Convergent community assembly among globally separated acidic cave biofilms

Daniel Jones[a,b#], Irene Schaperdoth[c], Diana E. Northup[d], Rodolfo Gómez-Cruz[e], and Jennifer L.

Macalady[c]

[a]Earth and Environmental Science, New Mexico Institute of Mining and Technology, Socorro,

NM, USA

[b]National Cave and Karst Research Institute, Carlsbad, NM, USA

[c]Department of Geosciences, The Pennsylvania State University, University Park, PA 16802

[d]Department of Biology, University of New Mexico, Albuquerque, NM, USA

[e]Biological Sciences Academic Division, Autonomous University Juárez de Tabasco (UJAT),

Villahermosa, Centro, Tabasco 86150, Mexico

#Address correspondence to Daniel S. Jones, daniel.s.jones@nmt.edu

Running head: Extreme acidophile community biogeography

Abstract

Acidophilic bacteria and archaea inhabit extreme geochemical 'islands' that can tell us

when and how geographic barriers affect the biogeography of microorganisms. Here we describe

microbial communities from extremely acidic (pH 0-1) biofilms known as "snottites" from

hydrogen sulfide-rich caves around the world. Given the extreme acidity and subsurface location

of these biofilms, and in light of earlier work showing strong geographic patterns among snottite

Acidithiobacillus populations, we investigated their structure and diversity in order to understand

how geography might impact community assembly. We used 16S rRNA gene cloning and

fluorescence *in situ* hybridization (FISH) to investigate 26 snottite samples from four sulfidic

caves in Italy and Mexico. All samples had very low biodiversity and were dominated by sulfur-

oxidizing bacteria in the genus *Acidithiobacillus*. *Ferroplasma* and other archaea in the

Thermoplasmatales ranged from 0 to 50% of total cells, and relatives of the bacterial genera

Acidimicrobium and *Ferrimicrobium* were up to 15% of total cells. Rare phylotypes included

Sulfobacillus spp. and members of the *Dependentiae* and *Saccharibacteria* (formerly TM6 and

TM7). Although the same genera of acidophiles occurred in snottites on separate continents,

most members of those genera represent substantially divergent populations with 16S rRNA

genes that are only 95-98% similar. Our findings are consistent with a model of community

assembly where sulfidic caves are stochastically colonized by microorganisms from local

sources, which are strongly filtered through selection for extreme acid tolerance, and these

different colonization histories are maintained by dispersal restrictions within and among caves.

Importance

Microorganisms that are adapted to extremely acidic conditions, known as extreme

acidophiles, are catalysts for rock weathering, metal cycling, and mineral formation in naturally

acidic environments. They are also important drivers of large-scale industrial processes such as

biomining and contaminant remediation. Understanding the factors that govern their ecology and

distribution can help us better predict and utilize their activities in natural and engineered

systems. However, extremely acidic habitats are unusual in that they are almost always isolated

within circumneutral landscapes. So where did their acid-adapted inhabitants come from, and

how do new colonists arrive and become established? In this study, we took advantage of a

unique natural experiment in Earth's subsurface to show how isolation may have played a role in

the colonization history, community assembly, and diversity of highly acidic microbial biofilms.

Introduction

Extreme acidophiles drive important biogeochemical processes in natural and engineered systems. Acidophilic sulfur- and iron-oxidizing bacteria and archaea speed up the weathering of sulfide minerals in natural ores and mine waste (1, 2), control metal cycling in acidic drainages (3-5), and create secondary mineral deposits in surface and subsurface environments (6, 7). Acidophilic consortia are used for large-scale bioremediation of acidic mine drainage and other heavy metal contaminated environments (8-11), and for industrial processes such as biomining and sulfuric acid production (12-14). Understanding the ecology of organisms in naturally acidic environments can inform microbial behavior in engineered systems and provide ideas for new bioremediation strategies (e.g., 11, 15). Furthermore, acidic environments are useful model systems for studying microbial evolution and ecology because they often contain low biodiversity (16) and represent extreme geochemical "islands" in a sea of neutrophilic habitats (17).

Studies conducted at local scales in acidic systems have identified geochemical factors that influence microbial distribution, including pH, conductivity, and metal concentrations (e.g., 18-25). However, microbial spatial distribution might not only be a result of natural selection by contemporary environmental factors, but also a legacy of past environmental conditions and stochastic colonization events (26). Dispersal barriers limit the movement of new species into an environment, and as a result, communities from the same geographic region are more likely to contain the same organisms because they share a similar biotic history (27-29).

Extremely acidic microbial biofilms form on the walls and ceilings of sulfide-rich caves, in areas where hydrogen sulfide degasses from springs and streams to the cave atmosphere (Figure 1). These biofilms, commonly known as "snottites," have pH values between 0 and 1.5, and are found hanging from the surface of gypsum corrosion residues that isolate them from buffering by the parent limestone. Snottites have been consistently observed where $H_2S(g)$ concentrations are between 0.2 to 25 parts-per-million by volume (ppmv), and rarely occur where concentrations are outside of this range (30, 31). Previous research has shown that snottites are inhabited by very low biodiversity communities that contain abundant *Acidithiobacillus* spp., as well as representatives or close relatives of the genera *Acidimicrobium*, *Sulfobacillus*, *Ferroplasma*, and *Cuniculiplasma* (30-35).

Sulfidic cave snottites present an exceptional opportunity to test how geographic barriers

affect microbial communities. Because snottites occur in stable subsurface environments and

only form on overhanging surfaces within a specific range of $H_2S(g)$ concentrations,

geochemical variability among snottite locations is relatively limited (Figure 1a). Furthermore,

their extremely low pH is a potential barrier to microbial dispersal in addition to the physical

barrier imposed by their subsurface location. In previous research, Jones et al. (36) used high-

resolution genetic analyses to resolve relationships among geographically separated populations

of *Acidithiobacillus spp.* from cave snottites. That work showed that *Acidithiobacillus*

populations from separate cave locations are divergent, and identified a distance-decay

relationship among the most abundant *Acidithiobacillus* spp. that was consistent with restricted

dispersal among cave locations. We therefore hypothesized that geographic isolation would also

affect other organisms in the snottite biofilms, and thereby impact the structure and diversity of

the whole biofilm community. We tested this hypothesis by sampling snottites from four caves

on two continents, and quantitatively comparing community structure and composition using 16S

rRNA gene sequencing and fluorescence *in situ* hybridization (FISH).

Results

Field observations and geochemistry. Snottites were collected from multiple locations

in four sulfidic caves: Cueva de Villa Luz and Cueva Luna Azufre in Tabasco, Mexico, and the

Grotte del Fiume (Frasassi) and Grotta Nuova di Rio Garrafo (Acquasanta Terme) in the Marche

region, Italy. $H_2S(g)$ concentrations at sampling sites ranged from 0.2 to 24 ppmv (Table 1),

except for samples GS07-35 and AS08-5 where $H_2S(g)$ was below detection. Snottites were also

observed (but not collected) in the Yellow Roses room of Cueva de Villa Luz, at $H_2S(g)$

concentrations between 30-50 ppm. $H_2S(g)$ commonly exceeds 80 ppm in the atmosphere of this

enclosed room, and snottites are not observed under these conditions (30, 35). Similarly, no

snottites were observed near a thermal cave stream in Acquasanta, where $H_2S(g)$ concentrations

commonly exceed 100 ppm (37, 38). We have observed that snottites form on a time scale as

short as months to weeks in Frasassi and Acquasanta, and at Villa Luz they have been observed

to form in days (39).

