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# MICROBIAL METABOLIC STRUCTURE IN A SULFIDIC CAVE HOT SPRING: POTENTIAL MECHANISMS OF BIOSPELEOGENESIS

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*Glenwood Hot Springs, Colorado, is a sulfidic hot-spring that issues from numerous sites. These waters are partially responsible for speleogenesis of the nearby Fairy Cave system, through hypogenic sulfuric-acid dissolution. To examine whether there may have been microbial involvement in the dissolution of this cave system we examined the present-day microbial flora of a cave created by the hot spring. Using molecular phylogenetic analysis of the 16S small subunit ribosomal RNA gene and scanning electron microscopy, we examined the microbial community structure within the spring. The microbial community displayed a high level of microbial diversity, with 25 unique phylotypes representing nine divisions of the Bacteria and a division of the Archaea previously not identified under the conditions of temperature and* p*H found in the spring. By determining a putative metabolic network for the microbial species found in the spring, it appears that the community is carrying out both sulfate reduction and sulfide oxidation. Significantly, the sulfate reduction in the spring appears to be generating numerous organic acids as well as reactive sulfur species, such as sulfite. Even in the absence of oxygen, this sulfite can interact with water directly to produce sulfuric acid. Consequently, such metabolic activity may represent a mechanism by which biospeleogenesis can lead to passage enlargement through sulfuric acid production without the influx of oxygen or oxygen-rich waters. Such activity may lead to higher levels of sulfuric acid production than could be accounted for by inorganic hydrogen sulfide oxidation. Therefore, rather than generating localized pockets of speleogenesis within cave systems, such biogenic sulfuric acid production may have a regional impact on water chemistry and subsequent speleogenesis of large cave systems.*

The theory of hypogenic cave formation through hydrogen sulfide-rich groundwater and subsequent sulfuric acid dissolution was first proposed by Egemeier (1981, 1987) in the early 1970s, with further refinement by Jagnow (1978), Davis (1980), Palmer (1991) and Queen *et al.* (1977). This theory proposes that, given an appropriate geological setting, caves can form through the dissolution activity of ascending hydrogen sulfide ($H_2S$)-rich ground water (Palmer 1991; Palmer & Palmer 2000). As the dissolved $H_2S$ approaches the oxic zone at the water table, the gas reacts with oxygen to form sulfuric acid ($H_2SO_4$) which subsequently dissolves the carbonate rocks, producing carbon dioxide and gypsum ($CaSO_4 \cdot 2H_2O$) (Hill 1987; Palmer & Palmer 2000). This hypothesis is supported in caves, such as Carlsbad Cavern, New Mexico, by the presence of numerous minerals known to be formed through sulfuric acid dissolution, including endellite and gypsum (Hill 1990). The fact that gypsum in these caves is also isotopically light suggests a biogenic component to the sulfuric acid speleogenesis theory (Canfield 2001; Canfield *et al*. 1998; Hill 1990; Klimchouk *et al*. 2000).

As the theory of sulfuric acid dissolution for caves in the Guadalupe Mountains of New Mexico became accepted as a valid process of speleogenesis, the number of such cave systems being similarly described around the world began to rise. Caves formed through sulfuric acid dissolution have now been described in countries as geographically diverse as Italy, Mexico, Romania and Turkmenistan (Hose & Pisarowicz 1999; Klimchouk *et al*. 2000; Klimchouk *et al*. 1995; Maltsev & Malishevsky 1990; Sarbu *et al*. 1996). Included within this group are caves such as Movile Cave, Romania, and Cueva de Villa Luz, Mexico, that are still undergoing active sulfuric acid speleogenesis. Of note is that both these caves demonstrate active microbial activity, with chemolithotrophic microbial communities growing within the cave systems (Hose *et al*. 2000; Sarbu *et al*. 1996; Sarbu *et al*. 1994). In these systems, acid production by the microbial communities may have a significant impact on the cave environment, leading to localized dissolution of the carbonate bedrock (Hose *et al*. 2000). Interestingly, within Movile Cave, such activity is occurring within a geologically confined area without a conduit to allow for the exchange of oxygen with the surface atmosphere. However, the inorganic interaction of oxygen with hydrogen sulfide is widely thought to be an important factor for cavern enlargement in sulfuric acid speleogenesis (Egemeier 1981, 1987; Hill 1987; Palmer & Palmer 2000; Sarbu *et al*. 1996). This would therefore suggest that microbial dissolution in these systems results in a localized secondary speleogenesis

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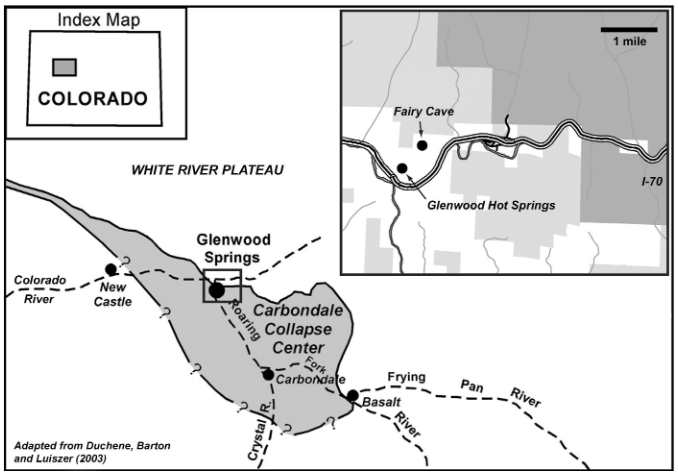


**Figure 1. Location map of Fairy Cave and Glenwood Hot Springs with respect to the Carbondale Collapse Center, Colorado. Modified from Duchene, Barton and Luiszer (2003).**

and may be an important factor in primary cave formation and enlargement.

In this study we examined the microbial community found in a sulfidic hot spring thought to be associated with active cave formation at Glenwood Springs, Colorado. The spring in question may be the hydrological conduit for the $H_2S$-rich ground water that formed the nearby Fairy Cave (commercially known as Glenwood Cavern and Fairy Caves). Morphologically, Fairy Cave appears to have been formed through a combination of carbonic acid and sulfuric acid speleogenesis; the sulfuric acid component is supported by the presence of gypsum deposits within the cave (Hill 1987). These deposits are isotopically light, also suggesting a biogenic component to speleogenesis within this system (H.R. DuChene, personal communication 2002). The aim of this study is to determine the metabolic activity of microorganisms found within this cave hot spring, and whether this activity plays any role in large-scale (regional) carbonate dissolution, or whether it is purely a localized phenomenon. Our results suggest that the primary component of cavern enlargement in sulfuric acid caves may be accounted for by microbial activity that results in increased sulfuric acid production and a greater role for biogenic speleogenesis of such cave systems.

