MICROBIAL SPELEOLOGY: OPPORTUNITIES AND CHALLENGES

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Abstract

In caves, microorganisms (algae, bacteria, archaea, fungi, protoza, and viruses) are major producers and consumers of organic matter and contribute to the formation of several types of minerals. However, with the notable exception of sulfide-based ecosystems, little is known about community composition, their specific adaptations to the subterranean ecosystem, their biogeographical distribution or their ecology. Interdisciplinary studies, using recently developed techniques, are now providing the tools with which to make great strides in elucidating aspects of subterranean microbial ecology that go beyond the traditional "who's home" studies. As we come to realize the value of microorganisms in cave ecosystems, we are also realizing the impact that humans can have on these microbial communities. Advances in our understanding of the functioning of microorganisms in caves and of the means to protect and preserve them are critical to the health and beauty of caves and their ecosystems.

Introduction

Much remains to be learned about microbial communities in caves compared to what is known about vertebrate and invertebrate communities that inhabit caves. Several intriguing and fascinating areas of research concerning the nature of microorganisms that exist in caves include:

1. Are there indigenous species of microorganisms in caves that would exist in caves whether humans were ever present or not? Are there similarities among these indigenous microbial species from caves across the planet?
2. Much of the research on microorganisms in caves has been conducted using traditional culturing techniques. Research from other fields of microbiology, using molecular biology techniques, has shown that we are able to culture only a small fraction of what's out there in the environment. These techniques are now being applied to microbial communities in caves to greatly expand the ability of biotic surveys to detect the rich microbial life present in caves.
3. Recent research has revealed the presence of unique communities of microorganisms in lava tubes, iron and manganese deposits in caves, sulfur-dominated caves, and low-nutrient environments of caves. Further research into these

intriguing habitats promises to help fill in the branches of the tree of life.
4. Within these unusual microbial habitats in caves we're learning that microorganisms interact with the mineral surfaces, particularly in iron–manganese, sulfur, and moonmilk environments.
5. If life exists on other planets in our solar system and beyond, it will likely be found in the subsurface of these extraterrestrial environments because of harsh surface conditions. Caves serve as an excellent analog for extraterrestrial subsurface environments.
6. For primary school children, caves serve as a wonderful vehicle for learning earth and life sciences. New efforts are turning the results from scientific research into creative activities and content to engage students in learning science.

Associated with these great research opportunities into microbial communities in caves, are significant challenges in carrying out effective research. These include:

1. Most microbial research studies in caves center around the question of "Who's Home?" We must move beyond this question to questions centering on the roles that microorganisms play in the cave ecosystem and the interactions among microorganisms.
2. Culture-based biotic surveys remain important, but we must incorporate more culture-independent studies, making effective use of molecular methods. These must be integrated into culture-dependent studies that allow us to study the physiology of the newly discovered species that molecular methods reveal.
3. Discovering and creating new ways to fund this research are critical. These kinds of studies are expensive to carry out.
4. The number of microbial biospeleologists in the United States is extremely small compared to the work that needs to be done. Training the new generation of microbial speleologists is important.
5. To conduct microbial speleology studies effectively, we need to develop best practices, drawing upon mainstream microbiology and molecular biology, tempered by the constraints of working effectively with cave microorganisms.

Opportunities: Exploring the Existence of an Indigenous Microbial Community in Caves

Early studies of microorganisms in caves relied entirely on culture-based studies and tended to reveal microbial species that were closely related to organisms already known from surface studies. This is not surprising given that we haven't learned how to grow most microorganisms from most environments. With the advent of molecular biology techniques pioneered by Norm Pace, we can now study microorganisms through their genetic sequences and a huge amount of diversity is now being revealed from the microbial world. Studies of sulfur-dominated caves are revealing the presence of a diverse community of *Epsilonproteobacteria* (Engel *et al.* 2003), almost all of which is novel (that is the species of bacteria are new to science). Comparison of genetic sequences from studies by Engel and others (Engel *et al.* 2003, 2004a, Lower Kane Cave in Wyoming; Engel *et al.* 2001, Cesspool Cave in Virginia; Vlasceanu *et al.* 2000, Frasasi caves in Italy; and Moville Cave in Romania) to those of Northup *et al.* (2004; unpublished data, Cueva de las Sardinas in Tabasco, Mexico) reveal an amazingly close similarity among genetic sequences, hinting that at least among the *Epsilonproteobacteria*, an indigenous community may exist in sulfur springs and caves. These comparisons, done by Annette Summers Engel and Megan Porter begin to address the issue of whether there is an indigenous community in caves.