16S rRNA gene libraries. We cloned archaeal and bacterial 16S rRNA genes from six

snottite samples (Table S1). *Acidithiobacillus* phylotypes (Figure 2) were the most abundant

clones in all bacterial libraries, and were the only bacterial sequences retrieved from samples

PC05-1, AS07-3, and AS08-5. The other three libraries contained relatives of the bacterial

genera *Acidimicrobium* and *Ferrimicrobium* (Figure 3). Libraries from samples RS05-2 and

VL07-20 also contained phylotypes of the genus *Sulfobacillus* and candidate phylum

Dependentiae (formerly TM6 (40)) (Figure S1, Figure S2). Samples VL07-20 and LA07-10 also

contained members of the *Saccharibacteria* (formerly candidate phylum TM7 (41)).

Ferroplasma relatives are the most abundant archaeal clones in universal and archaeal-specific

clone libraries (Figure 4). Other archaeal clones are related to "C-plasma" and *Cuniculiplasma*

(formerly "G-plasma" (3, 42)) in the order *Thermoplasmatales* (Figure 4).

Although many of the same microbial genera were present in snottites from both Italy

and Mexico, snottite clones from the two countries have less than 97% 16S rRNA gene sequence

identity (Table S1). *Acidithiobacillus* clones are <95% similar (Figure 2), clones affiliated with

Acidimicrobium/Ferrimicrobium are <95% similar (Figure 3), *Sulfobacillus* clones are <92%

similar (Figure S1), and *Ferroplasma* sequences are <97% similar (Figure 4). The only exception

are C-plasma clones VLSa30 and DSJa51, which share 99.6% similarity (Figure 3, Table S1),

and clone DSJb30 in the *Dependentiae*, which is 97.9-98.1% similar to Villa Luz clones VLSb7,

VLSb29, and VLSb87 (Figure S2).

All of the *Acidithiobacillus* clones from Italy are members of the genus *Acidithiobacillus*

thiooxidans, while all *Acidithiobacillus* clones from the Mexican caves represent a separate

species of *Acidithiobacillus* (referred to as "*At.* group II" in Figure 2, after Jones et al. (2016))

that is more closely related to *At. caldus* than *At. thiooxidans*. In order to see if deeper

sequencing would reveal the presence of *At. thiooxidans* in Cueva de Villa Luz, we generated

much larger rRNA gene libraries from samples collected during a later expedition to Villa Luz.

At. thiooxidans sequences were not detected among 143,944 and 102,229 rRNA gene amplicon

sequences from two snottite samples. Like the clone sequences, *Acidithiobacillus* spp. in these

samples formed a single operational taxonomic unit (OTU) that was similar to clone sequences

in *At.* group II. Other abundant OTUs in the deeper amplicon datasets include members of

groups that were identified in clone libraries (*Acidimicrobiaceae*, *Sulfobacillus*,

Saccharibacteria, *Dependentiae*), as well as other members of the *Gamma-* and

Alphaproteobacteria that were not identified in the clones libraries, including an abundant OTU

of *Metallibacterium* and multiple OTUs from the *Acidimicrobiaceae* (Figure 2, 3, S1, S2, Table

S2).

148   **Fluorescence *in situ* hybridization (FISH).** We sampled 26 snottite communities from

14 cave locations using FISH (Table 2). Representative FISH photomicrographs are shown in

Figures 5 and 6. All samples contain over 50% THIO1-positive cells (*Acidithiobacillus*), with the

exception of AS05-5. In sample AS05-5, over 25% of DAPI-strained cells did not hybridize with

any FISH probes, possibly indicating that a large proportion of the community was inactive at

the time of collection. ACM732-positive cells (*Acidimicrobium/Ferrimicrobium*) are present in

most but not all samples, and make up to 13.7% of total cells. In most samples, bacterial cells

that do not hybridize with either THIO1 or ACM732, hereafter referred to as "other bacteria,"

are <3% of cells. However, samples RS08-31, LA07-10, and VL07-26 have between 5.9-6.9%

other bacteria, and sample VL07-20 contains 15% other bacteria. At least some archaea are

present in all samples, although some samples contain less than 2% ARCH915-positive cells.

Twelve samples contain >2% FER656-positive cells (*Ferroplasma*), including four Acquasanta

samples with between 25-49% FER656. However, *Ferroplasma* are absent from some samples

that have abundant ARCH915-positive cells. Non-*Ferroplasma* archaea make up 15-30% of total

cells in several samples, including AS08-6 and several Frasassi RS samples.

163   We used principal component analysis (PCA) to explore the major axes of variance in

community composition based on the FISH dataset (Figure 7). For this and other statistical

analyses using FISH data (Table 2), our "species" were cell counts of THIO1, ACM732,

FER656, "other bacteria" (EUBMIX – (THIO1 + ACM732)), and "other archaea" (ARCH915 –

FER656). Principal components 1 and 2 combined explain more than 75% of the total variance.

The first principal component in Figure 7 appears to be related to location, with Acquasanta

samples plotting opposite Villa Luz samples. Species loadings show that the taxa driving this

clustering are FER656-positive cells, which were most abundant in the Acquasanta samples, and

ACM732 and "other bacteria" that were more abundant in Villa Luz.

172   ANalysis Of SIMilarities (ANOSIM; McCune and Grace, 2002) of FISH data indicates

that communities from different caves are significantly different (ANOSIM R = 0.268, p =

0.013), and this result remains significant if Villa Luz and Luna Azufre samples are pooled

(ANOSIM R = 0.287, p = 0.006). Mantel tests show that there is a statistically significant

correlation between community dissimilarity and geographical distance, both for all samples and

for Italian samples alone, if rank correlation is used (Mantel test with Spearman's *rho*: all

samples, *rho*=0.174, p=0.045; Italian samples only, *rho*=0.358, p<0.001; Frasassi samples only,

rho=0.158, p=0.065).

We also examined whether community composition is related to the concentration of

gases in the cave air. When these variables are fitted to the ordination analysis, CO_2 and SO_2

concentration align with the first ordination axis in the direction of the Acquasanta samples

(Figure 7), consistent with the high gas concentrations in that cave. These two variables are

statistically significantly correlated with the ordination axes ($r^2 = 0.33$, $p = 0.02$ for CO_2; $r^2 =$

0.31, $p = 0.02$ for SO_2), while H_2S concentration and temperature are not ($r^2 = 0.15$, $p = 0.22$ for

H_2S; $r^2 = 0.10$, $p = 0.34$ for temperature).

