Geomicrobiology of highly acidic, pendulous biofilms (“snottites”) from the Frasassi Caves, Italy

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Senior Integrative Exercise
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ABSTRACT

Limestone caves that result from the oxidation of hydrogen sulfide to sulfuric acid, sulfidic caves, harbor isolated ecosystems supported by lithotrophic sulfur-oxidizing microorganisms. The actively corroding Frasassi Caves in Italy are a perfect site to study such microbial communities. Here, we report on the geomicrobiology of extremely acidic, subaerial biofilms known as snottites.

We used 16S rDNA clone library construction and FISH analyses to assess microbial species richness and population structure of three different snottite types. The snottite microbial communities have very low species diversity and are predominantly composed of sulfur oxidizing microorganisms. A small number of organotrophic prokaryotes and Eukaryotes are present as well. Despite differences in location and lithologic substrate, all three snottite types had similar microbial communities. *Acidithiobacillus* sp., sulfur oxidizing primary producers, dominate all snottite types, and are an important trophic base for other snottite microorganisms and even cave macroinvertebrates. Furthermore, *Acidithiobacillus* may be responsible for snottite formation. Enrichment cultures of Frasassi *Acidithiobacillus* in thiosulfate media produced acid and extracellular polymers (slime). Other snottite microorganisms include representatives of Archaeal genus *Ferroplasma*, and Bacterial genus *Acidimicrobium*. Members of Bacterial lineage TM6, genus *Sulfobacillus*, and Archaeal genus *Thermoplasma* were also present in lower quantities.

We found that snottite microbial communities changed very little between May and August of 2005, despite pH variations. Furthermore, Frasassi snottite microbial communities are similar to those of other sulfidic cave snottites. In general, snottites are some of the least diverse biological communities known, likely due to their simple geochemistry and extreme acidity. Because snottite microorganisms are responsible for generating this extreme acidity, they contribute to sulfuric acid speleogenesis.

**Keywords:** Geomicrobiology, sulfide oxidation, hydrogen sulfide, sulfuric acid, cave ecology, karstification
INTRODUCTION

Most limestone caves are products of epigenic, carbonic acid-based dissolution processes (Palmer, 1991; Gillieson, 1996; Drever, 1997; Fetter, 2001). Caves formed in this manner are generally oligotrophic environments, and any life within is reliant on either minerals in the bedrock or surface inputs of organic matter for energy (Cunningham et al., 1995; Cañaveras et al., 2001; Northup and Lavoie, 2001; Auler and Smart, 2003). However, between five and ten percent of limestone caves form by sulfuric acid dissolution processes (Palmer, 1991; Forti et al., 1999). Sulfuric acid speleogenesis (sulfuric acid cave formation) occurs intensely at the water table, where hydrogen-sulfide in reduced, rising phreatic water is oxidized to sulfuric acid when it reacts with oxygen from recent surface recharge ($\text{H}_2\text{S} + 2\text{O}_2 \rightarrow \text{H}_2\text{SO}_4$) (Egemeier, 1981; Galdenzi and Maruoka, 2003; Hose and Macalady, 2006). In sharp contrast to carbonic acid-formed caves, these “sulfidic” caves harbor abundant microbial life, which thrives on the energy available at redox interfaces (Sarbu et al., 1996). Furthermore, caves formed by this process are among the biggest in the world. For example, the massive caves of the Guadalupe Mountains, Carlsbad and Lechuguilla Caves, were formed by sulfuric acid speleogenesis (Davis, 1981; Hill, 2000; Jagnow et al., 2000).

Microbial communities inhabiting sulfidic caves are important for geomicrobiological studies. Because such communities are generally well-isolated and founded on lithotrophic energy, sulfidic cave biota is similar to that of other sulfide-based
ecosystems such as those found around hydrothermal vents. For similar reasons, they are
good analogues for Proterozoic life (Sarbu et al. 1996; Forti et al. 1999; Boston et al.,
2001). Furthermore, the sulfide-oxidizing microorganisms play an important role in
catalyzing sulfuric acid formation and thereby enhancing speleogenesis (Engel et al.
2004b). Several different types of microbial communities are typically associated with
sulfidic caves. Microbe-packed vermiculations of organic-rich sediment cover ceilings
and walls; viscous, highly acidic biofilms called “snottites” drip from overhangs; varying
morphologies of white stream biofilms fill cave streams, and less-conspicuous
communities cover gypsum wall-crusts and inhabit stream sediments. Of these different
communities, the white stream mats have been the most studied, and have been shown to
contain complex microbial communities (Brigmon et al., 1994; Sarbu et al., 1996; Angert
et al., 1998; Engel et al., 2001; Engel et al., 2003; Engel et al. 2004a; Barton and Luiszer,
2005; Macalady et al. pers. com.).

This project examines the ecology of microorganisms in snottites from the
Frasassi Caves, Italy. Previous research on snottites is sparse, as most research on
microbial cave communities has focused on the subaqueous communities. One study on
Frasassi snottites was conducted by Vlasceanu et al. (2000), who reported finding three
different genera of known sulfide-oxidizing Bacteria, *Sulfobacillus, Acidithiobacillus,*
and *Halothiobacillus* (both reported as *Thiobacillus*, since reclassified as genera
*Acidithiobacillus* and *Halothiobacillus*; see Kelly and Wood, 2000). Lyon et al. (2004)
also investigated Frasassi snottites, and noted the presence of abundant prokaryotic as
well as eukaryotic life (fungi). Hose et al. (2000) sampled snottites from Cueva de Villa
Luz, Tabasco, Mexico (2000; see also Hose and Pisarowicz, 1999), and reported finding
Bacterial genera *Acidimicrobium* and *Acidithiobacillus* as well as some macroinvertebrates. Analogous acidic biofilms form in subaerial acid-mine drainage environments. However, microbial life forms in such environments exhibit iron-based rather than sulfur-based metabolisms (Bond et al., 2000a; Bond et al., 2000b).

In this research, we use 16S rDNA phylogenetic techniques and fluorescence *in situ* hybridization to provide a rigorous analysis of the prokaryotic species diversity and community composition of three types of Frasassi snottites. Additionally, we discuss the occurrence and formation of snottites and the role that these subaerial microbial communities play in cave formation.

**METHODS**

*Study Area and Sampling Sites*

Le Grotte di Frasassi (Frasassi Caves) are located in the Appennine Mountains in the Marches Region, central Italy (fig. 1). The caves were known as early as 1948, but quickly became famous in 1971 when local cavers discovered a huge stalactite-packed chamber (180 x 120 meters) now known as Grotta Grande del Vento (big cave of the wind) (Bunnell, 2004). It was quickly developed for tourism, and currently attracts around 350,000 visitors each year (Sarbu et al., 2000; Bunnell, 2004).

The Frasassi Caves comprise over 20 km of irregular, ramifying passages in the nearly-pure limestones of the Jurassic-aged Calcare Massiccio formation (fig. 2) (Sarbu et al., 2000; Galdenzi and Maruoka, 2003). Sulfuric acid speleogenesis is actively occurring in the lowermost levels near the water table. The upper levels of the cave
Figure 1. Map of the Frasassi cave system, showing sampling locations. Courtesy of Sandro Mariani.
Figure 2. (A) Cross-section of the Frasassi Gorge. Courtesy of Sandro Montanari. (B) Regional stratigraphic column, modified from Galdenzi and Maruoka (2003). The Frasassi cave system is actively forming in the Calcare Massiccio limestone formation. Sulfides in rising groundwater likely originate from microbial reduction in the Calcari a Raethavicula Fm.
display evidence of a sulfidic origin, most notably abundant gypsum deposits and ramiform passage morphology (Hose and Macalady, 2006), although they are no longer enlarging by hypogenic processes. At the water-table, anoxic, saline groundwater rises through faults and meets oxygen-rich vadose waters. The phreatic waters are originally meteoric, with a brief, decade-scale underground residence time (Tazioli et al., 1990; reported in English in Galdenzi and Maruoka, 2003). Sulfides and other reduced species in the rising groundwater are likely derived from microbial reduction in an organic-rich limestone (Calcari a Raethavicula Fm.) after the groundwater first gathers sulfate while rising through a Triassic anhydrite layer (Burano Fm.) (fig. 2) (Galdenzi and Maruoka, 2003).