Preliminary studies by Northup *et al.* (unpublished data) of genetic sequences from actinomycete communities on walls of Four Windows Cave, a lava tube in El Malpais National Monument, New Mexico, demonstrate groupings of genetic sequences with Mammoth Cave bacterial genetic sequences. Among the sequences studied, one of the Four Windows sequences groups with a *Chloroflexi* sequence from Mammoth Cave and another groups with a *Betaproteobacteria* sequence, also from Mammoth Cave. In both these instances, there are no other close relatives, suggesting that these are novel organisms, most closely related to each other.

But these studies are like a jigsaw puzzle in which you've put in the first pieces in a final picture for which you have no image to guide you. We know so little about cave microbial communities that it is too early to know whether a true indigenous community of microorganisms exists in caves — just tantalizing hints!

Opportunities: Using Molecular Techniques to Study Microbial Communities and Discover Novel Organisms

As will be discussed below in culture-independent versus culture-dependent challenges section, there are strong advantages to using culture-independent, molecular techniques to study cave microbial communities. One of the very first studies of cave microorganisms to use molecular methods is that of Vlasceanu and colleagues (1997) who studied the microbial mat organisms in Movile Cave. Using these techniques, Summers-Engel and Porter (for example, Engel *et al.* 2003), Barton (for example, Barton, Taylor, and Pace 2004), Northup (for example, Northup *et al.* 2003; Spilde *et al.* 2005), and others have begun to study low nutrient and mineral-rich environments, revealing the diverse communities of microorganisms associated with caves. Chelius and Moore (2004) and Northup *et al.* (2003) discovered rich communities of mesophilic Archaea in Wind Cave and Lechuguilla Cave respectively. Up until mid-2005, no one had succeeded in growing any of the mesophilic Archaea, which were discovered for the first time in 1992 (DeLong 1992). To discover many archaeal genetic sequences in caves was a revelation. However, it's a revelation that will become commonplace as we increase our use of molecular biology techniques to study cave microbial communities. One thing that strikes you when you look at family trees of bacterial genetic sequences (that is phylogenetic trees) from caves and their nearest relatives is that many of the genetic sequences from caves have no really close relatives, especially among the known cultured bacteria. Many of the organisms whose sequences group with cave sequences are uncultured and represent novel biodiversity. Thus, culture-independent techniques provide us with the opportunity to discover many new microorganisms in caves.

Opportunities: Studying Microbe-Mineral Interactions in Caves

The international Breakthroughs in Karst Geomicrobiology and Redox Geochemistry meeting (Sasowsky and Palmer 1994) brought together scientists who study caves, microorganisms in caves, and interactions between microorganisms and rock substrates. This landmark conference heralded the beginning of a wealth of studies using cave ecosystems to study microbe-rock interactions. Northup and Lavoie (2001) reviewed these studies and described how microbes play both active and passive roles in the formation and weathering of the interior lithology of caves. The true significance and the exciting developments, however, lie in the combining of forces by geologists and biologists to effectively study how microbes influence geology and vice versa. For several decades we have suspected and begun to document that microorganisms play a role in dissolution and precipitation reactions in speleothems, especially those of a sulfur, manganese, iron, nitrogen, or carbonate nature.

Several studies highlight the involvement of microorganisms in oxidizing sulfides to sulfuric acid, which has been shown to be a powerful force in speleogenesis and cave enlargement (Barton and Luiszer 2005; Engel *et al.* 2004b; Hose *et al.* 2000). Several potential new species of sulfide-oxidizing bacteria in the *Epsilonproteobacteria* and the *Gammaproteobacteria* have been discovered in caves with strong inputs of hydrogen sulfide. The biodiversity associated with these environments is revealing many new species as detailed above and is likely to shed light on similar sulfur-dominated reactions in other environments.