Discussion

Snottite microbial communities. *Acidithiobacillus* spp. were more than 50% of total

cells in all except one of the 26 snottite samples analyzed with FISH, and they were frequently

more than 80% of total cells (Table 2). *Acidithiobacillus* was also the only group present in all of

the biofilms sampled. These findings are consistent with other studies of snottite biofilms from

Frasassi and Cueva de Villa Luz (30, 31, 34, 35). Based on previous metagenomic and culture-

based analyses, *Acidithiobacillus thiooxidans* in Italian snottites are autotrophic sulfide-oxidizers

with the capacity to form extracellular polymeric substances, and were described as possible

'architects' of the biofilms (32, 36). The predominance of *Acidithiobacillus* spp. across the large

number of samples analyzed here is further evidence that *Acidithiobacillus* are responsible for

forming the acidic biofilms, and that they are important organisms for sulfide oxidation and

sulfuric acid production in sulfidic caves.

Close relatives or members of the bacterial genera *Acidimicrobium* and *Ferrimicrobium*

were present in most samples. Based on metagenomic analyses of Frasassi snottites, these

populations do not have the capacity to fix inorganic carbon, and are likely

chemoorganoheterotrophs or chemolithoheterotrophs (32, 33). However, the coverage of these

populations in previous snottite metagenomes was very low, and 16S rRNA genes recovered

from snottite populations are divergent from known isolates within the *Acidimicrobiaceae* family

(Figure 2), so inferences about their physiology are necessarily limited.

209   Other bacterial members included *Sulfobacillus* and members of the candidate phyla

Dependentiae (formerly TM6) and *Saccharibacteria* (formerly TM7). We hypothesize that,

based on their closest relatives in phylogenetic analyses and other members of their genera, the

Sulfobacillus represent another inorganic sulfur-oxidizing or organoheterotrophic population

(e.g., 43, 44) and the *Dependentiae* and *Saccharibacteria* are likely obligate symbionts or

parasites (40, 45, 46) (Figure 2; Figure S1). The protists and fungi that are commonly observed

in snottites (Figures 5, 6, S3; (31)) could be hosts for symbiotic *Dependentiae* and

Saccharibacteria. We observed nematodes and arthropods in Villa Luz and Luna Azufre

snottites that are additional potential hosts for symbionts (Figure S3). These likely represent the

extremely acid-tolerant bacteriophagous nematode (*Mesorhabditis acidophila*) and nematode-

eating mites previously described from Villa Luz snottites (47).

220   Archaea were present in all of the snottites sampled (Table 2). Members of the genus

Ferroplasma were detected in 18 out of the 26 samples analyzed using FISH, and were up to

50% of total cells. However, we also observed significant numbers of ARCH915-positive cells

that did not hybridize with FER656. Other archaeal populations identified in this and previous

studies of snottites include 'C-plasma' and *Cuniculiplasma* (formerly 'G-plasma') in the

Thermoplasmatales (Figure 4) (32, 33). In previous metagenomic analysis, *Ferroplasma* from

the Acquasanta caves had the genetic potential for sulfur oxidation, and *Cuniculiplasma* from

Frasassi had a homologue of sulfide quinone oxidoreductase (33), so these archaeal members

may have some capacity for lithotrophy in addition to organotrophic growth.

229   Villa Luz snottites were more diverse than snottites from other caves (Table S1). The

230   abundance of "other bacteria" in Villa Luz (Figure 7, Table 2) indicates that additional diversity

231   in these samples remains to be explored, and the deeper rRNA gene amplicon libraries from this

232   cave contain several taxa that haven't previously been described from snottites (see also D'Auria

233   et al. (35)). More work is needed to reveal the diversity of extreme acidophiles in this cave

234   community.

235   **Implications for community assembly.** Sulfidic cave snottites from across the globe are

populated by extreme acidophiles from the same few genera and families. Within those clades,

however, populations are divergent. The *Acidithiobacillus*, *Sulfobacillus*, and *Acidimicrobiaceae*

from snottites in the Mexican caves are less than 95% similar to those from the Italian caves, and

Ferroplasma spp. are less than 97% similar (Figure 2, 3, 4, S1). Furthermore, based on FISH cell

counts, differences among snottite communities are statistically significantly related to cave

location (Figure 7), so communities are site specific.

This pattern is consistent with a model of snottite community assembly in which caves

are stochastically colonized by microorganisms from local sources, followed by selective

filtering for those organisms that can survive in the extremely acidic subaerial cave environment.

If colonization events are sufficiently rare, then differences among snottite communities could

reflect differences in the founding community and/or later colonizers. Dispersal rates of extreme

acidophiles into these caves should be very low: snottites are analogous to extremely acidic

"islands" in a "sea" of alkaline limestone bedrock, so survival of extreme acidophiles during

transport into the cave environment would be rare. Consistent with this constraint, previous

research on the population structure of *Acidithiobacillus* spp. provides strong evidence for

restricted dispersal within and among sulfidic caves (36), and studies of other subsurface

communities have shown that they retain a site-specific "fingerprint" of their founding

communities (e.g., 28). If this model is correct, then differences in the composition of snottite

communities are partially the result of historical contingency. Whatever extreme acidophiles

happened to enter the cave were those that went on to form and colonize the extremely acidic

biofilms. Restricted dispersal could lead to novel diversity in this situation as those populations

are free to occupy new niche space and evolve and adapt *in situ*.

Differences among snottite communities could also result from environmental selection.

Earlier research found that *Acidithiobacillus* spp. from Frasassi and Acquasanta had differences

in their sulfur oxidation pathways and other functional genes (36), which indicates distinct

metabolic capabilities that could confer selective advantages in the environment. These two

Acidithiobacillus populations from Frasassi and Acquasanta had identical 16S rRNA genes.

Therefore, the far more divergent *Acidithiobacillus*, *Ferroplasma*, *Sulfobacillus,* and

Acidimicrobiaceae in Mexico versus Italy likely have substantial physiological differences that

the cave environment could select for. Snottites were chosen for this study because they occur in

a very specific geochemical niche in sulfidic caves (overhanging subaerial cave surfaces, $H_2S(g)$

between 0.2-25 ppm, extremely low pH; Figure 1a), so environmental variability among them

should be limited. However, we measured subtle but important geochemical differences: Frasassi

was substantially cooler than Acquasanta, Villa Luz, and Luna Azufre, $SO_2(g)$ was present in the

atmosphere of Acquasanta but not the other caves at the time of sampling (Figure 7, Table 1),

and there are likely other differences in nutrient or energy sources that we did not measure.

Villa Luz snottites were dominated by *Acidithiobacillus* spp. "group 2" that are more

closely related to *At. caldus* than *At. thiooxidans*. If *At. thiooxidans* populations were rare but

present in Villa Luz snottites, that would be evidence that they were being selected against.