Snottite samples for this study were collected in two different sulfidic regions of the caves, Ramo Sulfureo (Sulfurous Branch) and Pozzo dei Cristalli (Crystal Pit). Three different snottite types (fig. 3) were sampled: two different types from Ramo Sulfureo (RS1 and RS2) and one type from Pozzo dei Cristalli (PC1) (see fig. 1 for sample locations). RS1 snottites were suspended from micro- and macro-crystalline gypsum, while RS2 snottites hung from rosette-shaped elemental sulfur globules. RS1 and RS2 sample sites are in adjacent chambers connected subaqueously by several meters of groundwater conduits. PC1 snottites hung from gypsum wall crusts in a separate region of the caves. Pozzo dei Cristalli and Ramo Sulfureo are separated by several hundred meters of passages.
Figure 3. The three different snottite types sampled in this study. PC1 snottites, from Pozzo dei Cristalli (see fig. 1 for sample locations), hang from gypsum wall crusts. RS1 and RS2 samples are from Ramo Sulfureo; RS1 snottites hang from gypsum crystals, and RS2 snottites hang from rosette-shaped crystals of elemental sulfur. Scale bars are 2 cm. White arrow indicates spider webs. (RS1 and RS2 photos by J. Macalady.)
**Geochemistry**

Cave atmosphere gas measurements were collected during August 2005. H$_2$S, CO$_2$, NH$_3$, and N$_2$O were measured using Dräger short-duration tubes and an Accuro hand pump (Dräger Safety Inc., Germany). When possible, multiple measurements were taken to test consistency in readings. When appropriate, measurements were taken at different heights above the cave stream. Snottite and cave-wall pH were measured using pH paper (range 0-2.5).

Cave water chemistry was measured as follows: conductivity, redox potential, pH, and temperature were measured on site, using a Multimeter 350i probe (WTW, Weilhelm, Germany). Dissolved sulfide, oxygen, and ammonium were measured on site, using the methylene blue, indigo carmine, and salicylate methods, respectively. Anions were measured at the Osservatorio Geologico di Coldigioco microbiology lab with a portable spectrophotometer within 12 hours of collection (stored at 4ºC) according to the manufacturer’s instructions (Hach Co., Loveland CO, USA). Additional cave water geochemistry data comes from Cocchioni et al. (2003).

**Sample Collection and Preservation**

Samples of each of the three snottite types were collected in sterile plastic microfuge or centrifuge tubes in May and August of 2005. The pH of each snottite was measured using pH paper, and only snottites of pH <1 were collected. Snottite samples will be referred to as PC1, RS1, and RS2 (fig. 3).

DNA was extracted from samples intended for future clone library construction within 12 hours after collection. We used the MoBio Soil DNA extraction kit (MoBio,
USA), according to the manufacturers instructions. DNA extracts were stored in 1x TE buffer solution at 4°C for transport.

Samples intended for FISH were adjusted to pH 7 with 1N NaOH and fixed in 4% paraformaldehyde (PFA) within 12 hours after collection. PFA fixation was performed as follows: cells were pelleted in a microfuge and supernatant was decanted. Cells were then resuspended in a 4% paraformaldehyde solution and incubated for 2-3 hours, then washed in 1x phosphate buffer solution (PBS). Finally, cells were suspended in a 1:1 PBS/ethanol solution and stored at –20°C.

Samples intended for either future DNA extraction or FISH were preserved with a ratio of 1 part sample to 5 parts RNAlater (Ambion Inc., USA), and stored at –20°C. Samples intended for culturing were collected in 50 mL falcon tubes and refrigerated at 4°C.

16S rDNA Clone Library Construction

Two 16S rDNA clone libraries were constructed from RS2-type snottite samples collected in Ramo Sulfureo in May 2005. Sequences from a previously constructed clone library from PC1-type snottites (collected in May 2005, identical cloning procedure) is also analyzed in this paper. 16S rDNA genes were amplified by PCR, using either Bacterial specific forward primer 27f, or modified universal forward primer 533f. Both forward primers were coupled with universal reverse primer 1492r (see table 1 for primer information). Each PCR reaction contained 1.25 units ExTaq polymerase (TaKaRa Bio Inc., Shiga, Japan), 0.2 mM of each dNTP, 1X ExTaq PCR buffer, 0.2 μM of each forward and reverse primers, and snottite DNA. Reactions with forward primer 27F were
### TABLE 1. PCR PRIMERS AND FISH PROBES USED

#### PCR primers

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Target</th>
<th>Sequence (5’-3’)</th>
<th>direction</th>
<th>Specificity</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>27F</td>
<td>27-46</td>
<td>AGA GTT TGA TCC TGG CTC AG</td>
<td>forward</td>
<td>Bacterial Domain</td>
<td>Lane, D.J. 1991</td>
</tr>
<tr>
<td>1492R</td>
<td>1492-1510</td>
<td>GGT TAC CTT GTT ACG ACT T</td>
<td>reverse</td>
<td>Universal</td>
<td>Delong, E.F. 1992</td>
</tr>
<tr>
<td>533F mod</td>
<td>533-551</td>
<td>GTG CCA GCC GCC GCG GTA A</td>
<td>forward</td>
<td>Universal</td>
<td>Mod. from, Hugenholtz et al. 1998</td>
</tr>
</tbody>
</table>

#### FISH probes

<table>
<thead>
<tr>
<th>Probe name</th>
<th>Target</th>
<th>Sequence (5’-3’)</th>
<th>% Formamide</th>
<th>Specificity</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>EUB338†</td>
<td>16S</td>
<td>GCT GCC TTC CGT AGG AGT</td>
<td>20%</td>
<td>most Bacteria</td>
<td>Amann et al., 1990</td>
</tr>
<tr>
<td>EUB338-II</td>
<td>16S</td>
<td>GCA GCC ACC CGT AGG TGT</td>
<td>0-50%</td>
<td>Planctomycetales</td>
<td>Daims et al., 1999</td>
</tr>
<tr>
<td>EUB338-III</td>
<td>16S</td>
<td>GCT GCC ACC CGT AGG TGT</td>
<td>0-50%</td>
<td>Verrucomicrobiales</td>
<td>Daims et al., 1999</td>
</tr>
<tr>
<td>ARCH915</td>
<td>16S</td>
<td>GTG CTC CCC CGC CAA TTC CT</td>
<td>20%</td>
<td>most Archaea</td>
<td>Stahl and Amann, 1991</td>
</tr>
<tr>
<td>GAM42a†</td>
<td>23S</td>
<td>GCC TTC CCA CAT CGT TT</td>
<td>35%</td>
<td>most Gammaproteobacteria</td>
<td>Manz et al. 1992</td>
</tr>
<tr>
<td>THIO1</td>
<td>16S</td>
<td>GCG CTT TCT GGG GTC TGC</td>
<td>35%</td>
<td>Acidithiobacillus spp.</td>
<td>Gonzalez-Toril et al. 2003</td>
</tr>
<tr>
<td>ACM732</td>
<td>16S</td>
<td>GTA CCG GCC CAG ATC GCT G</td>
<td>35%</td>
<td>Acidimicrobium spp.</td>
<td>Bond et al. 2001</td>
</tr>
<tr>
<td>FER856</td>
<td>16S</td>
<td>CGT TTA ACC TCA CCC GAT C</td>
<td>25%</td>
<td>Ferroplasma spp.</td>
<td>Edwards et al. 2000</td>
</tr>
</tbody>
</table>