## METHODS

### SITE DESCRIPTION AND SAMPLE COLLECTION

The water at Glenwood Springs comes primarily from snowmelt, which percolates through the Pennsylvanian Eagle Valley Evaporites and is heated as it descends down-dip. Some of the thermal waters percolate through the Belden Formation of the Carbondale Collapse Center to the Leadville Aquifer, whereupon a complex set of faults allows upward flow along the dip of the south flank of the White River Uplift (Figure 1). The sulfidic water then reaches the surface through artesian flow, generating Glenwood Hot Springs (Geldon 1989).

Microbial filament samples were taken using aseptic techniques from the cave spring on May 16, 2000, followed by preservation in either 70% ethanol for phylogenetic analysis, or 4% paraformaldehyde in phosphate-buffered saline for microscopy. All samples were stored on ice for transport. DNA samples were processed within 24 hours and the microscopy samples were stored at 4°C.

Water chemistry at the spring was carried out on November 10, 2000. *p*H and conductivity were measured by an Orion 290A *p*H meter and a Hach conductivity/TDS meter. Bicarbonate was measured by titration with sulfuric acid and bromcresol green-methyl red indicator. Hach AccuVac Ampuls and a Hach DR/2000 portable spectrophotometer were used to measure nitrite, nitrate and phosphate; nitrate was measured using the cadmium reduction method; nitrite was measured using the diazotization method; phosphate (orthophosphate) was measured using the ascorbic acid method; and total iron was measured using the FerroVer method. In the laboratory, major cations were measured with an ARL-3410+ inductively coupled plasma optical emission spectrometer and trace metals were measured with an a Varian UltraMass-700 inductively coupled plasma mass spectrometer.

### DNA EXTRACTION

All DNA protocols were carried out in a laminar-flow hood (Nuaire, Inc.), using aerosol resistant pipette tips (Molecular Bio-Products, Inc.) to reduce the likelihood of contamination. Negative control isolations were carried out in parallel to measure any contamination of the final samples. DNA was extracted from the samples using the standard bead beating protocol (Kuske *et al*. 1997). Briefly, 0.5 g of microbial filament was resuspended in 500 µL 2x buffer A (200 mM Tris [*p*H 8.0], 50 mM EDTA, 200 mM NaCl, 3 mg/mL lysozyme) in a 2 mL screw-cap Eppendorf tube and incubated at 37°C for 30 min. Proteinase K (to 1.2 mg/mL) and sodium dodecyl sulfate (SDS to 0.3% wt/vol) were added and the mixture was further incubated at 50°C for 30 min. SDS was then increased to 5% and the samples were disrupted on a Mini-bead beater (Biospec) at low speed for 2 min and high speed for 30 s in the presence of 50% (vol/vol) phenol-chloroform-isoamyl alcohol (24:24:1) and approximately 0.1 g of zirconium-silica beads (0.1 mm diameter). Lysates were extracted with phenol-chloroform. Nucleic acids were precipitated with 0.3M sodium acetate and 2 volumes of ethanol. The nucleic acids were spun down at 13,000 x *g*, dried and resuspended in 20 µL $dH_2O$.

### POLYMERASE CHAIN REACTION (PCR) AND CLONING

Community rDNAs were PCR amplified from approximately 50 ng of template (bulk) DNA in reaction mixtures containing (final concentrations) 1 x PCR buffer (Perkin Elmer), 2.5 mM $MgCl_2$, 200 µM of each deoxynucleoside triphosphate, 300 nM of each forward and reverse primer, and 0.025 U of AmpliTaq Gold (Perkin Elmer) per µL. Reaction mixtures

were incubated on a Mastercycle Gradient thermal cycler (Eppendorf Scientific) at 94°C for 12 min for initial denaturation and activation of the AmpliTaq Gold. PCR was then carried out for 30 cycles at 94°C for 1 min, 53°C for 45 s, and 72°C for 1 min 30 s, and then a final extension period of 8 min at 72°C. Community rDNA was amplified with the Bacterial specific forward primer 8F (5' – AGA GTT TGA TCC TGG CTC AG – 3') and the universal reverse primer 805R (5' – GAC TAC CAG GGT ATC TAA T – 3'). For amplification of Archaeal rDNAs, the same PCR amplification conditions were used, along with the Archaeal specific forward primer 333Fa (5' – TCC AGG CCC TAC GGG – 3') and universal reverse primer 1391R (5'- GAC GGG CGG TGW GTR CA – 3'). The PCR products were separated on a 1% agarose gel, excised and purified on a Quiaquick gel purification column (Quiagen), and eluted in 30 µL $dH_2O$. The purified PCR products were cloned into a TOPO TA Cloning Kit according to the manufacturer's recommendations (Invitrogen Corp.).

### SCREENING OF RDNA CLONES BY RESTRICTION FRAGMENT LENGTH POLYMORPHISM (RFLP)

rDNA inserts from recombinant clones were reamplified by PCR with reaction mixtures containing (as final concentrations) 1x PCR reaction buffer (50 mM KCl, 100mM Tris, *p*H 8.3), 2.5 mM $MgCl_2$, 100 µM of each deoxynucleoside triphosphate, 150 nM of each vector specific forward and reverse primer (T3 and T7 respectively) and 0.01 U of Pfu DNA polymerase per µL. 100 ng of purified plasmid vector was used as the template. PCR was then carried out for 30 cycles at 94°C for 1 min, 52°C for 45 s, and 72°C for 1 min 30 s, with a final extension period of 8 min at 72°C. 20 µL of crude rDNA product was then digested with 1.5 U of the 4-base-specific restriction endonucleases *HindPI*1 and *Msp*I in 1x NEB buffer 2 (New England Biolabs), in a final volume of 25 µL, for 2 hours at 37°C. Digested fragments were separated by electrophoresis on a 2% SeaKem LE agarose gel (FMC BioProducts) and visualized by ethidium bromide staining and UV illumination. RFLP patterns were grouped visually and representatives were selected for sequencing.