However, deep amplicon sequencing did not identify any *At. thiooxidans* out of 246,173

amplicon sequences from Villa Luz snottites, although that does not exclude the possibility that

At. thiooxidans have had other opportunities to colonize the cave or that they exist elsewhere in

the cave. Interestingly, this is not true of the *Acidimicrobiaceae*. The most abundant

Acidimicrobiaceae OTU from the Villa Luz amplicon libraries clusters with the Villa Luz clones

(24.8% and 13.7% of VL13-1 and VL13-2 libraries, respectively), but there is a second rare

OTU that is more similar to the Frasassi clones that represents 1.1% and 0.003% of total

sequences from these libraries (OTU_9 in Figure 3). Nevertheless, this rare OTU is still >3%

different from the Frasassi *Acidimicrobiaceae* clones. Evolutionary rates for 16S rRNA genes are

0.025 to 0.091% per million years (48), so these differences represent at least tens of millions of

years of evolution, which highlights how different the Villa Luz and Luna Azufre populations

are compared to those in Frasassi and Acquasanta. The formation and colonization of snottites is

apparently an adaptation to the sulfidic cave environment that can be exploited by substantially

different organisms.

Methods

Sample collection and geochemistry. Snottite biofilm samples were collected from four

sulfidic caves: Cueva de Villa Luz and Cueva Luna Azufre in Tabasco, Mexico, and the Grotte

del Fiume (Frasassi) and Grotta Nuova di Rio Garrafo (Acquasanta Terme) in the Marche region,

Italy. Villa Luz is located 2 km south of Tapijulapa in Tabasco, Mexico, and contains roughly 2

km of passage in a Cretaceous limestone. The entrance to Luna Azufre is located 320 meters

southeast from Villa Luz, and Luna Azufre has over 500 m of passage in the same formation.

Both caves are fed by phreatic water containing up to 15 mM $H_2S(aq)$ that could originate either

from nearby volcanic or petroleum sources (30, 49, 50). The Frasassi Caves are located 3 km

southeast of Genga in the Marche region, Italy, and contain over 25 km of passage in a Jurassic

platform limestone. Sulfidic waters entering Frasassi contain up to 600 μM $H_2S(aq)$ that likely

originates from sulfate reduction in an underlying evaporite formation (51, 52). Grotta Nuova di

Rio Garrafo is located 2 km south of Acquasanta Terme, and 80 km to the southeast of Frasassi.

The Acquasanta cave consists of over 1 km of passage in a marly Eocene limestone, and is fed

by thermal sulfidic waters that reach temperatures up to 50°C with over 800 μM $H_2S(aq)$ (37,

53).

Samples were collected from 2-16 locations within each cave. At each sampling site,

multiple snottites (approximately 0.5-1.5 grams of biofilm) were collected into sterile centrifuge

tubes from an area of cave wall no larger than one square meter. At the time of collection, the pH

values of at least 5-10 snottites from the collection area were measured with pH paper (range 0-

2.5). The pH of all samples was between 0-1.5.

$H_2S(g)$ concentration was measured at the time of collection using Dräger tubes (range

0.2-60 ppm) (Dräger Safety Inc, Germany) and by an EN-MET MX2100 portable gas detector

(range 1-120 ppm) (ENMET corp, USA). $SO_2(g)$ and $CO_2(g)$ concentrations were also measured

by ENMET MX2100 (lower detection limits 0.1 ppm and 0.1 %, respectively), and $CO_2(g)$ was

also measured by Dräger tubes (100 to 6000 ppm) at some sites. In Villa Luz and Luna Azufre,

samples for $CO_2(g)$ concentration were collected in sealed 60 mL serum bottles (2 bottles per

site, flushed 10 times each with 1 volume cave air) and measured with a SRI 310 gas

chromatograph (GC) equipped with a thermal conductivity detector and a PoraPak Q packed

column (3' x 1/8") with helium carrier gas at 80°C (SRI instruments, USA). $NH_3(g)$ and $N_2O(g)$

were measured using Dräger tubes at Acquasanta and Frasassi in 2005-2007, but those gases

were never detected (lower detection limit 0.25 and 0.5 ppm, respectively). The total range of

multiple gas measurements at each site is given in Table 1.

16S rRNA gene libraries. For DNA extraction, approximately 1 gram of snottite biofilm

was preserved in 5 parts RNAlater (Ambion, USA) within 4 hours of collection, and stored at -

20°C. DNA extraction of samples RS05-2 and PC05-1 were described in Macalady et al. (2007).

For samples RS05-24, VL07-20, LA07-10, AS07-3, AS08-5, and RS09-1, DNA was extracted

via bead beating and phenol-chloroform extraction as described in (32, 54). RNAlater-preserved

samples were first diluted with 3 parts 1x phosphate-buffered saline (PBS). To remove

polysaccharides from the DNA extract, prior to final elution the DNA was resuspended in 200

µL tris (200 mM, pH 8.0), 100 µL NaCl (5 M), and 600 µL ethanol (100%), incubated at -20°C

for 30 minutes, and pelleted by centrifugation.

Bacterial, universal, and archaeal 16S rRNA gene clone libraries were constructed from

environmental DNA templates. For bacterial and universal libraries, 16S rRNA genes were

amplified by polymerase chain reaction (PCR), ligated into pCR4-TOPO vector, and transformed

into OneShot Mach1 *E. coli* as described in (31). Archaeal clones were sequenced from sample

VL07-20 using forward primer 344f (ACG GGG YGC AGC AGG CGC GA) (55) and reverse

primer 1492r (GGT TAC CTT GTT ACG ACT T) (56). Archaeal clones were sequenced from

samples RS05-24 and RS09-1 using 344f and reverse primer deg1392r (ACR GGC GGT GTG

TRC) (32). Amplification, ligation, and transformation of the archaeal sequences was performed

as in Jones et al. (32). Some additional 16S rRNA gene clones were recovered from Frasassi

samples GS08-6, GB08-30, and RS08-31 using 27F and primer Sag2 for the *Acidithiobacillus*

16S-23S intergenic transcribed spacer region, as described in (36). Inserts were extracted from

RS05-2 and PC05-1 via plasmid extraction as described in (31). Inserts for all other samples

were amplified by colony PCR as described in (57). Clones were sequenced at the Penn State

University Nucleic Acid Facility.

High-throughput rRNA gene "amplicon" libraries were generated from two snottite

samples from Villa Luz collected in 2013 (58). Samples were preserved in sucrose lysis buffer

(59), and DNA was extracted using the MoBio PowerSoil™ DNA Isolation Kit (MoBio,

Carlsbad, CA). Amplicon libraries were created and sequenced by Molecular Research LP (MR

DNA) (Shallowater, TX), using bacterial-specific primers 46f (5′-

GCYTAAYACATGCAAGTCG-3′) and 1409r (5′-GTGACGGGCRGTGTGTRCAA-3′) (59,

60).

