over a biogenically precipitated  $\text{CaSO}_3$  (gypsum) crystal, have the consistency of dumplings. The buoyant density of this highly soluble mineral allows these microbial mats to float at the water-air interface, presumably to allow sulfide- and hydrogen-oxidation for energy metabolism, while the microbial biofilm and EPS prevent the water from dissolving the mineral.

### **Applications within Archaeology**



**Figure 5. Ancient Paleolithic paintings within Altamira Cave, Spain, are at risk from the impact of microbial growth and metabolic activity, which could destroy the pigment. (Photo courtesy of Dr. Tesareo Saiz-Jimenez.)**

Without sunlight and with reduced organic carbon, caves provide places for preserving a wide variety of archaeological materials—from human bones to the Dead Sea scrolls. Nonetheless, microbial species are affecting the integrity of the 15,000-year-old Paleolithic paintings that are found along the walls of Altamira Cave in Spain (Fig. 5). Analysis of those microbial species indicates that humans altered their community structure and activities, enabling them to colonize the pigments of the wall paintings.

Artificial lighting, body heat, and increases in CO<sub>2</sub> and moisture from breathing, increase these detrimental effects.

Such findings led officials to close of Altamira Cave and to construct a museum that includes a replica of the paintings. Based on archaeological and geological information, the replica depicts the cave as it was 15,000 years ago, allowing tourists to appreciate what the cave contains while avoiding further intrusions that can prove so detrimental to these ancient works of art. Such findings are helping others managers to protect similar ancient works of art, such as 1,200- year-old Mayan hieroglyphs in Naj Tunich Cave in Guatemala. Here, the local community transformed a nearby cave into a replica of Naj Tunich before many tourists visited the area, thus protecting the ancient artworks from detrimental changes that follow upsets to the delicate microbial balance within such cave-based systems.

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