### SEQUENCING OF RDNA CLONES

Plasmid templates from representative clones were sequenced on a Long ReadIR 4200 DNA sequencer (Li-Cor, Inc.), using the Thermo Sequenase Cycle Sequencing Kit (USB Corp.) in accordance with the manufacturer's instructions. Primers for sequencing were the vector primers T3 and T7, with sufficient coverage to sequence the 800 base insert of the bacterial and 1,000 base inserts of the archaeal rDNAs in both directions.

### PHYLOGENETIC ANALYSES

Sequences were compared to available databases by use of the BLAST (Basic Local Alignment Search Tool) network service (http://www.ncbi.nlm.nih.gov/BLAST; (Altschul *et al*. 1997)). Partial sequences of the 16S rRNA gene were compiled using the AlignIR 2.0 Fragment Assembly and Contig Editor software (Li-Cor, Inc). Compiled sequences were aligned using the ARB Software Package (http://mpi-bremen.de/molecol/arb), with additional sequences from the Ribosomal Database Project (Cole *et al.* 2003). Before further phylogenetic analysis, those sequences displaying similar BLAST hits were directly compared using the pairwise BLAST alignment tool [http://www.ncbi.nlm.nih.gov/blast/bl2seq/bl2.html]. Any sequences that demonstrated ≥98% identity toward each other were considered representatives of the same phylotype and grouped accordingly. All presented dendrograms were constructed by use of ARB with evolutionary distance (neighbor-joining) algorithms. The robustness of inferred topologies was tested by bootstrap resampling of phylogenetic-trees, calculated with evolutionary distance using PAUP* software (Sinauer Associates Inc., Sunderland, MA). The sequences obtained from the rDNA clones in this study were deposited in the Genbank database, accession numbers AY702823-AY702856.

### STATISTICAL APPROACHES

The statistical analyses were performed using EstimateS version 6.01b (http://viceroy.eeb.uconn.edu/estimates; Colwell 1997). Each clone represented a separate sample without replacement, and 100 randomizations were performed to obtain the Chao1 estimator for each sample size. Using the singletons and doubletons calculated for each sample collection by EstimateS, we used the log transformation of Chao to calculate the 95% confidence intervals (Chao 1987).

### MICROSCOPY

Approximately 0.1 g of microbial filament material was critically-point dried prior to gold-sputtering and imaging on an ISI-30 scanning electron microscope at a magnification of 3000x.

## RESULTS

### DESCRIPTION OF SAMPLING SITES

The springs at Glenwood are produced from snowmelt water percolating through the Carbondale collapse center, containing the Pennsylvanian Eagle Valley Evaporites, several miles to the south (Figure 1). This water is heated as it descends, dissolving halite (NaCl) and gypsum ($CaSO_4 \cdot 2H_2O$), whereupon faults in the limestone bedrock allow the water to ascend as artesian springs (H. DuChene 2002, personal communication; Geldon 1989). These springs have been commercialized as Glenwood Hot Springs, leading to the containment of the main spring and its diversion into a bathing pool. The cave spring emerges near the main spring in an underground conduit and continues to flow through a cave. The waters of this spring have undermined the soil in one spot, producing an entrance collapse that allows access to the cave. We measured the volume of water issuing from the cave spring as approximately 25–30 L/s.

**Table 1. Physical and chemical parameters of the water measured at the cave spring.**

| Parameter | Spring Water (SD) |
|---|---|
| PHYSICAL | |
| Temperature | 49.6°C |
| *p*H | 6.39 |
| CHEMICAL (mg/L) | |
| Dissolved oxygen | 0.048 |
| Total dissolved solids | 18755 (±144) |
| Total alkali | 544 |
| $HCO_3$ | 664 |
| $NO_3$ | 0.166 (±0.018) |
| $SO_4$ | 1102 (±2.83) |
| $NO_2$ | 0.0100 (±0.000) |
| $PO_4$ | 0.238 (±0.167) |
| Fe (total) | 0.093 (±0.007) |
| Na | 6574 (±125) |
| Cl | 9706 (±19.8) |
| K | 125.0 (±6.5) |
| Mg | 84.60 (±3.05) |
| Ca | 490.4 (±0.22) |
| $SiO_4$ | 30.23 (±0.11) |
| $H_2S$* | 1.65 (±0.63) |

* Average readings of the waters at Glenwood Springs according to Geldon (1989).

The water flowing through the cave was tested using several chemical and physical parameters (Table 1). The concentrations of dissolved solutes ($Na^+$, $Cl^-$, $Ca^{2+}$, and $SO_4^{2-}$) are comparable to the chemical characteristics of waters that pass through evaporative sedimentary rocks in a karstic setting (Hose *et al*. 2000). The spring water is essentially anoxic, with the sampling site being close to the spring source and giving the water insufficient time to become oxygenated. Isotopic fractionation analysis indicates that the sulfates present in the water do not have a biogenic origin, with a $\delta^{34}S$ of 15.7‰ (C. Bern 2002, personal communication), which agrees with the postulated source of the $SO_4^{2-}$ being the Pennsylvanian Eagle Valley Evaporites (Geldon 1989). Hydrogen sulfide ($H_2S$) has previously been measured in the spring at 1.2–2.1 mg/L, and is probably a by-product of microbial activity in the anaerobic, sulfate-rich subterranean water (Geldon 1989).

### MOLECULAR PHYLOGENETIC ANALYSIS OF SAMPLES

To determine the community composition of the cave spring microbial filaments we carried out comparative phylogenetic analysis of the the small subunit ribosomal RNA (16S rDNA) gene. Community DNA was isolated from approximately 0.5 g of microbial filament material, and 16S rDNA gene fragments for both bacterial and archaeal species were amplified using the polymerase chain reaction (PCR). Amplification of gene products for both archaeal and bacterial sequences was successful (data not shown). The PCR products were cloned and 96 representative clones each from the Archaea and Bacteria were isolated, generating two clone libraries: the DZ, dark zone bacterial library and the DZ_Ar, dark zone archaeal library.