†EUBMIX is a mixture of EUB338, EUB338-II, and EUB338-III
‡requires competitor oligo cGam42a GCCCTCCACTTCGTTT
run in an Eppendorf Master Cycler Gradient PCR machine as follows: 5 minutes at 94°C initial denaturation, 25 cycles of 1 minute denaturation at 94°C, 25 seconds annealing at 50°C, and 2 minutes elongation at 72°C, followed by a final elongation for 20 minutes at 72°C. Libraries with forward primer 533F were incubated in a similar fashion but with a 45 second annealing at 45°C. Successful PCR results were verified by gel electrophoresis. PCR products were inserted into pCR4-TOPO® plasmids and subsequently transformed into chemically competent OneShot®TOP10 E. Coli using the TOPO TA cloning kit (Invitrogen, USA), according to the manufacturers instructions. The E. coli were grown at 37°C on LB agar plates containing 50 µg/mL kanamycin, to select for those cells containing plasmids. Individual colonies were picked and used to inoculate liquid TB dry® (MoBio, USA) media, and regrown in a shaking incubator at 37°C and 200 rpm. Plasmids were isolated from liquid cultures using the MoBio UltraClean Mini Plasmid Prep kit (MoBio USA), according to the manufacturers instructions. Plasmid extractions were verified by gel electrophoresis. Glycerol stocks (10% w/v) of each liquid culture were preserved and stored at -80°C.

**Sequencing and Phylogenetic Analysis**

16S rDNA clones were sequenced at the Pennsylvania State University Biotechnology Center using T3 and T7 plasmid-specific primers. Sequences were assembled using CodonCode Aligner v.1.4.1 (CodonCode Corp., USA) with Phred base calling, and hand checked for accuracy. Full sequences were added into ARB (Ludwig et al. 2004) using the parsimony quick add function, and carefully aligned manually with similar sequences. Chimera checks were performed online using CHIMERA_CHECK.
Similar sequences in public databases were identified using BLAST software (Altschul et al., 1990). Phylogenetic analyses were then performed using closely related, full-length clones from sulfidic caves, other closely related species, and isolated species representative of major groupings in addition to snottite sequences. Sequences were aligned in ARB and exported to PAUP* version 4.0b10 (Swofford, 2000) and MrBayes version 3.0b4 (Huelsenback and Ronquist, 2000) where Bayesian analysis, neighbor joining, and maximum parsimony phylogenetic analyses were performed. Bayesian analyses were run for 500,000 generations with 4 independent chains, under the general time reversible model and a gamma distributed rate variation. Trees were produced every 100 generations, and the first 20% of the trees were discarded. A consensus tree was computed in *PAUP by 50% majority rule. Maximum parsimony (heuristic search) and neighbor joining bootstrap trees were created in PAUP*, each with 500 bootstrap replicates. Phylogenetic trees were created following suggestions by Hall (2001).

Rarefaction analyses and Chao1 analysis of the clone libraries were computed using EstimateS 7.5.0 (Colwell, 2005). Operational taxonomic units were defined by >98% sequence similarity. Confidence intervals were calculated by log transformation, and are based on 100 randomizations.

**Fluorescence In Situ Hybridization (FISH) and Cell Counting**

After fixation in paraformaldehyde (PFA), samples were applied to 10 well Teflon coated slides, allowed to dry, and then dehydrated in three washes, three minutes
each, in 50%, 80%, and 90% ethanol. Positive and negative control cells for each FISH probe were included on the slide. Hybridization of each slide was performed as follows: 8 μL of hybridization buffer (0.9M NaCl, 20mM Tris/HCL pH 7.4, 0.01% sodium dodecyl sulfate (SDS), and a varying formamide stringency) were added to each well, followed by 25 ng of each probe. See table 1 for a full list of FISH probes and required formamide stringency. Probes were manufactured at Sigma-Genosys (USA), and the specificity of each probe was checked using the PROBE_MATCH tool in ARB. Slides were incubated for two hours at 46°C in a “hybridization chamber,” a 50mL tube with a tissue soaked in hybridization buffer. Slides were then moved to a wash buffer (20 mM Tris/HCL pH 7.4, 0.01% SDS, and NaCl of varying concentration, depending on formamide stringency of the hybridization buffer (determined by Lathe, 1985)) for 15 minutes at 48°C. Slides were rinsed with distilled water, air dried, and then counterstained with 4’,6’-diamidino-2-phenylindole (DAPI), which fluorescently labels DNA. Slides were rinsed, air dried, and then mounted with Vectashield H-1000 mounting medium for fluorescence (Vectashield Laboratories, USA), and viewed with a Nikon E800 epifluorescence microscope. Photos were taken with a CCD black and white digital camera.

Counting of fluorescently labeled cells was done from photographs taken under 1000x magnification. Black and white photos of the same field under different filter sets were merged in Adobe Photoshop. We chose to do counts from photographs because we could easily check that probe-marked cells were also DAPI-labeled, without the DAPI filter weakening the fluorescent probe signals. To insure objectivity in counts, photos for counting were taken without prior examination of the field of view under more than one
filter. Probe-labeled cells were only counted if they were stained by DAPI as well. Probe-labeled cells were calculated as a percentage of the total DAPI stained cells, and these percentages were averaged over the number of photos counted. Because of high cell densities in most samples, only 25% of the field was counted (divided using the grid function in Photoshop). Between 717 and 1225 DAPI stained cells were counted for each probe combination for each sample. Fewer cells, as low as 439, were counted from the PC1 May sample, due to very low cell densities in this sample.

**Culturing and Isolation**

We cultured and isolated microorganisms from RS2 snottites. Samples for cloning were collected in May 2005 and stored at 4°C. The cultures were grown in 1353 ATCC *Thiobacillus albertensis* media. Liquid media (1x) was created as follows: 5.0 g Na₂S₂O₃, 3.0 g KH₂PO₄, 0.4 g (NH₄)₂SO₄, 0.5 g MgSO₄•7H₂O, 0.25 g CaCl₂•2H₂O, and 0.010 g FeSO₄•7H₂O were mixed in 1 L 18MΩ H₂O. The media was adjusted to a final pH of 4.5 using 1N NaOH, and was autoclaved at 121°C for 20 minutes. Liquid cultures were inoculated, and incubated at 30°C and shaken at 100 rpm. Solid media were created following the same recipe, 2X strength with 3% agar at pH 4, and cooled in Petri dishes.

Cultures were initially grown in liquid media, and passaged every 5 or 6 days. For isolation, we plated three different volumes (1, 5, and 10 mL) of liquid media onto solid media plates, grew them up, and individual colonies were picked and regrown in liquid media. This was repeated 3 times. Isolation was verified with FISH.
RESULTS

Geochemistry

Cave water chemistry data is summarized in table 2. \( \text{H}_2\text{S} \) concentrations were generally high (50-390 µM) in cave waters while \( \text{O}_2 \) was generally low (below 12 µM). Cave air chemistry data is summarized in table 3.

Both physical and chemical constraints appear to govern snottite occurrence. Snottites were only found suspended from overhanging surfaces, and they were never found below the high water mark. Furthermore, they were only found in regions with high cave air \( \text{H}_2\text{S} \) concentration. RS2 snottites were very abundant, and coated most available overhanging surfaces at the sample site. Concentration of \( \text{H}_2\text{S} \) was 24 ppm where snottites were most abundant, one meter above the stream. RS1 snottites occurred about 4 meters above the cave stream, where \( \text{H}_2\text{S} \) concentration was 3.4 ppm (concentration measured 20 ppm one meter above the stream). No overhangs were present below four meters at RS1. \( \text{H}_2\text{S} \) concentrations near PC1 snottites were the lowest of the three snottite types, between 0.2 and 2 ppm. PC1 snottites occurred in Pozzo dei Cristalli along the cave stream between the high water mark and 1.5 meters above the cave stream, where \( \text{H}_2\text{S} \) concentrations dropped below 0.2 ppm.