Fluorescence *in situ* hybridization (FISH). Approximately 0.25-0.5 grams of biofilm

was fixed for FISH analysis. Samples were either fixed for 3-4 hours in 3% (w/v)

paraformaldehyde within 10 hours of collection, or stored in RNAlater (Ambion, USA) as

described above and later fixed in paraformaldehyde. After fixation, samples were stored in 1:1

PBS/ethanol at -20°C. Fluorescence *in situ* hybridization followed the procedure outlined by

Hugenholtz et al. (61). Environmental samples were applied to 10-well Teflon coated slides,

dried, and dehydrated for 3 minutes each in 50%, 80%, and 90% ethanol washes. Hybridization

buffer contained 0.9 M NaCl, 20 mM of Tris/HCl pH 7.4, 0.01% sodium dodecyl sulfate (SDS),

25 ng of each fluorescently labeled probe (Table 3), and variable formamide concentrations

depending on the probe (Table 3). Slides were incubated at 46°C for 2 hours, followed by a 15

minute incubation at 48°C in wash buffer containing 20 mM Tris/HCl pH 7.4, 0.01% SDS, and

variable NaCl concentrations following Lathe (62). Slides were rinsed with deionized water,

counterstained with 4',6'-diamidino-2-phenylindole (DAPI), dried, and mounted with

Vectashield (Vector Laboratories Inc, Burlingame, CA, USA). Slides were viewed at 1000x

magnification with a Nikon Eclipse 80i epifluorescence microscope, and photomicrographs were

taken with a monochrome Photometrics CoolSNAP ES^2 CCD camera using NIS-Elements AS

3.0 software.

Between 800-1200 DAPI-stained cells were counted for each probe combination. Cells

were counted from at least six photos, each representing a different microscope field, from at

least two slide wells. Standard deviation was calculated from differences in proportion of probe-

labeled cells among each photo. To ensure objectivity in counts, photos for counting were taken

without prior examination of the field of view under more than one filter (EUBMIX whenever

possible).

Phylogenetic, bioinformatic, and statistical analyses. Clone sequences were assembled

and edited using CodonCode Aligner v.2.0.4 (CodonCode Corp., USA). Chimeric sequences

were identified and removed using Bellerophon 3 (63). Contiguous sequences were aligned using

the SINA aligner (64) (https://www.arb-silva.de) and loaded into an ARB (65) database with

SILVA database v. 132 (66, 67). Alignments were manually refined using the ARB_Edit4

aligner.

For phylogenetic analysis, alignments were trimmed to equalize sequence length, and

positions with <50% gaps were masked. Maximum likelihood phylogenies of full-length

sequences were calculated with RAxML v.8.2.12 (68), using the general time reversible

nucleotide substitution model, gamma distributed rates, the proportion of invariant sites and base

frequencies estimated from the data, and 100 rapid bootstrap replicates. Percent nucleotide

sequence similarity was calculated in ARB.

High throughput amplicon libraries were processed using the pipeline described in (69),

except that only the first 200 bp of the R1 (forward) read was used for OTU clustering and

taxonomic identification. Taxonomic classification of representative OTU sequences was

determined with mothur v.1.3.2 using the SILVA database v.132 and a confidence cutoff of 50

(70). Short amplicon sequences were then placed into phylogenetic trees using the evolutionary

placement algorithm (EPA) with default parameters (71).

For statistical analyses of FISH cell counts, we used average proportions of THIO1,

ACM732, FER656, "other bacteria" (total EUBMIX – (THIO1 + ACM732)), and "other

archaea" (ARCH915 – FER656). Principal component analyses (PCA), ANOSIM and Manel

tests (72) were performed using the vegan package in R (73, 74). The geographic distance matrix

used for Mantel tests included linear distances among individual caves and linear distances

between sampling locations within the same cave system, either measured at the time of

sampling or from cave maps (Table S3). Mantel statistics were evaluated with Spearman's rank

correlation and 1000 randomizations. For PCA analyses, cross-product matrices were calculated

with Pearson's correlation coefficients.

Accession number(s). 16S rRNA gene clones referenced in this study are available in the

GenBank database under accession numbers KU341124-KU341208, OP269604-OP269649,

KC582420-KC582530, and DQ499162-DQ499330. Amplicon libraries are available in NCBI's

Sequence Read Archive under project PRJNA878455.

Acknowledgements

Thanks to A. Montanari for logistical support and the use of facilities and laboratory space at the

Osservatorio Geologico di Coldigioco (Italy). S. Mariani, S. Galdenzi, S. Cerioni, and M.

Mainiero provided expert advice and field assistance with research in Italy, and F. Baldoni, S.

Recanatini, S. Dattagupta, and members of the Gruppo Speleologico CAI di Fabriano and the

Gruppo Speleologico Marchigiano CAI di Ancona assisted with sample collection and provided

additional excellent field support. We thank L. Rosales-Lagarde, L. Hose, and S. Dattagupta for

logistical support, insightful discussion, and field assistance with sample collection in Mexico.

Thanks to the municipal government of Tacotalpa, Tabasco, Mexico, and to C. Rogers Morales

Mendez and L. Felino Arevalo Gallegos, for granting permits for cave access and sample

collection. C. Alberto Cordero Martinez provided lodging and logistical support in Villa Luz

Park. The two 2013 Villa Luz samples were collected during an expedition supported by

National Geographic grant #EC0644-13 to P. Boston, and sequencing of these two libraries was

supported by a Vehslage Grant from the National Speleological Foundation. This work was

supported by a graduate research fellowship to DSJ from the Cave Conservancy Foundation, and

grants to JLM from the National Science Foundation (EAR 0311854 and EAR 0527046) and

NASA NAI (NNA04CC06A).