The RFLPs in each clone library were grouped according to similarity, and one or more representatives from each group were sequenced. Within the bacterial clone library, six sequences were identified as chimeras using the CHIMERA_CHECK program (http://rdp.cme.msu.edu/html/) and unstable phylogenetic placement. These sequences were removed from further analysis. The remaining 90 representative bacterial clones from the cave spring were compared by pairwise BLAST, allowing us to identify 25 unique phylotypes within the community that were widely distributed through nine divisions of the bacteria, including the *Nitrospira, Cytophagales/Flavobacteria/Bacteroides* (CFB), Green non-sulfur relatedness-group, and the OP11 division (Table 2). Of the 96 archaeal clones isolated from the cave, sequencing revealed that 75 contained ambiguous sequences. These were removed from consideration, resulting in 21 representative 16S archaeal rDNA phylotypes. Once again, direct sequence comparisons were made, resulting in the grouping of the archaeal clones into five unique phylotypes (Table 2).

### PHYLOGENETIC DISTRIBUTION

Phylogenetic analyses not only allow us to determine the distribution of phylotypes identified within an environment, but by phylogenetically aligning a phylotype with its nearest cultivated relative we may make some assumptions as to the role of this organism in the environment (Pace 1997). Therefore, dendograms were created for the phylotypes identified in this study (Figs. 3 & 4). The resultant dendograms indicate that the phylotypes generally group with the divisions identified via the BLAST search (Table 2), the exception being DZ_C11 and DZ_B10, which were identified as members of the Green Non-Sulfur group via BLAST, but belong to the OP11 division by sequence alignment.

Several of the phylotypes identified within the cave had low sequence identity to previously cultivated organisms, allowing us to make only general assumptions about their metabolic activity based on the genera to which these phylotypes belong. However, all the phylotypes demonstrated identity to organisms from environments characteristically devoid of light: benthic and lacustrine sediments, caves, hydrothermal vents and activated sludges, many of which rely on sulfidic compounds for energy conservation (Angert *et al*. 1998; Bond *et al*. 1995; Donachie *et al*. 2002; Lopez-Garcia *et al*. 2002; Teske *et al*. 2002).

Among the *Gammaproteobacteria* phylotypes isolated, DZ_A2 shows identity with the *Thiothrix* spp. Members of this group are usually found living at the boundary of oxidation/reduction gradients, such as where oxygen-rich waters run over $H_2S$-sediments. These bacteria oxidize

**Table 2. Phylogenetic affinities of rDNA sequences identified from Glenwood cave hot spring.**

| Division (% representation) | Clone | # clones/ group | Closest identified relative | % Sequence identity[a] | NCBI Accession # |
|---|---|---|---|---|---|
| **BACTERIA** | | | | | |
| *Gammaproteobacteria* (9.4%) | DZ_A2 | 1/8 | *Thiothrix fructosivorans* | 92% | AY702830 |
| | DZ_B12 | 1/8 | *Methylobacter whittenburyi* | 88% | AY702833 |
| | DZ_C5 | 2/8 | Unidentified sediment bacterium | 92% | AY702855 |
| | DZ_C8 | 3/8 | *Alkalispirillium mobile* | 94% | AY702842 |
| | DZ_G3 | 1/8 | *Achromatium sp.* HK6 | 93% | AY702849 |
| *Deltaproteobacteria* (1.2%) | DZ_H2 | 1/1 | Uncultured sludge bacterium A11b | 92% | AY702850 |
| *Epsiloproteobacteria* (60.0%) | DZ_A5 | 6/51 | Uncultured hydrocarbon seep bacterium | 87% | AY702853 |
| | DZ_A7 | 2/51 | Uncultivated bacterium VC2.1 | 95% | AY702832 |
| | DZ_B1 | 27/51 | Uncultured *Proteobacterium* a1b030 | 94% | AY702834 |
| | DZ_B2 | 12/51 | *Wolinella succinogenes* | 90% | AY702835 |
| | DZ_C10 | 1/51 | Uncultivated sediment Proteobacterium | 92% | AY702838 |
| | DZ_D3 | 1/51 | Uncultured *Proteobacterium* a1b030 | 94% | AY702843 |
| | DZ_E6 | 1/51 | Uncultured *Proteobacterium* R103-B43 | 97% | AY702845 |
| | DZ_G10 | 1/51 | *Wolinella succinogenes* | 90% | AY702847 |
| OP11 (2.3%) | DZ_A3 | 1/2 | Uncultivated candidate OP11 | 87% | AY702831 |
| | DZ_H3 | 1/2 | Uncultured lake bacterium | 86% | AY702851 |
| *Actinobacteria* (14.1%) | DZ_A1 | 10/12 | *Propionibacterium acnes* | 98% | AY702829 |
| | DZ_A4 | 1/12 | Uncultured *Propionibacterium* PH-B24N | 98% | AY702852 |
| | DZ_D6 | 1/12 | Uncultured *Propionibacterium* PH-B24N | 98% | AY702844 |
| Low G+C Group (4.7%) | DZ_B9 | 1/4 | Uncultured travertine eubacterium | 97% | AY702837 |
| | DZ_C7 | 3/4 | Uncultured sediment bacterium | 99% | AY702841 |
| Green Non-Sulfur (5.9%) | DZ_C11 | 1/5 | Uncultured sludge bacterium SRB109 | 96% | AY702839 |
| | DZ_B10 | 4/5 | Uncultured sludge bacterium SRB109 | 93% | AY702854 |
| *Flexistipes* (1.2%) | DZ_E9 | 1/1 | *Flexistipes sp.* Vp180 | 91% | AY702846 |
| *Nitrospira* (1.2%) | DZ_G1 | 1/1 | *Saltmarsh clone* LCP-6 | 89% | AY702848 |
| **ARCHAEA** | | | | | |
| *Euryarchaeota* | | | | | |
| *Thermoplasmata* | DZ_ArA2 | 11/21 | *Picrophilus oshimae* | 91% | AY702826 |
| | DZ_ArB2 | 1/21 | *Thermoplasma acidophilium* | 92% | AY702827 |
| | DZ_ArA3 | 7/21 | *Ferroplasma acidarmanus* | 96% | AY702823 |
| | DZ_ArE3 | 1/21 | *Thermoplasma acidophilium* | 91% | AY702824 |
| | DZ_ArF4 | 1/21 | *Thermoplasma acidophilium* | 90% | AY702828 |