On the May sampling trip, all snottite types had pH values ranging between 0 and 2. Average pH was 1.5 for RS2 snottites, and 1.8 for RS1 snottites. Snottites were more acidic on the August sampling trip; RS1 and RS2 snottites all had pH values <1, and PC1 snottites had pH between 0 and 1.5. Some PC1 snottites had higher pH values (up to 3) but these snottites were not collected.
### TABLE 2. SUMMARY OF RAMO SULFUREO AND POZZO DEI CRISTALLI CAVE WATER CHEMISTRY

<table>
<thead>
<tr>
<th>Location</th>
<th>PC1 (8/5/2005)</th>
<th>PC1 (8/18/2005)</th>
<th>PC1 (5/23/2005)</th>
<th>RS1 (8/7/2005)</th>
<th>RS1 (5/27/2005)</th>
<th>Ramo Sulfureo (11/2000-12/2001)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>7.5</td>
<td>7.3</td>
<td>7.3</td>
<td>7.3</td>
<td>7.4</td>
<td>7.2-7.6</td>
</tr>
<tr>
<td>cond (µS/cm)</td>
<td>2550</td>
<td>2530</td>
<td>2480</td>
<td>1823</td>
<td>1370</td>
<td>1052-1849</td>
</tr>
<tr>
<td>temp (°C)</td>
<td>13.3</td>
<td>13.2</td>
<td>13.8</td>
<td>13.5</td>
<td>13.3</td>
<td>13.5 (average)</td>
</tr>
<tr>
<td>(O_2) (µM)</td>
<td>5.2</td>
<td>-</td>
<td>0.8</td>
<td>2.6</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>(S^{2-}) (µM)</td>
<td>379</td>
<td>307</td>
<td>159</td>
<td>212</td>
<td>&lt;0.2</td>
<td>50-390</td>
</tr>
<tr>
<td>(NH_4^+) (µM)</td>
<td>233</td>
<td>116</td>
<td>83</td>
<td>67</td>
<td>35</td>
<td>-</td>
</tr>
<tr>
<td>(SO_4^{2-}) (µM)</td>
<td>781</td>
<td>1750</td>
<td>1708</td>
<td>1302</td>
<td>1427</td>
<td>800-1700</td>
</tr>
<tr>
<td>(NO_3^-) (µM)</td>
<td>&lt;0.7</td>
<td>&lt;0.7</td>
<td>-</td>
<td>&lt;0.7</td>
<td>&lt;2.0</td>
<td>-</td>
</tr>
<tr>
<td>(NO_2^-) (µM)</td>
<td>&lt;2.0</td>
<td>&lt;2.0</td>
<td>-</td>
<td>&lt;2.0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>(Ca^{2+}) (µM)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>2.5-3.0</td>
</tr>
<tr>
<td>(Mg^{2+}) (µM)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.4-0.9</td>
</tr>
<tr>
<td>(Na^+) (µM)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>6.1-13.3</td>
</tr>
<tr>
<td>(K^+) (µM)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.2-0.4</td>
</tr>
</tbody>
</table>

\(\dagger\)Data from Cocchioni et al. (2003)

### TABLE 3. CAVE ATMOSPHERE \(H_2S(g)\) AND \(CO_2(g)\) MEASUREMENTS \(\dagger\)

<table>
<thead>
<tr>
<th>Location (\dagger)</th>
<th>PC1 (\dagger)</th>
<th>RS1 (\dagger)</th>
<th>RS2 (\dagger)</th>
<th>Upper #</th>
</tr>
</thead>
<tbody>
<tr>
<td>(H_2S) (ppm)</td>
<td>0.2-1.9</td>
<td>3.4</td>
<td>24</td>
<td>not detected</td>
</tr>
<tr>
<td>(CO_2) (ppm)</td>
<td>1200</td>
<td>&gt;3000</td>
<td>&gt;3000</td>
<td>1300</td>
</tr>
</tbody>
</table>

\(\dagger\)NH4 and NO2 were also sampled, but were not present in detectable quantities at these sites.

\(\dagger\)PC1 measurements were taken 8/5/2005, and the other three sites on 8/7/2005

\(\dagger\)Measurements taken near areas of highest snottle density: PC1 at 0.5-1.5 m above cave stream, RS1 at 4 m, and RS2 at 1 m.

\#Measurements from non-sulfidic cave region (~40 meters above water table).
Because snottites were found in a distinct “band” (height 0.5 m to 1.5 m) along the cave wall at PC1, it made sense to measure the sulfide concentration at the lower and uppermost heights. Results are shown in figure 4: hydrogen sulfide concentration decreased rapidly from above 6 ppm at the water surface to 0.2 ppm at 1.5 meters. No snottites were found above this height. No snottites were found on the cave wall below the high-water mark, at about 0.5 meters. (This height marked an abrupt transition between exposed limestone below (pH 6-7) and gypsum wall coatings above (pH 2).)

Because the cave stream at PC1 is narrow and flows linearly from its resurgence point for several meters, we were able to calculate sulfide loss over time by a simple “wine cork” experiment. Sulfide concentration was measured at different points in the stream, surface flow velocity was calculated by timing a wine cork between two points, and average stream velocity was estimated by multiplying average surface velocity by 0.8. Results are shown in figure 5. Hydrogen sulfide concentration appears to decrease linearly with time from the resurgence point (R$^2 = 0.88$).

16S rDNA Sequence Analysis

Two clone libraries were created from RS2 snottites, one using a Bacteria-specific primer set and one using a universal primer set. We obtained 106 complete sequences total, 55 and 51 from each library, respectively. A third Bacterial clone library was created from PC1 snottites, from which we obtained 71 complete sequences. Taxonomy of 16S rDNA sequences, based on BLAST results and phylogenetic analyses, are summarized in table 4. Placement of clones in a tree of life showing major lineages in all
Figure 4. A graph of cave air hydrogen sulfide concentration by height above the stream, in Pozzo dei Cristalli. Approximate best-fit curve is shown.

Figure 5. Graph showing hydrogen sulfide loss from the cave stream by time since resurgence, in Pozzo dei Cristalli. Aqueous hydrogen sulfide measurements were taken at 4 different distances, and time was calculated based on a mean flow rate of the stream (wine cork experiment). Best-fit line is shown ($r^2 = 0.88$).
domains is shown in figure 6. Detailed taxonomic analyses using Bayesian, maximum parsimony, and neighbor joining methods are shown in figures 7 and 8.

All 71 clones from PC1 snottites represent a single phylotype (>98% sequence similarity) in the genus *Acidithiobacillus*. RS2 snottites revealed a more diverse species assemblage. Within the Bacterial domain, one phylotype each grouped within the genera *Acidithiobacillus* (γ-proteobacteria), *Acidimicrobium* (Actinobacteria), and *Sulfobacillus* (Firmicutes). There were also five clones, representing three phylotypes, which grouped within the Bacterial lineage TM6 from which no species have been isolated. We also retrieved three mitochondria of unknown Eukaryote hosts. RS2 snottites also returned one phylotype from each of two different lineages within Archaeal family Thermoplasmatales, genera *Ferroplasma* and *Thermoplasma*. Clones from the Bacterial genus *Acidithiobacillus* made up the majority of RS2 sequences, followed by clones from *Acidimicrobium*. In total, seven Bacterial and two Archaeal phylotypes were found in RS2 snottites, and one Bacterial phylotype in PC1 snottites. Of the RS2 snottites, three phylotypes (*Sulfobacillus* and two TM6 phylotypes) were only represented by only one clone.