REFERENCES
432
433   1.   Vera M, Schippers A, Sand W. 2013. Progress in bioleaching: fundamentals and
434   mechanisms of bacterial metal sulfide oxidation—part A. Appl Microbiol Biotechnol
435   97:7529-7541.
436   2.   Johnson DB. 2014. Biomining—biotechnologies for extracting and recovering metals
437   from ores and waste materials. Curr Opin Biotechnol 30:24-31.
438   3.   Baker BJ, Banfield JF. 2003. Microbial communities in acid mine drainage. FEMS
439   Microbiol Ecol 44:139-152.
440   4.   Rowe OF, Sánchez-España J, Hallberg KB, Johnson DB. 2007. Microbial communities
441   and geochemical dynamics in an extremely acidic, metal-rich stream at an abandoned
442   sulfide mine (Huelva, Spain) underpinned by two functional primary production systems.
443   Environ Microbiol 9:1761-1771.
444   5.   Santelli CM, Pfister DH, Lazarus D, Sun L, Burgos WD, Hansel CM. 2010. Promotion of
445   Mn(II) oxidation and remediation of coal mine drainage in passive treatment systems by
446   diverse fungal and bacterial communities. Appl Environ Microbiol 76:4871-4875.
447   6.   Mansor M, Harouaka K, Gonzales MS, Macalady JL, Fantle MS. 2018. Transport-
448   induced spatial patterns of sulfur isotopes (δ^{34}S) as biosignatures. Astrobiology 18:59-72.
449   7.   Burgos WD, Borch T, Troyer LD, Luan F, Larson LN, Brown JF, Lambson J, Shimizu
450   M. 2012. Schwertmannite and Fe oxides formed by biological low-pH Fe(II) oxidation
451   versus abiotic neutralization: Impact on trace metal sequestration. Geochim Cosmochim
452   Acta 76:29-44.
453   8.   Grettenberger CL, Pearce AR, Bibby KJ, Jones DS, Burgos WD, Macalady JL. 2017.
454   Efficient low-pH iron removal by a microbial iron oxide mound ecosystem at Scalp Level
455   Run. Appl Environ Microbiol:e00015-17.
456   9.   Johnson DB, Hallberg KB. 2005. Acid mine drainage remediation options: a review.
457   Science of the Total Environment 338:3-14.
458   10.   Kumar RN, Nagendran R. 2007. Influence of initial pH on bioleaching of heavy metals
459   from contaminated soil employing indigenous *Acidithiobacillus thiooxidans*.
460   Chemosphere 66:1775-1781.
461   11.   Larson LN, Sánchez-España J, Kaley B, Sheng Y, Burgos WD. 2014. Thermodynamic
462   controls on the kinetics of microbial low-pH Fe(II) oxidation. Environ Sci Technol
463   48:9246-9254.
464   12.   Johnson DB. 2018. The evolution, current status, and future prospects of using
465   biotechnologies in the mineral extraction and metal recovery sectors. Minerals 8:343.
466   13.   Pakostova E, Johnson DB. 2019. Microbial generation of sulfuric acid from granular
467   elemental sulfur in laboratory-scale bioreactors. Hydrometallurgy 190:105152.
468   14.   Rawlings DE, Johnson DB. 2007. The microbiology of biomining: development and
469   optimization of mineral-oxidizing microbial consortia. Microbiology 153:315-324.
470   15.   Rowe OF, Johnson DB. 2008. Comparison of ferric iron generation by different species
471   of acidophilic bacteria immobilized in packed-bed reactors. Syst Appl Microbiol 31:68-
472   77.
473   16.   Tyson GW, Chapman J, Hugenholtz P, Allen EE, Ram RJ, Richardson PM, Solovyev
474   VV, Rubin EM, Rokhsar DS, Banfield JF. 2004. Community structure and metabolism
475   through reconstruction of microbial genomes from the environment. Nature 428:37-43.

17.   Whitaker RJ, Banfield JF. 2005. Population dynamics through the lens of extreme
environments. Rev Mineral Geochem 59:259-277.
18.   Palacios C, Zettler E, Amils R, Amaral-Zettler L. 2008. Contrasting microbial
community assembly hypotheses: a reconciling tale from the Rio Tinto. PLoS ONE
3:e3853.
19.   Lear G, Niyogi D, Harding J, Dong Y, Lewis G. 2009. Biofilm bacterial community
structure in streams affected by acid mine drainage. Appl Environ Microbiol 75:3455-
3460.
20.   Kuang J-L, Huang L-N, Chen L-X, Hua Z-S, Li S-J, Hu M, Li J-T, Shu W-S. 2013.
Contemporary environmental variation determines microbial diversity patterns in acid
mine drainage. ISME J 7:1038-1050.
21.   Sheng Y, Bibby K, Grettenberger C, Kaley B, Macalady JL, Wang G, Burgos WD. 2016.
Geochemical and temporal influences on the enrichment of acidophilic iron-oxidizing
bacterial communities. Appl Environ Microbiol 82:3611-3621.
22.   Jones DS, Kohl C, Grettenberger C, Larson LN, Burgos WD, Macalady JL. 2015.
Geochemical niches of iron-oxidizing acidophiles in acidic coal mine drainage. Appl
Environ Microbiol 81:1242-1250.
23.   Bond PL, Druschel GK, Banfield JF. 2000. Comparison of acid mine drainage microbial
communities in physically and geochemically distinct ecosystems. Appl Environ
Microbiol 66:4962-4971.
24.   Edwards KJ, Gihring TM, Banfield JF. 1999. Seasonal variations in microbial
populations and environmental conditions in an extreme acid mine drainage environment.
Appl Environ Microbiol 65:3627-3632.
25.   Schrenk MO, Edwards KJ, Goodman RM, Hamers RJ, Banfield JF. 1998. Distribution of
Thiobacillus ferrooxidans and *Leptospirillum ferrooxidans*: Implications for generation
of acid mine drainage. Science 279:1519-1522.
26.   Martiny JBH, Bohannan BJ, Brown JH, Colwell RK, Fuhrman JA, Green JL, Horner-
Devine MC, Kane M, Krumins JA, Kuske CR. 2006. Microbial biogeography: putting
microorganisms on the map. Nature Reviews Microbiology 4:102-112.
27.   Langenheder S, Lindström ES, Tranvik LJ. 2006. Structure and function of bacterial
communities emerging from different sources under identical conditions. Appl Environ
Microbiol 72:212-220.
28.   Marshall IPG, Ren G, Jaussi M, Lomstein BA, Jørgensen BB, Røy H, Kjeldsen KU.
2019. Environmental filtering determines family-level structure of sulfate-reducing
microbial communities in subsurface marine sediments. ISME J 13:1920-1932.
29.   Takacs-Vesbach C, Mitchell K, Jackson-Weaver O, Reysenbach A-L. 2008. Volcanic
calderas delineate biogeographic provinces among Yellowstone thermophiles. Environ
Microbiol 10:1681-1689.
30.   Hose LD, Palmer AN, Palmer MV, Northup DE, Boston PJ, DuChene HR. 2000.
Microbiology and geochemistry in a hydrogen-sulphide-rich karst environment. Chem
Geol 169:399-423.
31.   Macalady JL, Jones DS, Lyon EH. 2007. Extremely acidic, pendulous microbial biofilms
from the Frasassi cave system, Italy. Environ Microbiol 9:1402-1414.
32.   Jones DS, Albrecht HL, Dawson KS, Schaperdoth I, Freeman KH, Pi Y, Pearson A,
Macalady JL. 2012. Community genomic analysis of an extremely acidophilic sulfur-
oxidizing biofilm. ISME J 6:158-170.