Another forefront of activity centers around studies of iron and manganese-oxidizing bacteria and their ability to dissolve carbonate rocks in caves (Northup *et al.* 2003; Spilde *et al.* 2005). Extensive deposits of ferromanganese deposits in Spider and Lechuguilla Caves appear to form an underground soil on cave walls and ceilings, hence the name speleosols. Bacterial species present in these deposits can be cultured on site and we have demonstrated that these cultures can produce similar mineral morphologies in the laboratory. These reactions produce acidity, which can contribute to carbonate dissolution and the formation of the underlying punk rock. A wealth of novel biodiversity is being discovered in these deposits also.

These examples are just some of the fascinating new studies that explore the interactions of microorganisms and cave minerals. Many additional studies can be found in the special issue on "Cave Geomicrobiology" in the August 2001 issue of *Geomicrobiology Journal* and in subsequent issues of this journal and in *Journal of Cave and Karst Studies.*

Opportunities: Caves as Laboratories for Developing Life Detection Strategies for the Search for Extraterrestrial Life

Lava tubes and other caves are an important analogue for habitable environments on Mars. During the earliest history of Mars, a time during which biological processes may have been initiated, similar life could have been sustained in the vadose zone environments offered by short-term habitable zones. The unique environmental niche represented by life found underground in the vadose zone on the Earth, as represented by microbial life found around the world on the walls of caves, especially lava tubes, represents a superlative opportunity for studying easily accessible subsurface microbial communities and associated materials.

Are there biosignatures left by these extant and extinct microorganisms that can be used to detect life on Mars and elsewhere? Boston *et al.* (1992) and McKay *et al.* (1994) have suggested the possibility of life in the subsurface of Mars. Lava tubes provide an excellent analog for the study of life on Mars, not only because there are known lava tubes on Mars (Boston 2003), but because the tubes provide access to the subsurface, where cracks, fractures, and voids of all sizes may exist and may provide hospitable and protected conditions for microorganisms. Investigation of these environments on Earth is therefore important for creating the tools and techniques for detecting life on Mars and other extraterrestrial environments. Lava tubes contain frequent occurrences of biofilms called "lava wall slime" that represent an untapped resource for detecting and characterizing life in the subsurface (for example Northup *et al.* 2004). The existence of subsurface caves or voids that could provide similar geological environments on Mars is likely, based on the evidence for young lava flows (Boston 2003; Boston *et al.* 2003, 2004). The evidence for transient or sustained sources of water throughout geological history for such environments on Mars has become dramatically more likely with several recent discoveries on Mars.

The work of Boston *et al.* (2001) is establishing a suite of biosignatures from cave studies that will help guide life detection on Mars and other extraterrestrial bodies.

Opportunities: Using Studies of Microbes in Caves to Captivate Young Learners

To primary school students and young adults, caves are particularly intriguing and fun. We are using the results of our scientific studies in caves to create Web-based content available to students through formal education avenues and informally through home access to the Internet. We began by creating a Web site for our team, the **S**ubsurface **L**ife **I**n **M**ineral **E**nvironments (**SLIME**) Team (www.caveslime.org). This Web site provides information about studies being conducted by the SLIME Team and findings of interest (we hope) to others. One of the ways we are expanding the site involves a collaboration with Janet Shagum, a microbiologist and instructor for the science writing course in the English Department at the University of New Mexico. Her students write new material for the Web site after interviewing project scientists. For example, in the spring of 2005 one of the students wrote a story of Penny Boston's experiences in the Mars Simulation in Utah. The students write creative pieces that provide good, popular science looks at the research going on in caves and associated habitats.

Another venture as been the collaboration with the EPSCOR program to create a Virtual Center for the Environment (http://vce.inram.org/), which included a Cave Journey (www.caveslime.org/cavejourney). The Cave Journey includes content written by Northup and Tamara Montoya, a professional writing staff member of EPSCOR and features information about the earth and life sciences of New Mexico caves. The content is keyed to New Mexico Science Benchmarks and Standards and includes activities for teachers or parents, which were written by New Mexico high school teachers Patsy Jones and Ray Bowers. For students, there are also species accounts, a photo gallery of cave biota and speleothems for use in presentations, and a glossary of terms used in the Web site content. Initial response of New Mexico high school teachers attending a workshop on the Cave Journey was enthusiastic. The results coming out of cave research represent an exciting way to interest young adults in learning about caves and science through caves. An important way to create a desire to protect and conserve caves is to demonstrate their intrinsic fascinating nature.