Rarefaction of PC1 and RS2 clone libraries show that that snottites are the lowest-diversity microbial communities sampled from Frasassi (fig. 9a). RS2 is represented by two rarefaction curves, one representing only the Bacterial clone library (labeled RS2 B) and one including all clones from both the Bacterial and universal libraries (labeled RS2 all). Other Frasassi clone libraries included in the rarefaction, Mat1, 2, and biovermiculation clone libraries, are all Bacterial libraries (from Macalady et al., pers. com.). The error bars on the rarefaction curves indicate 95% confidence intervals,
<table>
<thead>
<tr>
<th>Snottite</th>
<th>PCR forward primer$^1$</th>
<th>Taxonomy of nearest relatives$^2$</th>
<th>No. of clones</th>
<th>Representative clones$^3$</th>
<th>Inferred physiology $^4$</th>
</tr>
</thead>
<tbody>
<tr>
<td>PC1</td>
<td>Bacterial 27F</td>
<td><strong>Bacteria</strong>&lt;br&gt;Gamma-Proteobacteria&lt;br&gt;Genus <em>Acidithiobacillus</em></td>
<td>71</td>
<td>FS5, FS34</td>
<td>Sulfur oxidizer, primary producer</td>
</tr>
<tr>
<td>RS2</td>
<td>Bacterial 27F</td>
<td><strong>Bacteria</strong>&lt;br&gt;Gamma-Proteobacteria&lt;br&gt;Genus <em>Acidithiobacillus</em>&lt;br&gt;Actinobacteria&lt;br&gt;Genus <em>Acidimicrobium</em>&lt;br&gt;Firmicutes&lt;br&gt;Genus <em>Sulfobacillus</em>&lt;br&gt;TM6 lineage</td>
<td>34, 17, 3</td>
<td>DSJB6, DSJB16, DSJB2, DSJB22, DSJB94, DSJB30</td>
<td>Sulfur oxidizer, primary producer, sulfur oxidizer? Unknown</td>
</tr>
<tr>
<td>RS2</td>
<td>Universal 533F</td>
<td><strong>Bacteria</strong>&lt;br&gt;Gamma-Proteobacteria&lt;br&gt;Genus <em>Acidithiobacillus</em>&lt;br&gt;Actinobacteria&lt;br&gt;Genus <em>Acidimicrobium</em>&lt;br&gt;TM6 lineage&lt;br&gt;Alpha-Proteobacteria&lt;br&gt;Mitochondria</td>
<td>34, 5, 2, 3</td>
<td>DSJB2, DSJB22, DSJB94, DSJB30, DSJA62, DSJA4, DSJA51</td>
<td>Sulfur oxidizer, primary producer, unknown, organelle</td>
</tr>
</tbody>
</table>

$^1$ The universal reverse primer 1492R was used with all libraries
$^2$ Based on BLAST searches and phylogenetic analyses
$^3$ Clones that represent the phylotype in phylogenetic analyses
$^4$ Based on phylogenetic analyses
Figure 6. Phylogenetic 16S rDNA tree representing major lineages in all domains of life. Placement of snottite clones is indicated by red circles. Modified from Macalady and Banfield (2003).

Figure 7. Bayesian analysis phylogram showing the placement of Archaeal 16S rDNA sequences (in bold) within the Thermoplasmales family. GenBank accession numbers, when available, are listed with the sequence names. DSJA sequences are from snottite sample RS2. Black circles (●) indicate nodes supported by Bayesian posterior probability >75%, and small red circles (○) indicate nodes that are supported by Bayesian posterior probability, maximum parsimony bootstrap, and neighbor joining bootstrap values >75%.

Figure 8. Bayesian analysis phylograms showing the placement of Bacterial 16S rDNA sequences (in bold) within the A) γ-Proteobacteria, B) Actinobacteria, C) Mitochondria (α-Proteobacteria), D) TM6 lineage, and E) Firmicutes. DSJA and DSJB sequences are from snottite sample RS2, and FS sequences are from snottite sample PC1. GenBank accession numbers, when available, are listed with the sequence names. In the mitochondrial tree (C), the taxa of the host Eukaryotic cells are labeled at the right. Black circles (●) indicate nodes supported by Bayesian posterior probability >75%, open triangles (△) indicate support by maximum parsimony bootstrap values >75%, open circles (○) indicate support by neighbor joining bootstrap values >75%, and small red circles (●) indicate nodes that are supported by all three methods >75%.

Figure 9. Statistical measures of snottite microbial diversity, based on 16S rDNA clone libraries. Operational taxonomic units (OTUs) are defined by 98% sequence similarity. (A) Rarefaction curves comparing observed microbial richness of different cave microbial communities (other clone libraries from Macalady et al., pers. com.). Snottite libraries are represented as follows: PC1 is the PC1 Bacterial library (open light diamonds, n = 71, 1 OTU), RS2 B is the RS2 Bacterial library (closed light diamonds, n = 54, 5 OTUs), and RS2 A1 is a combination of both the RS2 Bacterial and universal clone libraries (black diamonds, n = 107, 9 OTUs). The other cave communities are represented as follows: A biovermiculation library (closed triangles, n = 67, 48 OTUs), and two stream biofilm libraries, mat 1 (closed circles, n = 86, 56 OTUs) and mat 2 (open circles, n = 78, 36 OTUs). Error bars represent 95% confidence intervals based on variance of OTUs drawn (y-axis) by clone library size (x-axis), from 100 randomizations/sample size. (B) Chao1 estimates of total community diversity as a function of number of clones sampled. Samples PC1 and RS2 A1 (similar parameters as in A, only positive error bars are shown) are represented, as is mat 1 (only negative error bars shown) for comparison. Error bars are 95% confidence intervals.
Figure 6

Figure 7

Thermoplasmata

Thermoplasma acidophilum M20822
Thermoplasma volcanium AJ299215

Picrophilus oshimae X84901

Copper bioleaching clone AJ003138
Ferroplasma acidarmanus AF145441
Ferroplasma acidophilium AJ224936

DSJA4

Acid mine drainage clone AF225459
Hot spring clone AF232925

DSJA51

Ferroplasma

Sulfolobus solfataricus X03235

Pyrobaculum aerophilum L07510

50 changes

Out-groups
Figure 8 (A-B)

A

- *Halothiobacillus hydrothermalis* M90662
- *Halothiobacillus kellyi* AF170419
- Snotite isolate Fras1C AF213054
- *Psychrobacter immobiliis* U39399
- *Moraxella lacunata* B64049
- *Beggiaota alba* L40994
- *Thioploca ingrica* L40998
- *Nitrosococcus oceanus* M96395
- Acid mine drainage clone X86769
- *Acidithiobacillus ferrooxidans* M79402
- *Acidithiobacillus thiooxidans* M79396
- Snotite clone Fras2 AF213056
- FS5
- FS34
- DSJB6
- DSJB16
- *Acidithiobacillus caldus* Z29975
- *Thiobacillus tepidarius* M79424
- *Desulfovibrio desulfuricans* M34113
- *Desulfobulbus rhadoformis* U12253
- 50 changes

B

- *Escherichia coli* L10328
- *Rhizobium leguminosarum* D12782
- *Mycobacterium chitae* M29560
- *Propioniferax innocua* S93388
- *Streptomyces lavendulae* D85112
- *Microthrix parvicella* X82546
- *Acidimicrobium ferrooxidans* U75647
- DSJB2
- DSJB22
- Peat bog clone TM62
- Peat bog clone TM56
- Aquaculture pond clone GCM079
- Deep-sea sediment clone AB015539
- *Atopobium minutum* X67145
- *Eubacterium fssor* L34620
- *Rubrobacter radiotolerans* U65647
- Wall surface clone AJ298579
- 50 changes

Out-groups

Out-groups

Halothiobacillus

Acidithiobacillus

Out-groups

Acidimicrobium
determined from variance of the observed number of operational taxonomic units (OTUs, equivalent to phylotypes) by number of clones sampled based on 100 randomizations. In short, they indicate variance within the clone library but do not describe how well-sampled the community is. Chao1 analysis (fig. 9b), a nonparametric total-community diversity estimator, estimated that RS2 snottites contain 10 ± 5 OTUs. PC1 snottites were estimated to contain 1 Bacterial phylotype. Error bars in figure 9b are 95% confidence intervals.