33.   Jones DS, Schaperdoth I, Macalady JL. 2014. Metagenomic evidence for sulfide
oxidation in extremely acidic cave biofilms. Geomicrobiol J 31:194-204.
34.   Vlasceanu L, Sarbu SM, Engel AS, Kinkle BK. 2000. Acidic cave wall biofilms located
in the Frasassi Gorge, Italy. Geomicrobiol J 17:125-139.
35.   D'Auria G, Artacho A, Rojas RA, Bautista JS, Méndez R, Gamboa MT, Gamboa JR,
Gómez-Cruz R. 2018. Metagenomics of bacterial diversity in Villa Luz Caves with sulfur
water springs. Genes 9:55.
36.   Jones DS, Schaperdoth I, Macalady JL. 2016. Biogeography of sulfur-oxidizing
Acidithiobacillus populations in extremely acidic cave biofilms. ISME J 10:2879.
37.   Galdenzi S, Cocchioni F, Filipponi G, Morichetti L, Scuri S, Selvaggio R, Cocchioni M.
2010. The sulfidic thermal caves of Acquasanta Terme (central Italy). J Cave Karst Stud
72:43-58.
38.   Jones D, Tobler D, Schaperdoth I, Mainiero M, Macalady J. 2010. Community structure
of subsurface biofilms in the thermal sulfidic caves of Acquasanta Terme, Italy. Appl
Environ Microbiol 76:5902-5910.
39.   Hose LD, Macalady JL. 2006. Observations from active sulfidic karst systems: is the
present the key to understanding Guadalupe Mountain speleogenesis, p 185-194. *In* Land
L, Lueth V, Raatz B, Boston P, Love D (ed), New Mexico Geological Society
Guidebook, 57th Annual Fall Field Conference, Caves and Karst of Southeastern New
Mexico, vol 57.
40.   Yeoh YK, Sekiguchi Y, Parks DH, Hugenholtz P. 2015. Comparative genomics of
candidate phylum TM6 suggests that parasitism is widespread and ancestral in this
lineage. Mol Biol Evol 33:915-927.
41.   Albertsen M, Hugenholtz P, Skarshewski A, Nielsen KL, Tyson GW, Nielsen PH. 2013.
Genome sequences of rare, uncultured bacteria obtained by differential coverage binning
of multiple metagenomes. Nat Biotechnol 31:533-538.
42.   Golyshina OV, Lünsdorf H, Kublanov IV, Goldenstein NI, Hinrichs K-U, Golyshin PN.
2016. The novel extremely acidophilic, cell-wall-deficient archaeon *Cuniculiplasma*
divulgatum gen. nov., sp. nov. represents a new family, *Cuniculiplasmataceae* fam. nov.,
of the order *Thermoplasmatales*. Int J Syst Evol Microbiol 66:332-340.
43.   Norris PR, Clark DA, Owen JP, Waterhouse S. 1996. Characteristics of *Sulfobacillus*
acidophilus sp. nov. and other moderately thermophilic mineral-sulphide-oxidizing
bacteria. Microbiology 142:775-783.
44.   Panyushkina AE, Babenko VV, Nikitina AS, Selezneva OV, Tsaplina IA, Letarova MA,
Kostryukova ES, Letarov AV. 2019. *Sulfobacillus thermotolerans*: new insights into
resistance and metabolic capacities of acidophilic chemolithotrophs. Sci Rep 9:15069.
45.   Murugkar PP, Collins AJ, Dewhirst FE. 2019. Complete genome sequence of strain
PM004, a novel cultured member of the human oral microbiome from the candidate
phylum *Saccharibacteria* (TM7). Microbiology Resource Announcements 8:e01159-19.
46.   Deeg CM, Zimmer MM, George EE, Husnik F, Keeling PJ, Suttle CA. 2019.
Chromulinavorax destructans, a pathogen of microzooplankton that provides a window
into the enigmatic candidate phylum *Dependentiae*. PLoS Path 15:e1007801.
47.   Borgonie G, Dierick M, Houthoofd W, Willems M, Jacobs P, Bert W. 2010. Refuge
From predation, the benefit of living in an extreme acidic environment? The Biological
Bulletin 219:268-276.

48. Kuo CH, Ochman H. 2009. Inferring clocks when lacking rocks: the variable rates of
molecular evolution in bacteria. Biol Direct 4:35.
49. Hose LD, Rosales-Lagarde L. 2017. Sulfur-rich caves of Southern Tabasco, Mexico, p
803-814. *In* Klimchouk A, N. Palmer A, De Waele J, S. Auler A, Audra P (ed),
Hypogene Karst Regions and Caves of the World. Springer International Publishing,
Cham. (doi:10.1007/978-3-319-53348-3_54)
50. Rosales Lagarde L, Boston PJ, Campbell AR, Hose LD, Axen G, Stafford KW. 2014.
Hydrogeology of northern Sierra de Chiapas, Mexico: a conceptual model based on a
geochemical characterization of sulfide-rich karst brackish springs. Hydrogeol J 22:1447-
1467.
51. Galdenzi S, Jones DS. 2017. The Frasassi Caves: A "classical" active hypogenic cave, p
143-159. *In* Klimchouk A, N. Palmer A, De Waele J, S. Auler A, Audra P (ed),
Hypogene Karst Regions and Caves of the World. Springer International Publishing,
Cham. (doi:10.1007/978-3-319-53348-3_8)
52. Jones DS, Polerecky L, Galdenzi S, Dempsey BA, Macalady JL. 2015. Fate of sulfide in
the Frasassi cave system and implications for sulfuric acid speleogenesis. Chem Geol
410:21-27.
53. Galdenzi S. 2017. The thermal hypogenic caves of Acquasanta Terme (Central Italy), p
169-182. *In* Klimchouk A, N. Palmer A, De Waele J, S. Auler A, Audra P (ed),
Hypogene Karst Regions and Caves of the World. Springer International Publishing,
Cham. (doi:10.1007/978-3-319-53348-3_10)
54. Bond PL, Smriga SP, Banfield JF. 2000. Phylogeny of microorganisms populating a
thick, subaerial, predominantly lithotrophic biofilm at an extreme acid mine drainage site.
Appl Environ Microbiol 66:3842-3849.
55. Raskin L, Stromley JM, Rittmann BE, Stahl DA. 1994. Group-specific 16S rRNA
hybridization probes to describe natural communities of methanogens. Appl Environ
Microbiol 60:1232-1240.
56. Lane D. 1991. 16S/23S rRNA sequencing, p 115-175. *In* Stackebrandt E GM (ed),
Nucleic Acid Techniques in Bacterial Systematics. Wiley, New York.
57. Macalady JL, Dattagupta S, Schaperdoth I, Jones DS, Druschel GK, Eastman D. 2008.
Niche differentiation among sulfur-oxidizing bacterial populations in cave waters. ISME
J 2:590-601.
58. Kimble JC. 2018. Ph.D. thesis. The University of New Mexico.Comparison of bacterial
and archaeal communities in the subsurface versus surface: Implications for nitrogen
cycling.
59. Northup DE, Melim LA, Spilde MN, Hathaway JJM, Garcia MG, Moya M, Stone FD,
Boston PJ, Dapkevicius MLNE, Riquelme C. 2011. Lava cave microbial communities
within mats and secondary mineral deposits: Implications for life detection on other
planets. Astrobiology 11:601-618.
60. Kimble JC, Winter AS, Spilde MN, Sinsabaugh RL, Northup DE. 2018. A potential
central role of Thaumarchaeota in N-Cycling in a semi-arid environment, Fort Stanton
Cave, Snowy River passage, New Mexico, USA. FEMS Microbiol Ecol 94.
61. Hugenholtz P, Tyson GW, Blackall LL. 2002. Design and evaluation of 16S rRNA-
targeted oligonucleotide probes for fluorescence in situ hybridization, p 29-42. *In* de
Muro MA, Rapley R (ed), Gene Probes: Principles and Protocols. Humana Press,
Totowa, NJ. (doi:10.1385/1-59259-238-4:029)