[a]Data obtained via an on-line BLAST search (Altschul *et al*. 1997).

reduced forms of sulfur and directly fix $CO_2$ for biosynthesis via a circumvented Calvin cycle (Dworkin 2002). DZ_B12 is related to members of the *Methylobacter,* which oxidize biogenic methane to formate and $CO_2$. These organisms are a natural component of most microbial ecosystems, but optimally grow below 40°C and are generally not found in hot spring environments. DZ_C8 is related to *Alkalispirillium mobile*, a moderately halophilic, obligate anaerobe that uses organic anions such as acetate and succinate as carbon sources. *A. mobile* is also unable to grow at the temperatures or pH encountered in the cave spring, preferring *p*H values > 9.0 and temperatures in the 35–38°C range. The final *Gammaproteobacteria* identified, DZ_G3, is related to *Achromatium* sp. These organisms are characterized by large $CaCO_3$ inclusions and are generally found in areas where calcium is plentiful, so their presence in this karstic spring is not

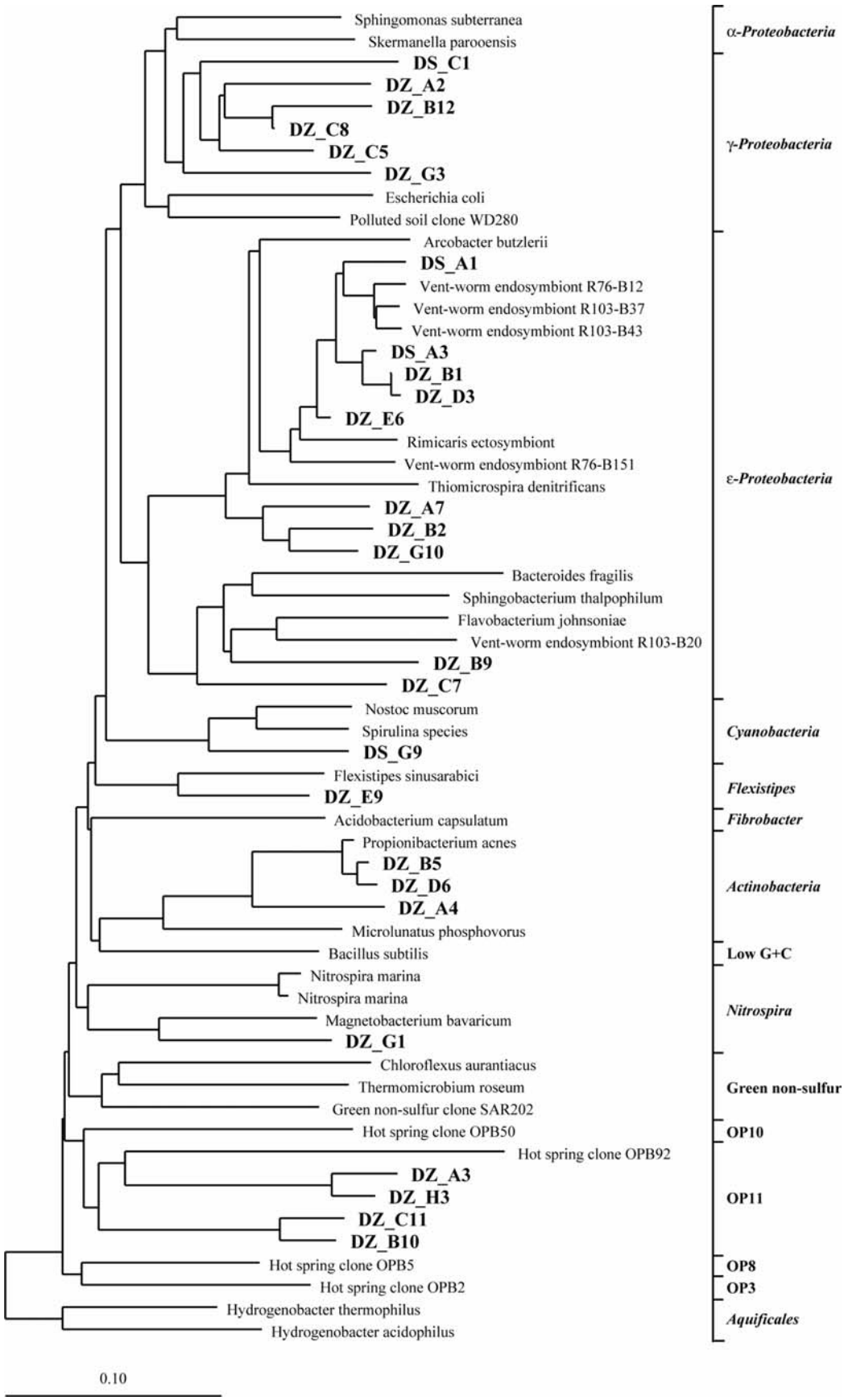


**Figure 2. Evolutionary distance consensus dendogram of the Bacterial domain and the bacterial DZ 16S SSU rDNA phylotypes identified within the Glenwood Hot Spring microbial community. Reference sequences were chosen to represent a broad diversity within the Bacterial domain, with members of the *Acquificales,* as the most deeply divergent members of the domain, as the outgroup for analysis. Branch points were supported by consensus agreement of phylogenetic analysis using neighbour-joining, parsimonious and maximum-likelihood algorithms. The bar indicates 10% sequence divergence.**