**Fluorescence Microscopy and Cell Counting**

We assessed microbial community composition of all three snottite types using FISH experiments. Community composition results from cell counts are shown in figure 10. Selected FISH photomicrographs are shown in figure 11. Bacteria-specific probe EubMix (a mixture of probes Eub338, Eub338-2, and Eub338-3) and Archaea-specific probe Arch915 were used to assess relative abundances of Bacteria versus Archaea. Genus-specific probes were used to measure the abundance of the dominant species found in our clone libraries. *Acidithiobacillus*-specific probe Thio1, *Acidimicrobium*-specific probe ACM732, and *Ferroplasma*-specific probe FER656 were all used (for full probe details, see table 1). The probe Gam42a, specific for Bacteria in the $ g \ $ proteobacteria lineage, confirmed that *Acidithiobacillus* were the only $ g \ $ proteobacteria species present. DAPI, which fluorescently labels DNA, was used as a counter stain in all samples.

Snottite samples were highly spatially variable. Many inactive regions with weakly fluorescent cells and empty sheaths were intermixed with active, brightly-
Figure 10. Microbial community composition results from FISH cell counts for all three Frasassi snottite types collected in May and August 2005. Count data are expressed as percentages of total DAPI stained cells. Error bars represent one standard deviation.

Figure 11. FISH images of Frasassi snottites. Photomicrographs are labeled with the snottite type and FISH probes used (see table 1 for probe details). Scale bar is 5 µm. (A and B) “Clumps” of coccoid and irregular Archaea, red. Note presence of protists and fungi in (B), indicated by white arrows. (C) High cell-density region with abundant coccoid-shaped Ferroplasma cells. (D and E) Good images of Eukaryotic cells, a fungal filament in (D) and fungal spores in (E), indicated by arrows. (F and G) High cell density regions with abundant thin, short filament-shaped Acidimicrobiurn (red). (F and I) Rod-shaped Acidithiobacillus forming filaments. Note the non-Thio1 labeled, fat rod-shaped cells, indicated by arrows.
fluorescing regions. Samples of the same snottite type were also spatially variable with respect to species distribution. Thio1-labeled cells were generally ubiquitous, but Arch915-, Fer656-, and Acm732-labeled cells often occurred in dense clusters (e.g. 11a, b, c, f). Interestingly, no obvious differences were noted between the May and August samples of same snottite type, except with respect to the presence vs. absence of Eukaryotes. Eukaryotes were absent in PC1 and RS1 May samples, and present in August samples.

In the active regions, most DAPI-stained cells (>75% in all samples) hybridized with either EubMix or Arch915. Three different EubMix morphologies were observed. Thio1-hybridizing cells were short rods (1-2μm) (fig. 11h, i), Acm732-hybridizing cells were short thin filaments, (3-6μm) (fig. 11f, g), and other EubMix-hybridizing Bacteria (not Thio-1 or Acm732) are generally fat rods (2μm), significantly larger than Thio1-hybridizing cells (fig. 11h, i). Two different Arch915 morphologies were observed. Large, irregularly-shaped cells (1-3μm) that generally hybridized with the Fer656 probe (fig. 11b), and coccoid-shaped cells of different sizes (<1-2μm) that only sometimes hybridized with Fer656 (fig. 11a, c). There were also different types of Eukaryotes (fig. 11b, d, and e.): large round cells (>5μm) with an obvious nucleus, presumably protists (fig. 11b); long, 2μm thick filaments with evenly spaced nuclei, likely fungi (fig. 11d); and round, autofluorescent (>5μm) spores (fig. 11e).

PC1-snottites were the least diverse, with nearly all active cells binding to Thio1. The May sample had no Eukaryotic cells present, but the August sample had both fungal filaments and spores, and abundant protists. The Thio1-hybridizing cells were
occasionally bound together into filaments in the August sample (fig. 11h, i), but this was never observed in the May sample.

RS1-snottites were more diverse. Thio1-hybridizing cells were the most abundant, but about 6% Acm732-hybridizing and other EubMix fluorescing-cells were present. Nearly 40% of the community was Arch915-hybridizing, but only half of the amount of Arch915-hybridizing cells fluoresced under Fer656. Three different Arch915 morphologies were observed: large, irregularly-shaped cells (fig. 11b), large coccoid-shaped cells (fig. 11a), and small coccoid-shaped cells (fig. 11c). Fer656 bound consistently to the large, irregularly-shaped cells, sometimes to the small coccoid-shaped cells, and rarely to the large-coccoid shaped cells. Eukaryotes were abundant in the August sample, but very few protists and no fungi were present in the May sample. Thio1-hybridizing cells sometimes formed filaments in the August sample, but never in the May sample.

RS2 snottites are similar in diversity to RS1 snottites. Thio1-labeled cells dominated this sample. However, Acm732-hybridizing cells were more abundant in RS2 snottites than the other two snottite types. Other EubMix-hybridizing cells made up approximately 3% of total DAPI stained cells. About 17% of DAPI-stained cells hybridized with Arch915, most of which bound to Fer656. However, there were a significant component (6%) of other Arch915-labeled cells that did not bind to Fer656. In the August sample, no Fer656 results are reported because we had problems with the probe for this particular sample. Eukaryotes were numerous in both August and May samples, and Thio1-labeled cells were commonly bound together in filaments in both August and May samples (fig. 11i, h).
**Culturing and Isolation**

We cultured and isolated species of *Acidithiobacillus*, which was verified using FISH probe Thio1. The isolated strain, which has not yet been sequenced, appears as small rod-shaped cells. In early stages of culturing, some *Acidithiobacillus* cells were observed with fluorescent microscopy to be producing extra-cellular polymers (slime).

**DISCUSSION**

**Microbial Ecology**

Based on 16S rDNA clone libraries and FISH experiments, the microbial communities of all three Frasassi snottite types are not only similar to each other, but also very similar to snottite communities from other sulfidic caves (Hose et al., 2000; Vlasceanu et al., 2000; Jones et al., 2005). Furthermore, the snottites all exhibit very low species diversity (fig. 9). PC1 snottites contain predominantly *Acidithiobacillus*, with a few Archaeal and Eukaryotic cells. RS1 and RS2 snottites, located in a separate section of Frasassi, are also dominated by *Acidithiobacillus*. However, *Acidimicrobium*, *Ferroplasma*, and other Archaea are also present, along with a few *Sulfobacillus* and TM6 representatives. In general, the three snottite types harbor very similar microbial communities even though they occur in different chemical environments (table 3) and hang from different substrates (fig. 3). Vlasceanu et al. (2000) sampled snottites from Frasassi as well, and found genera *Acidithiobacillus* (clone Fras1; see 8a), *Sulfobacillus* (clone Fras2; figure 8e), and *Halothiobacillus* (Strain1C; figure 8e). Hose et al. (2000)
studied snottites from Cueva de la Villa Luz near Tabasco, Mexico, and cloned Bacterial genera *Acidithiobacillus* and *Acidimicrobium*. Jones et al. (2005) found similar *Ferroplasma*- and *Acidithiobacillus*-dominated snottites in Rio Garaffo Cave, central Italy, approximately 75 km from Frasassi.