Challenges: Moving Beyond Who's Home Studies

Learning "who's home" using advanced molecular biological techniques and targeted enrichment cultures is an essential first step in studying the microbiology of caves. As discussed elsewhere in this paper, there are a plethora of studies of geomicrobiological interactions in caves being conducted, which are filling in this portion of the picture of how microorganisms function in the ecosystem. However, it's also time to beef up studies of the role of microorganisms in non-geomicrobiological ecosystem functioning. Key areas in need of further investigation include microbial transformations associated with water and caves and the interactions between geochemistry and microorganisms; the role of microorganisms in cycling carbon, nitrogen, and phosphorus in the subsurface environment of caves; microbial interactions within communities (competitive versus mutualistic interactions); the nature of microbial food webs; and important applied studies of how various anthropogenic impacts on karst systems affect microbial communities in the subsurface. The exciting part is how many interesting studies remain to be done; the challenging part is how many interesting studies remain to be done.

Challenges: Culture-independent versus culture-dependent studies

Scientists have discovered that we are able to grow in culture less than one percent of the organisms that are in the environment using standard culturing techniques (Amann *et al.* 1995). Several cave microbiologists have done significantly better than this by adapting their media recipes to the cave environment in which microorganisms live (Rusterholtz and Mallory 1994; Boston *et al.* 2001; Spilde *et al.* 2005). This represents what Boston calls the "Keeping the Zoo" part of cave microbiology. Molecular phylogenetic techniques have allowed us to significantly expand the groups of organisms found in caves as discussed elsewhere in this paper and have been a welcome addition to microbiologists' bag of tricks. By extracting DNA from the environment, amplifying the DNA to yield millions of copies of particular genes, cloning and sequencing, one can obtain a much less biased view of what microorganisms are present in a particular cave environment. There are relatively new community fingerprinting methods that allow us to compare communities and their biodiversity, another extremely valuable tool. These techniques are, however, more costly by orders of magnitude than are traditional enrichment culturing techniques.

We have developed an interleaved strategy that begins with initial molecular biological characterization to characterize genetic sequences of microorganisms present. These results then guide culturing efforts and allow us to learn more about the physiology and biochemistry of the microorganisms present. These cultures are then fingerprinted using the community molecular techniques to determine which enrichment cultures are worth characterizing with molecular techniques. Microcosm studies in which we mimic conditions present in the cave environment from which the microorganisms came further help us study the roles that these microorganisms are playing. You really need both culturing and molecular techniques, with geological techniques thrown in where needed, to answer many basic questions

Challenges: Funding

One of the biggest challenges is cave microbiology work, as in other fields, is funding these studies. Molecular biology, geochemistry, and imaging techniques are expensive. On the positive side is the successful funding of several cave microbiology proposals by the National Science Foundation in the last decade. However, funding is becoming much tighter and we must become innovative in exploring new funding sources and selling the importance of karst studies. Karst scientists must become experts in promoting the public understanding of the value of karst. Also, it is our hope that the

National Cave and Karst Research Institute will provide a strong lead in identifying and helping to create new funding sources.

Challenges: Need for New Microbial Speleologists

As established karst scientists gray, it's important to replenish and expand the work force to study these fascinating microbial systems. Things have improved on this front and there are now strong cave and karst academic programs at Western Kentucky University, University of South Florida, and New Mexico Tech. New researchers are now on faculty at other universities and are working to establish cave and karst programs. To retain students graduating from these programs in the field, jobs and opportunities to work and publish must be available, which will require efforts by established karst scientists to serve as grant reviewers, spokespeople for karst, and associate editors of karst and non-karst journals. As mentioned at the recent NCKMS symposium in Albany, we need to become leaders with the responsibility and authority to be able to promote karst and cave sciences.