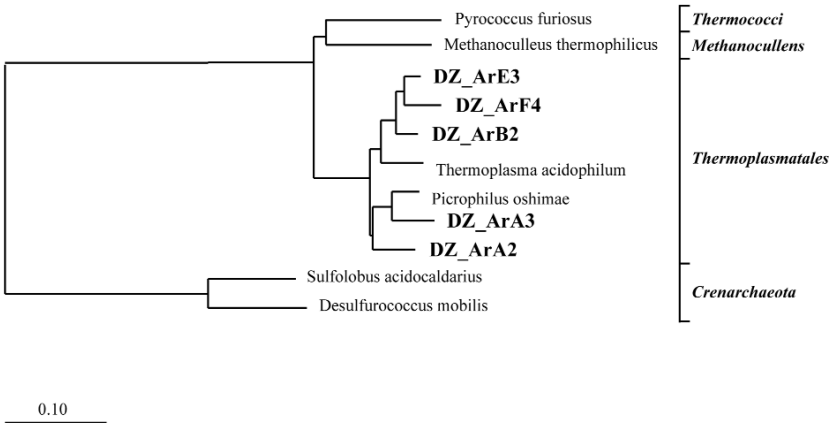


**Figure 3.**
**Evolutionary distance consensus dendogram of the Archaeal domain and the DZ_Ar 16S SSU rDNA phylotypes identified within the Glenwood Hot Spring microbial community. Reference sequences were chosen to demonstrate the lineage of the identified phylotypes. *Sulfolobus acidocaldarius* and *Desulfurococcus mobilis,* as members of the *Crenarchaeota,* were used as the outgroup for analysis. Branch points were supported by consensus agreement of phylogenetic analysis using neighbour-joining, parsimonious and maximum-likelihood algorithums. The bar indicates 10% sequence divergence.**

surprising (Dworkin 2002). Further, while the other identified *Gammaproteobacteria* in the cave spring carry out oxygenic respiration, *Achromatium* sp. are strict sulfate-reducing anaerobes. It is thought that the cellular $CaCO_3$ inclusions in this species are required to buffer the $H_2SO_4$ produced by the organism through incomplete sulfate reduction to sulfite (Dworkin 2002).

The predominant phylotype identified in the *Epsiloproteobacteria* is DZ_B1. This organism belongs to an uncultivated pyhlogenetic group previously seen in sulfidic, anoxic aquatic environments, including Parker's Cave, Kentucky, the Cariaco Basin of Venezuela and deep sea sediments, which suggests that these organisms are also involved in sulfate reduction (Angert *et al*. 1998; Madrid *et al*. 2001; Orphan *et al*. 2001; Teske *et al*. 2002). All of these environments are also aphotic. A large number of the identified *Epsiloproteobacteria* phylotypes (DZ_A7 and DZ_G10), share similarity to *Wolinella succinogenes*, a sulfate-reducing bacterium. *W. succinogenes* is commonly found in anaerobic muds where it ferments complex organic compounds, such as cellulose, with sulfate as an electron acceptor. When organic carbon sources are not readily available, this organism is capable of fixing $CO_2$ and surviving chemolithotrophically. Notably, *W. succinogenes* does not always fully reduce $SO_4^{2-}$ to $H_2S$, and produces thiosulfate and elemental sulfur that can be used by numerous other sulfate-reducing species (Dworkin 2002).

Beyond members of the *Proteobacteria,* another predominant group within the cave spring are the *Actinobacteria* (Fig. 2). The isolated phylotypes share identity with members of the *Propionibacteria,* from which the skin commensal *P. acnes* has been found as a PCR contaminant (Tanner *et al*. 1998). However, identification of *Propionibacteria* contamination in our laboratory is rare (<1% per clone library), and identified phylotypes share 100% identity with *P. acnes* via BLAST search. In this study, the *Propionibacteria* accounted for a much greater percentage of the clone library (14%) and displayed 98% identity with previously cultivated *Propionibacter* species, suggesting these clones did not represent contamination. Members of this group have also been previously identified in environmental samples (Donachie *et al*. 2002). The *Propionibacteria* are generally halotolerant anaerobes, although no growth has previously been observed at the temperatures of the cave spring. They also ferment lactate, carbohydrates and polyhydroxy-alcohols, to acetate, $CO_2$ and propionate (Dworkin 2002).

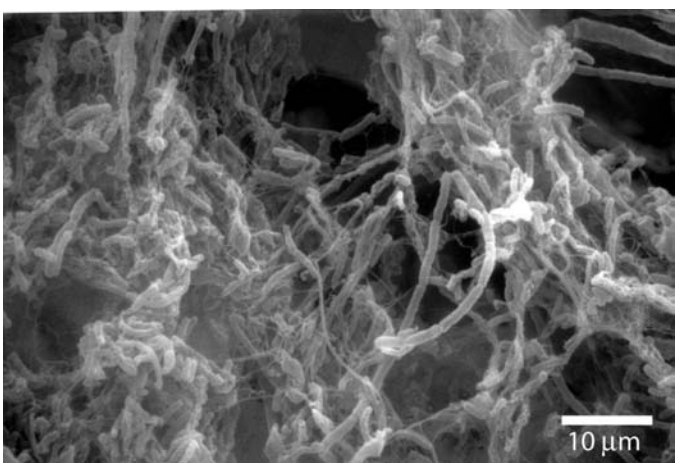


**Figure 4. Scanning electron micrograph of the filament community structural morphology. Numerous microbial cell structures can be observed, including coccoid, septate and filamentous morphotypes. A glutnous matrix appears to be holding the community together, which is likely responsible for the gross structural morphology of the microbial filaments. (Magnification 3000x).**

Other organisms identified in the cave spring include members of the anaerobic CFB group, which specialize in degrading complex biomolecules to organic acids, such as lactate. The *Nitrospira* oxidize nitrite to nitrate, and are capable of sulfate-reducing, chemolithotrophic and chemoorganotrophic growth. The *Flexistipes* are obligate anaerobes and are very adept at using a variety of electron acceptors for growth, which is unique given their anaerobic fermentation (Dworkin 2002; Madigan *et al*. 2000). Finally, DZ_C11 and DZ_B10 are mem-