The majority of sequences in each of our three clone libraries represent a single phylotype of the genus *Acidithiobacillus* (previously *Thiobacillus*), most similar to *Acidithiobacillus thiooxidans* (fig. 8a). FISH analyses with probe Thio1 show that *Acidithiobacillus* sp. are ubiquitous throughout all three snottite types. Members of genus *Acidithiobacillus* are acidophilic sulfur- and iron-oxidizing lithoautotrophs that can be easily cultured from a variety of acidic environments, and *Acidithiobacillus thiooxidans* is one sulfur-oxidizing member (Waksman and Joffe, 1922; Konishi et al., 1995; Kelly and Wood, 2000). In some snottite samples, *Acidithiobacillus* are linked together into filaments (fig. 11h, i). To our knowledge, this morphology has not been previously reported for *Acidithiobacillus*. Given its dominance not only in the snottites described here but also in those described by Hose et al. (2000) and Vlasceanu et al. (2000), *Acidithiobacillus* likely represents a keystone sulfide-oxidizing and carbon-fixing primary producer in snottite microbial communities.

We also retrieved a number of Bacterial sequences related to the genus *Acidimicrobium*. FISH analyses reveal that *Acidimicrobium* are an important component of RS1 and RS2 microbial communities (4 and 8%). The genus *Acidimicrobium* has only one named species, *A. ferrooxidans*, which is an iron-oxidizing, thermoacidophile with the capacity to grow both autotrophically and heterotrophically (Clark and Norris, 1996; Johnson, 1998; Stackebrandt and Schumann, 2000). *A. ferrooxidans* is the most similar
isolate to our sequences (fig. 8b), but is only 91% similar by ARB Neighbor Joining similarity analysis. Given this low sequence similarity and the lack of a significant iron source in Frasassi (Galdenzi and Maruoka, 2003), the Acidimicrobium phylotype cloned here is likely a novel species with different physiology.

A single sequence was related to Sulfolobus (fig. 8e), a genus of sulfur- and iron-oxidizing acidophiles that can be either auto- or heterotrophic (Tourova et al., 1995; Dufresne et al., 1996; Norris et al., 1996; Johnson, 1998). Sulfolobus species have been found in many different acidic environments, including one clone from Frasassi (Fras1) by Vlasceanu et al. (2000). By ARB neighbor joining similarity analysis, our sequence is only 93% similar to any Sulfolobus isolate, but is >98% similar to Fras1. Although we only retrieved a single sequence of this phylotype in our study, Vlasceanu et al. retrieved multiple sequences, indicating that Sulfolobus sp. may play a more significant role in other Frasassi snottites that were not sampled in this study.

The remaining Bacterial sequences group within the TM6 lineage (fig. 8d). TM6 is a lineage based purely on environmental clone DNA sequences. No members of this major lineage have been isolated, so it has yet to be assigned a true name (Hugenholtz et al., 1998). We retrieved three different TM6 phylotypes. Because no members of TM6 has yet been cultured, we cannot infer any physiology based on taxonomy.

We retrieved two different Archaeal phylotypes from the RS2 universal clone library. Both group within the Euryarchaeal family Thermoplasmales. One phylotype is most similar to genus Ferroplasma, and the second is most similar to genus Thermoplasma. Ferroplasma are iron-oxidizing acidophiles, with species that are either strictly autotrophic (Golyshina et al., 2000) or both auto- and heterotrophic (Edwards et
Thermoplasma isolates are organoheterotrophic acidophiles (Ruepp et al., 2000; Madigan et al., 2003), although *T. acidiphilum* is able to use elemental sulfur to grow anaerobically (Gonzalez-Toril et al., 2003). We started, but have not yet completed, carbon uptake experiments using $^{13}$C enriched bicarbonate. Results from this experiment will determine whether the snottite Archaea are primary carbon producers, as is *Acidithiobacillus*.

Archaea vary in abundance and diversity among all three snottite types. PC1 contains very few active Archaea detectable by FISH. RS2 contains roughly 18% Archaea detectable with probe Arch915, two-thirds of which hybridize with *Ferroplasma*-specific probe Fer656. RS1 contains a larger proportion Archaea (~38%), slightly more than half of which are *Ferroplasma*. A significant portion of non-*Ferroplasma* Archaea exist in RS1 and RS2, perhaps representing the *Thermoplasma* phylotype that we sequenced. Because no FISH analysis was performed with a *Thermoplasma*-specific probe, we cannot draw any conclusions about their taxonomy. In the future, we plan to perform 16S rDNA and FISH analyses specifically targeting the Archaeal constituent of the three snottite types, especially in sample RS1 where Archaea constitute almost 40% of the community. Interestingly, FISH analysis of small white snottites from the Rio Garaffo cave complex, a much warmer cave with higher sulfide levels near Acquasanta Terme, central Italy (Galdenzi and Menichetti, 1995), revealed a microbial community dominated (up to 80%) by *Ferroplasma* species (Jones et al., 2005).

The protists and fungi inhabiting snottites are likely organoheterotrophic, as are all known protists and fungi (Madigan et al., 2003), and so they must be preying on
snottite prokaryotes. Interestingly, *Acidithiobacillus* commonly formed filaments (fig. 11h, i) in all samples with Eukaryotes present (all except PC1 and RS2 May samples). Filament formation by prokaryotes is often a strategy to avoid predation (Sommaruga et al., 1995; Hahn et al., 1999), and our observations appear to support this theory.

The snottite microbial communities appear to be part of the lithotrophic base of the cave food web. Vlasceanu et al. (2000) performed $^{13}$C and $^{15}$N analyses on Frasassi snottite samples, white stream mats, cave invertebrates, and surface samples from outside the caves (fig. 12). Their analyses show that snottites have the lowest $^{15}$N ratios found in the cave (see also Stern et al., 2003). Cave wall macroinvertebrates also have low $^{15}$N values. From this, Vlasceanu et al. (2000) conclude that wall macroinvertebrates must be feeding on the snottites. Hose et al. (2000) found mites living amongst Cueva de Villa Luz snottites, and we observed invertebrates, commonly spiders, living in close proximity to the Frasassi snottites (note spider webs in figure 3).

**Microbial Community Diversity**

Snottite microbial communities are among the least diverse biological communities known (Hose and Macalady, 2006). The diversity observed in our clone libraries includes a single (Bacterial) phylotype in PC1 snottites and nine different Bacterial and Archaeal phylotypes in RS2. In comparison, Torsvik et al. (1990) found over 4000 species of Bacteria in several grams of beech forest soil. Even when compared to other microbial communities from Frasassi, snottites have the least number of phylotypes (Macalady et al., pers. com.). For example, note the relatively high diversity of biovermiculation and stream biofilm clone libraries shown by rarefaction in figure 9.
Figure 12. Carbon and nitrogen stable isotope ratios from cave biota. Snottite samples are labeled accordingly, and stream biofilm samples are labeled "mat." C and C* indicate values from cave invertebrates collected near stream biofilm and snottites, respectively. S indicates surface samples (collected outside of the cave). Modified from Vlasceanu et al. (2000).
We used two different methods to evaluate snottite community diversity and compare this to other cave communities. Rarefaction analyses (Colwell, 2005) (fig. 9a) were used to compare species richness of the snottite clone libraries created here to that of other Frasassi microbial communities (data from Macalady et al., pers. com.). Figure 9a shows rarefaction curves for three different snottite clone libraries, one biovermiculation library, and two stream biofilm clone libraries. These curves show that snottites exhibit the lowest diversity of the different cave communities. To estimate total species richness of the snottites, we used Chao1 (Colwell, 2005), an abundance-based nonparametric microbial diversity estimator (fig. 9b). Chao1 uses clone library data, i.e. phylotypes observed vs. number of sequences cloned, to estimate the total species diversity of the community sampled. Chao1 estimated that PC1 snottites contain 1 phylotype (we found 1), and that RS2 snottites contain 10 ± 5 phylotypes (we found 9). Therefore, our clone libraries adequately represent the total species diversity of RS2 and PC1 snottites, and so it is unlikely that many phylotypes remain to be discovered. For a review on the effectiveness and appropriateness of these two and other microbial diversity estimation techniques, see Hughes et al., (2001).