Challenges: Need for Best Practices

Rusterholtz and Mallory (1994) pioneered the idea that microbiological studies in caves needed to go beyond the traditional methods. They established that inoculating and incubating microbial cultures on site in caves is critical to being able to grow the more indigenous species of microorganisms. The removal of samples from caves for inoculating in the laboratory almost always guarantees that you'll be growing the weeds and the organisms that were likely transported into the cave by humans. Studies by Boston *et al.* (2001; Spilde *et al.* 2005) have shown the value of making low-nutrient media using water from the cave and rock dust from similar parent material as cave walls. When samples are removed for DNA extraction, some researchers believe that it's important to keep the samples on dry ice or in sucrose lysis buffer. Some of these strategies have been tested in the laboratory while others are based on experience and intuition of researchers. We need rigorous testing of various methods with subsequent publication of results. The National Cave Karst Research Institute will be taking the lead on sponsoring best practice workshops to bring experts together to hash out these proposed best strategies.

Summary

Microbial speleology provides a range of interesting and productive opportunities for expanding karst and cave sciences. We are discovering that many new microbial species can be identified from caves; evidence from Mallory and others (unpublished data) has revealed that many of these species produce useful chemical compounds of interest to pharmaceutical scientists. Molecular techniques can be applied with great success to cave microbial studies, greatly expanding our ability to accurately characterize microorganisms present in caves. These techniques and others are being used to expand our knowledge of how these newly identified microbial species interact with mineral surfaces, helping to precipitate and dissolve rocks in caves. Life detection on other planets is being aided by studies of cave microbial communities, which identify biosignatures for extant and extinct life in the subsurface. All of these studies provide rich fodder for education initiatives that use caves to teach earth and life sciences to children and the general public. Learning science through learning about caves is fun and exciting and you don't even have to get dirty if you use the Internet! All of these exciting opportunities also represent challenges as we work to expand funding and to recruit new scientists to the karst programs. We must expand existing studies into more aspects of how microorganisms function in the ecosystem and must determine the best practices for microbial work in the subsurface. We've got our work cut out for us, but we have an amazing array of opportunities in the field of microbial speleology.

References

Amann, R.I.; Ludwig, W.; and Schleifer, K.-H. 1995, Phylogenetic identification and in situ detection of individual microbial cells without cultivation. Microbiological Reviews v. 59, p. 143-169.

Barton, H.A. and Luiszer, F. 2005, Microbial metabolic structure in a sulfidic cave hot spring: Potential mechanisms of biospeleogenesis Journal of Cave and Karst Studies v. 67, p. 28-38.

Barton, H.A.; Taylor, M.R.; and Pace, N.R. 2004, Molecular phylogenetic analysis of a bacterial community in an oligotrophic cave environment. Geomicrobiology Journal, v. 21, p. 11-20.

Boston, P.J. 2003, Extraterrestrial caves, *In* Gunn J, ed., *Encyclopedia of Cave and Karst Science*, London, Fitzroy-Dearborn Publishers, Ltd., p. 355-358.

Boston, P.J.; Frederick, R.D.; Welch, S.M.; Werker, J.; Meyer, T.R.; Sprungman, B.; Hildreth-Werker, V.; and Thompson, S.L. 2004, Extraterrestrial subsurface technology test bed: Human use and scientific value of Martian caves, Space Technology & Applications Forum 2003 Proceedings AIP #654. American Institute of Physics, College Park, MD.

Boston, P.J.; Frederick, R.D.; Welch, S.M.; Werker, J.; Meyer, T.R.; Sprungman, B.; Hildreth-Werker, V.; Thompson, S.L.; and Murphy, D.L. 2003, Human utilization of subsurface extraterrestrial environments, Gravitational and Space Biology Bulletin v. 16, p. 121-131.

Boston, P.J., Ivanov, M.V., and McKay, C.P. 1992, On the possibility of chemosynthetic ecosystems in subsurface habitats on Mars, Icarus v. 95, p. 300-308.

Boston, P.J.; Spilde, M.N.; Northup, D.E.; Melim, L.A.; Soroka, D.S.; Kleina, L.G.; Lavoie, K.H.; Hose, L.D.; Mallory, L.M.; Dahm, C.N.; Crossey, L.J.; and Schelble, R.T. 2001, Cave biosignature suites, Microbes, minerals and Mars, Astrobiology Journal v. 1, p. 25-55.