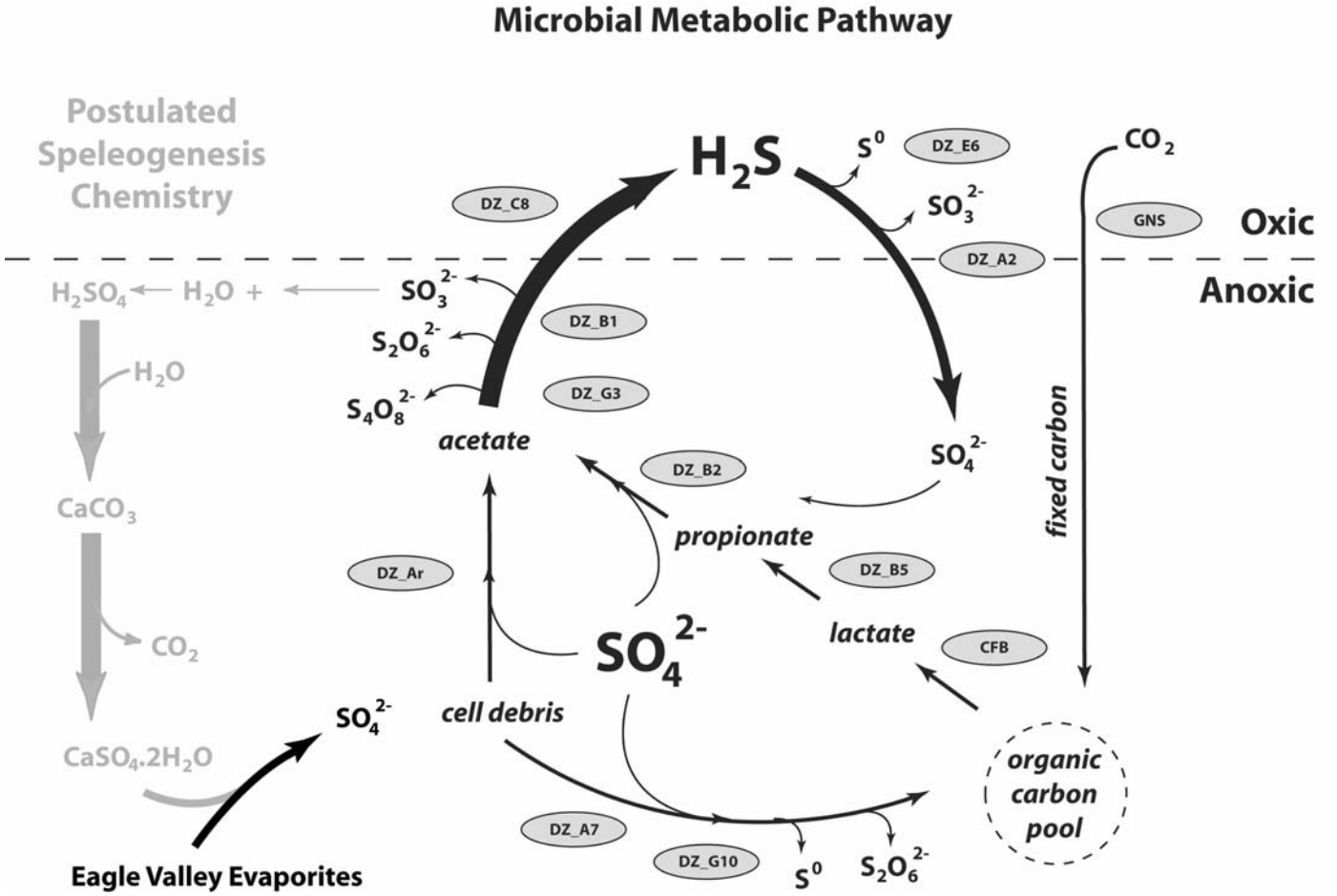


**Figure 5. Proposed metabolic network that sustains the microbial filament population identified in the Glenwood Hot Spring cave (only the major community phylotypes are shown). Each phylotype is shown with a shaded oval. The putative metabolic activity for each phylotype is indicated with an arrow, with thicker arrows indicating reactions that appear to drive the metabolic network. The source of sulfate into the system is dissolved gypsum from the Eagle Valley Evaporites. Metabolic by-products, such as acetate and propionate, and the major sulfur species present, are indicated. A presumed oxic/anoxic boundary that allows sulfur-cycling in this system is shown as a dotted line.**

bers of the green non-sulfur group, normally associated with anoxygenic photosynthetic growth in neutral to alkali hot springs (Ward *et al*. 1998). Members of this group can grow equally well autotrophically in the dark, using an aerobic lifestyle and have previously been identified in aphotic environments (Schabereiter-Gurtner *et al*. 2002; Teske *et al*. 2002). Members of the green non-sulfur group can oxidize $H_2S$ and reduce $CO_2$ to glyoxylate through the 3-hydroxypropionate pathway.

Finally, several representatives of the *Thermoplasmata* division within the Archaea were identified in the cave spring (Figure 3). Members of this group have previously been identified only in aphotic environments such as coal refuse piles and hot-spring sediments (Dworkin 2002; Madigan *et al*. 2000). The *Thermoplasmata* are obligate heterotrophs, generally fermenting the breakdown products of cellular decomposition with $SO_4^{2-}$ as an electron acceptor. While the temperature of the cave spring is favorable to growth of the *Thermoplasmata* (35–67 °C), members of this genus are not known to survive above pH values of 4.0 due to the loss of protons that maintain cellular integrity (Dworkin 2002). While the *p*H of the cave spring was 6.39, it is possible that extremely acidic microhabitats exist within the microbial filaments to allow survival of these organisms. Such microhabitats appear to be common within microbial consortia (Boetius *et al.* 2000; Pallud *et al.* 2004).

### COMMUNITY DIVERSITY

With the advent of molecular phylogenetic techniques, it is becoming possible to use the tools employed by traditional ecologists to measure microbial community diversity (Begon *et al*. 1998). Recently, Hughes *et al.* recommended the use of nonparametric estimators to reliably determine microbial diversity (Hughes *et al*. 2001). Using the assumption that each clone represented a sample, we used the species richness estimator Chao1 to estimate the true richness of the cave spring

microbial community (Chao 1987). This estimator was used due to the small sample size and the likelihood of underestimation (versus overestimation) of the true richness of the community. After sampling 90 clones the Chao1 estimator curve did not level off, suggesting that this sample size is insufficient to measure diversity at this spring. Ninty-five % confidence intervals indicate that in order to sufficiently sample the cave spring bacterial community more than 500 clones would need to be isolated.

### MORPHOLOGY OF CAVE SPRING MICROBIAL COMMUNITIES

The cave spring contained microbial communities in the form of filaments. On a gross morphological scale these filaments are approximately 2–5 mm in diameter and 35–50 mm in length. Electron microscopy was used to examine the morphological structure of the microbial filament communities. The results (Fig. 4) demonstrate a high morphological complexity in the cave spring community, which appears to be held together by a glutinous matrix, as seen in similar filamentous microbial communities (Angert *et al*. 1998; Hose *et al*. 2000). Numerous granules are interspersed between the cells, which presumably represent precipitated sulfur forms and agree with both the metabolic activity and community diversity postulated from this study (Angert *et al*. 1998; Hose *et al*. 2000; Knickerbocker *et al*. 2000). Several different cell morphologies were observed in the spring community, such as small individual rod and cocci forms, up to large cells forming long chains.