We hypothesize that the low species diversity observed in snottites is due to their simple geochemistry and very low pH. Hydrogen sulfide appears to be the sole electron donor consistently supplied to snottites in significant quantities, and primary producers produce organic carbon \textit{in situ}. Sulfur-oxidizing microorganisms are very diverse and widespread (Lane et al., 1992), so chemical environment is not the only diversity-limiting factor. The extreme acidity of snottites also likely limits microbial diversity. For example, comparably low community diversity has been observed in acid-mine drainage
environments (Baker and Banfield, 2003; Tyson et al., 2004). Factors affecting microbial diversity are, in general, poorly understood. Contrasting sulfidic cave snottites and higher-diversity cave communities presents an exciting opportunity to elucidate controls on microbial diversity in future research.

**Snottite Formation and Structure**

An interesting hypothesis for the low-microbial diversity in snottites was suggested by Phillip Bennett (pers. com., 12/5/05). He suggested that snottite formation is an adaptive strategy by *Acidithiobacillus*. Hanging separately from the cave walls allows snottites to avoid limestone and gypsum buffering and achieve extremely low pH values (Engel et al., 2001). Under such highly acidic conditions, *Acidithiobacillus* thrives and can outcompete less acid-tolerant species in the microbial community. This could explain the dominance of *Acidithiobacillus* in all snottites sampled by us and by others (Hose et al., 2000; Vlasceanu et al., 2000). Furthermore, in the early stages of culturing *Acidithiobacillus*, we noted that some *Acidithiobacillus* (identified with FISH probe Thio1) were generating abundant extracellular polymers (slime). The second most abundant species from Frasassi snottites, *Ferroplasma* spp., has been shown by Tyson et al. (2004) to lack genes encoding extracellular polymer production. Therefore, because *Acidithiobacillus* are highly abundant in snottites and have been shown to create slime, they are probably at least partially responsible for the formation of snottites.

When performing FISH analyses on Frasassi snottite samples, we noticed that the samples are highly variable combinations of both active and inactive regions. In active regions, cells fluoresce brightly with appropriate FISH probes, indicating the presence of
numerous ribosomes and thus, high cell activity. Inactive areas are either places where cells are dormant, indicated by weak or no fluorescence with FISH, or where empty filaments and sheaths but no cells are found. The presence of active and inactive regions suggests that there is a spatial structure to the snottites. It is reasonable to suggest that the most active areas of the snottites are on the outside, where both hydrogen-sulfide and oxygen diffuse easily. The central region of the snottite would be inactive, because oxygen and hydrogen sulfide would not reach the center as readily due to slow diffusion through the viscous matrix. If this hypothesis has merit, it suggests that snottites grow “outward,” or rather outward and downward due to gravity, because the slime-forming *Acidithiobacillus* would be most active around the perimeter.

**Snottite Chemical Environment**

Snottites are found in subaerial cave regions with high concentrations of hydrogen sulfide, provided there are overhanging surfaces from which to suspend (table 3). Hose et al. (2000) noted that in Cueva de Villa Luz, snottites occurred in regions with the highest hydrogen sulfide gas concentrations. The lowest measured hydrogen sulfide concentration at which snottites were found at Frasassi was 0.2 ppm at PC1. This may define the lower hydrogen sulfide limit for snottite occurrence.

RS1 snottites were suspended from elongate gypsum crystals, whereas RS2 snottites grew suspended from rosette-shaped elemental sulfur crystals. Microbial activity may be responsible for this difference in substrate. Elemental sulfur is often formed as an intermediary in the oxidation of hydrogen sulfide to sulfuric acid, and certain sulfur-oxidizing microorganisms will only oxidize hydrogen sulfide “partway” to elemental
sulfur. Many such organisms will then “finish” oxidizing the elemental sulfur to sulfate when hydrogen sulfide supplies run low (Madigan et al., 2003). The snottites at RS2 occur under much higher concentration of hydrogen sulfide than RS1 snottites, 24 ppm versus 3.4 ppm. Perhaps the sulfur-oxidizers in RS2 snottites have such a plentiful source of hydrogen sulfide that it is only necessary for them to oxidize it partway, whereas it is more favorable for RS1 sulfur-oxidizers to obtain all available energy from hydrogen sulfide by oxidizing it completely.

**Seasonal Variations**

We did not find any striking differences in the snottite prokaryotic community composition between the May and August sampling trips (fig. 10). One subtle difference was that Eukaryotes were present in all three snottite samples in August, but were conspicuously absent in the RS1 and PC1 May samples.

An important geochemical difference between the May and August samples is the higher pH (1-2) of the May snottites compared to the August samples (0-1). We propose that this results from a changing input of vadose water. The cave water chemistry in Frasassi changes with the seasonal wet/dry fluctuations of central Italy (Sarbu et al., 2000). Higher contribution of vadose water means increased dissolved oxygen input to the cave ecosystem. We propose that this higher oxygen input allows the white stream biofilm communities to flourish and consume more aqueous hydrogen sulfide. Thus, less hydrogen sulfide volatizes into the cave atmosphere, snottite sulfide oxidizers create less sulfuric acid, and snottite pH is higher.
**Snottite Contributions to Speleogenesis**

Previous literature has suggested that the white stream mat microorganisms play an important role in sulfuric acid speleogenesis (Engel et al., 2004a; Barton et al., 2005; Macalady et al., pers. com.). Engel et al. (2004b) provided particularly convincing evidence that stream biofilms in Kane Cave are responsible for the majority of sulfide loss from the stream, and hence acid formation and limestone dissolution. However, relatively less emphasis has been placed on the importance of subaerial communities (Hose et al., 2000; Vlasceanu et al., 2000).

It is highly likely that subaerial communities do play an important role in limestone dissolution. Egemeier’s (1981) original model of sulfuric acid speleogenesis specifically referred to subaerial cave regions as the areas where cave enlargement occurs. On subaerial surfaces, sulfuric acid reacts with limestone and replaces it with gypsum:

\[
2H^+ + SO_4^{2-} + CaCO_3 + H_2O \rightarrow CaSO_4\cdot2H_2O + CO_2
\]

Gypsum wall crusts build up, eventually detach, fall into the cave stream, and dissolve in the gypsum-undersaturated stream water. Hose et al., (2000) and Vlasceanu et al., (2000) reported that acidic drippings from snottites are a key player in this process. Such drippings have some of the lowest pH values measured in Frasassi. Because *Acidithiobacillus* is so abundant in snottites and has been shown to produce acid in culture in this study and others (Oprime et al., 2001), it and other sulfide oxidizers in the snottites must have an important role in acid creation and thus, cave enlargement.

Galdenzi et al. (1997; reported in English by Sarbu et al., 2000) performed a five-year limestone dissolution experiment in which limestone chips are suspended in both the
Figure 13. Limestone dissolution experiments. (A) Subaerial limestone chips suspended in Ramo Sulfero. Photo by J. Macalady. (B) Photograph of a corroded limestone chip, after it was suspended for 5 years. From Sarbu et al. (2000). (C) Current limestone dissolution experiment in Ramo Sulfero. Some chips are encased in 0.2 mm mesh to prevent microbial colonization.
cave air and in the stream (fig. 13). Subaerial and subaqueous chips dissolved at similar rates, about 15 mg/cm$^2$/yr or 0.05 mm/yr. The dissolution rates of the subaerial chips accelerated throughout the study. Currently, a new experiment to test the microbial contribution to dissolution is underway. Galdenzi et al.’s experiment is being repeated with some limestone chips enclosed in 0.02 mm mesh to prevent microbial colonization (fig. 13c). Results from this experiment will help to elucidate the extent to which microbial processes augment sulfuric acid speleogenesis.

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