Chelius, M.K. and Moore, J.C. 2004, Molecular phylogenetic analysis of archaea and bacteria in Wind Cave, South Dakota. Geomicrobiology Journal v. 21, p. 123-134.

DeLong, E.F. 1992, Archaea in coastal marine environments. Proceedings of the National Academy of Sciences U.S.A. v. 89, p. 5685-5689.

Engel, A.S.; Porter, M.L.; Kinkle, B.K.; and Kane, T.C. 2001, Ecological assessment and geological significance of microbial communities from Cesspool. Geomicrobiology Journal v. 18, p. 259-274.

Engel, A.S.; Lee, N.; Porter, M.L.; Stern, L.A.; Bennett, P.C.; and Wagner, M. 2003, Filamentous "Epsilonproteobacteria" dominate microbial mats from sulfidic cave springs. Applied and Environmental Microbiology v. 69, p. 5503-5511.

Engel, A.S.; Porter, M.L.; Stern, L.A.; Quinlan, S.; and Bennett, P.C. 2004a, Bacterial diversity and ecosystem function of filamentous microbial mats from aphotic (cave) sulfidic springs dominated by chemolithoautotrophic "Epsilonproteobacteria." FEMS Microbiology Ecology v.51, p. 31-53.

Engel, A.S.; Stern, L.A.; and Bennett, P.C. 2004b, Microbial contributions to cave formation: New insights into sulfuric acid speleogenesis. Geology, v. 32, p. 369-372.

Hose, L.D.; Palmer, A.N.; Palmer, M.V.; Northup, D.E.; Boston, P.J.; and DuChene, H.R. 2000, Chemical Geology, v. 169, p. 399-423.

McKay, C.P.; Ivanov, M.; and Boston, P.J. 1994, Considering the improbable: Life underground on Mars. Planetary Reports v. XIV, p. 13-15.

Northup, D.E.; Barns, S.M.; Yu, L.E.; Spilde, M.N.; Schelble, R.T.; Dano, K.E.; Crossey, L.J.; Connolly, C.A.; Boston, P.J.; Natvig, D.O.; and Dahm, C.N. 2003, Diverse microbial communities inhabiting ferromanganese deposits in Lechuguilla and Spider Caves. Environmental Microbiology, v. 5, p. 1071-1086.

Northup, D.E.; Connolly, C.A.; Trent, A.; Spilde, M.N.; Boston, P.J.; Peck, V.; and Natvig, D.O. 2004, Studies of bacterial communities from Four Windows Cave, El Malpais National Monument, New Mexico. Journal of Cave and Karst Studies, v. 66, p. 111.

Northup, D.E. and Lavoie, K.H. 2001, Geomicrobiology of caves: A review. Geomicrobiology Journal, v. 18, p. 199-222.

Rusterholtz, K.J. and Mallory, L.M. 1994, Density, activity and diversity of bacteria indigenous to a karstic aquifer. Microbial Ecology, v. 28, p. 79-99.

Sasowsky, I.D. and Palmer, M.V. 1994, Breakthroughs in karst geomicrobiology and redox chemistry: abstracts and field-trip guide for the symposium held February 16 through 19, 1994, Colorado Springs, Colorado. Special Publication 1, Karst Waters Institute, Inc., Charles Town, WV.

Spilde, M.N.; Northup, D.E.; Boston, P.J.; Schelble, R.T.; Dano, K.E.; Crossey, L.J.; and Dahm, C.N. 2005, Geomicrobiology of cave ferromanganese deposits: A field and laboratory investigation. Geomicrobiology Journal, v. 22, p. 99-116.

Vlasceanu, L.; Popa, R.; and Kinkle, B.K. 1997, Characterization of *Thiobacillus thioparus* LV43 and its distribution in a chemoautotrophically based groundwater ecosystem. Applied and Environmental Microbiology, v. 63, p. 3123-3127.

Vlasceanu, L.; Sarbu, S.M.; Engel, A.S.; and Kinkle, B.K. 2000, Acidic cave-wall biofilms located in the Frasassi Gorge, Italy. Geomicrobiology Journal v. 17, p. 125-139.