## DISCUSSION

It was the aim of this study to determine whether the activity of the microorganisms found in the Glenwood Hot Spring cave suggests a more regional biogenic influence on water chemistry and subsequent sulfuric acid speleogenesis, or whether such activity is a localized, secondary function that causes microscale cavern enlargement. Our results suggest that the community structure of the microorganisms found in this cave environment is complex, with the metabolic activity of the community resulting in the production of a number of different reactive sulfur species. To simplify the structure of this community, the metabolic activities of the primary phylotypes are presented in Figure 5; the exception are members of the OP11 division for which no metabolic function is presently known. The microbial community contains both sulfate-reducing and sulfide-oxidizing species, even though the filaments do not form classic stratified microbial communities (Cohen & Rosenberg 1989). Therefore, in order for the described metabolic network to function, an oxic/anoxic boundary must occur within the filaments. Such ordered community structure in microbial consortia has previously been demonstrated to account for anaerobic oxidation of methane in anoxic sediments (Boetius *et al*. 2000). If such an ordered structure does exist in the filaments, the metabolic network described in Figure 5 accounts for the activity of all identified phylotypes.

An important observation of the putative metabolic network found within the cave spring is the potential to generate numerous sulfur species, including sulfide, sulfite, thiosulfate, tetrathionate, sulfate and even elemental sulfur (Barrett & Clark 1987; Le Faou *et al*. 1990). While Palmer's model of sulfuric acid speleogenesis suggests that sulfide gas must interact with oxygenated waters for acid production, it does not take into account the potential for the production of such acid by anoxic sulfate reduction (Egemeier 1981, 1987; Hill 1987, Palmer & Palmer 2000). For example, sulfite can react with water directly to produce sulfuric acid, thereby bypassing the requirement of oxygen for sulfuric acid production. Such activity could explain the high levels of speleogenesis in deep groundwater systems, such as Lechuguilla Cave, where the high levels of oxygen required for large scale carbonate dissolution cannot readily be accounted for. Thus microbial activity may provide an important component to sulfuric acid production and speleogenesis on a large scale (Palmer & Palmer 2000). Such activity could also explain some of the local conditions of rapid speleogenesis not easily accounted for by $H_2S$ movement within groundwater. At localized zones where anoxic water may meet the surface or interact with oxygenated waters, there may be a very aggressive, localized speleogenesis. Microbial activity may be continually oxidizing and reducing sulfur species to increase the amount of acid produced in the system, and such activity is supported by the presence of the *p*H-sensitive *Thermoplasmata* (Palmer & Palmer 2000; Socki *et al*. 2001). Indeed, multiple rounds of microbial oxidation/reduction must be occurring within such systems to account for the level of isotopic fractionation seen in the gypsum deposits and therefore cannot reflect static inorganic processes (Canfield 2001; Canfield *et al*. 1998; Hill 1990; Hose *et al*. 2000). Localized increases in microbial activity through interactions with organic-rich surface water or an oxygenic atmosphere may explain the localized pockets of aggressive sulfuric acid dissolution seen in sulfidic-cave systems, including the presence of features such as rillenkarren and dissolution notches (Hose *et al*. 2000; Palmer & Palmer 2000). Such microbial metabolism, with the production of several different ionic forms of sulfur, can also help account for the numerous sulfur minerals found in Lechuguilla Cave. This includes elemental sulfur, which is produced as an energy storage molecule by many sulfate-reducing and sulfide-oxidizing species (Dworkin 2002).

Despite the metabolic activity within the cave spring appearing to suggest a biogenic component to sulfuric acid speleogenesis, there is sometimes a disparity between identification of species based on phylogenetic analysis and the metabolic function of that species in an ecosystem (Achenbach & Coates 2000). Therefore, further geomicrobial studies are needed to confirm our findings that there may be a broader biogenic component in the speleogenesis of sulfuric acid caves. Recently Engel *et al.* (2004) demonstrated localized dissolution of carbonates by *Epsiloproteobacteria* in Kane Cave, Wyoming. The bacteria locally produced sulfuric acid that dis-

solved the host rock, leaving behind obvious solution pockets from the microbial metabolic activity. Further, these investigators demonstrated that dissolved $H_2S$ in the cave spring water entering the cave was quickly consumed by sulfide-oxidizing bacteria before it could generate inorganic sulfuric acid. However, our results are based on a free-floating microbial community that does not interact with the host rock, suggesting that reactive sulfur species produced by these organisms must enter the water directly. It should be noted that in caves such as Cueva de Villa Luz, Mexico, the anoxic water entering the cave system, where the dissolved $H_2S$ has not yet had the opportunity to interact with $O_2$, is slightly acidic, while oxygenated inlets into the system have a pH above 7. As the most oxygenated waters entering this system have the highest pH, this suggests that the oxygen does not sufficiently interact with the hydrogen sulfide to acidify the water in these inlets (A. Palmer, personal communication 2004). Further, the large number of microbial filaments found within the Cueva de Villa Luz inlets indicates an active subsurface microbial community below the cave, with the anoxic nature of the water reflecting the microbial consumption of available oxygen (Lavoie, personal communication 2004). Such observations are in agreement with our hypothesis as, after the oxygen has been consumed within this system, sulfate will continue to be reduced to sulfite with the associated production of sulfuric acid.

Our results remain a preliminary, descriptive investigation of the metabolic activity of a microbial cave community that may be involved in biospeleogenesis. While comparisons are made with other sulfuric-acid cave systems, all these systems were formed through the activity of sulfidic waters well below the conditions of temperature found in Glenwood Hot Springs. While there may be significant differences in community structure between these systems, many of the identified phylotypes in our study are also capable of growth in more temperate conditions. Therefore, additional investigations are needed to confirm our hypothesis, including comparative studies with other springs, the distinct observation of reactive sulfur species production by such communities, and the direct observation of calcium carbonate dissolution by metabolic by-products of community growth.

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