62. Lathe R. 1985. Synthetic oligonucleotide probes deduced from amino acid sequence data:
Theoretical and practical considerations. J Mol Biol 183:1-12.
63. Huber T, Faulkner G, Hugenholtz P. 2004. Bellerophon; a program to detect chimeric
sequences in multiple sequence alignments. Bioinformatics 20:2317-2319.
64. Pruesse E, Quast C, Knittel K, Fuchs BM, Ludwig W, Peplies J, Glöckner FO. 2007.
SILVA: a comprehensive online resource for quality checked and aligned ribosomal
RNA sequence data compatible with ARB. Nucleic Acids Res 35:7188-7196.
65. Ludwig W, Strunk O, Westram R, Richter L, Meier H, Yadhukumar, Buchner A, Lai T,
Steppi S, Jobb G, Förster W, Brettske I, Gerber S, Ginhart AW, Gross O, Grumann S,
Hermann S, Jost R, König A, Liss T, Lüßmann R, May M, Nonhoff B, Reichel B,
Strehlow R, Stamatakis A, Stuckmann N, Vilbig A, Lenke M, Ludwig T, Bode A,
Schleifer K-H. 2004. ARB: a software environment for sequence data. Nucleic Acids Res
32:1363-1371.
66. Quast C, Pruesse E, Yilmaz P, Gerken J, Schweer T, Yarza P, Peplies J, Glöckner FO.
2012. The SILVA ribosomal RNA gene database project: improved data processing and
web-based tools. Nucleic Acids Res 41:D590-D596.
67. Yilmaz P, Parfrey LW, Yarza P, Gerken J, Pruesse E, Quast C, Schweer T, Peplies J,
Ludwig W, Glöckner FO. 2013. The SILVA and "All-species Living Tree Project (LTP)"
taxonomic frameworks. Nucleic Acids Res 42:D643-D648.
68. Stamatakis A. 2006. RAxML-VI-HPC: maximum likelihood-based phylogenetic analyses
with thousands of taxa and mixed models. Bioinformatics 22:2688-2690.
69. Jones DS, Lapakko KA, Wenz ZJ, Olson MC, Roepke EW, Sadowsky MJ, Novak PJ,
Bailey JV. 2017. Novel microbial assemblages dominate weathered sulfide-bearing rock
from copper-nickel deposits in the Duluth complex, Minnesota, USA. Appl Environ
Microbiol 83:e00909-17.
70. Schloss PD, Westcott SL, Ryabin T, Hall JR, Hartmann M, Hollister EB, Lesniewski RA,
Oakley BB, Parks DH, Robinson CJ. 2009. Introducing mothur: open-source, platform-
independent, community-supported software for describing and comparing microbial
communities. Appl Environ Microbiol 75:7537-7541.
71. Berger SA, Krompass D, Stamatakis A. 2011. Performance, accuracy, and web server for
evolutionary placement of short sequence reads under maximum likelihood. Syst
Biol:syr010.
72. McCune B, Grace JB, Urban DL. 2002. Analysis of Ecological Communities, vol 28.
MjM software design Gleneden Beach, OR.
73. R Development Core Team. 2007. R: A language and environment for statistical
computing., R Foundation for Statistical Computing, Vienna, Austria. http://www.R-
project.org.
74. Oksanen J, Blanchet FG, Friendly M, Kindt R, Legendre P, McGlinn D, Minchin PR,
O'Hara R, Simpson GL, Solymos P, Szoecs E, Wagner H. 2017. vegan: Community
Ecology Package. R package version 2.4-3. https://CRAN.R-project.org/package=vegan.
653
654

Figure 1. (A) Schematic depicting the snottite niche in sulfidic caves. Snottites hang from gypsum wall crusts on cave walls and ceilings in close proximity to sulfidic cave streams. (B-G) Representative photographs of snottite communities described in this study. Yellow scale bars are 2 cm, black bar in (b) is 1 cm, and the collection tube in (f) is 1.5 cm in diameter.

664
665
666 **Figure 2.** Phylogenetic analysis of 16S rRNA gene sequences from the genus *Acidithiobacillus*.
667 The base tree is a maximum likelihood phylogram created with nearly full-length sequences,
668 with shorter amplicon sequence (gray underline) placed using the EPA algorithm. Representative
669 sequences from 16S rRNA gene clones are in bold black. Bootstrap values >50% are provided
670 for each node.
671
672

673
674
Figure 3. Phylogenetic analysis of 16S rRNA gene sequences from the *Acidimicrobiaceae*
family. The base tree is a maximum likelihood phylogram created with nearly full-length
sequences, with shorter amplicon sequences (gray underline) placed after the fact using the EPA
algorithm. Representative sequences from 16S rRNA gene clones are in bold black. The
amplicon sequence in bold gray indicated by two asterisks (**) represents that most abundant
Acidimicrobiaceae OTU (24.8 and 13.7% of the VL13-1 and VL13-2 libraries, respectively);
other *Acidimicrobiaceae* OTUs are <2% relative abundance (Table S2). Bootstrap values >50%
are provided for each node.

686
687
688 **Figure 4.** Neighbor joining phylogram of 16S rRNA gene sequences from the Archaeal family
689 *Thermoplasmatales*. Snottite clones are in bold black. Bootstrap values >50% are provided for
690 each node. Group names for the 'alphabet plasmas' are after Baker and Banfield (3).
691

Figure 5. Representative fluorescence *in situ* hybridization (FISH) photomicrographs. The sample name and probes used for each photo are provided in the legend. White scale bars are 5 μM. Abundant DAPI-labeled cells in AS08-6 are nearly all archaea, as are abundant DAPI-labeled cells in RS08-31.

Figure 6. Representative fluorescence *in situ* hybridization (FISH) photomicrographs. The sample name and probes used for each photo are provided in the legend. White scale bars are 5 µM. Note the abundant non-THIO1 labeled cells (white arrows) in VL07-20. Fat arrow in GS08-6 indicates THIO1-labeled cells linked into filaments, and thin arrows indicate non-THIO1-labeled bacterial cells.

710
711
712 **Figure 7.** Principal component analysis (PCA) of FISH cell counts (Table 3). Sample scores are
713 coded by cave (communities from different caves are significantly different by ANOSIM). Fitted
714 vectors of environmental variables are included as an overlay; CO_2 and SO_2 gas concentrations
715 are statistically significantly correlated with the ordination axes ($p < 0.05$), H_2S gas and
716 temperature are not ($p > 0.1$).
717

718 **Table 1.** Summary of snottite samples and associated environmental data.

Sample	Cave	Site	Wall minerology	Temp (^{o}C)	[$H_2S(g)$] (ppmv)	[$CO_2(g)$]	[$SO_2(g)$] (ppmv)
GS07-35	Frasassi	GS2	gypsum	13-14	bd[b]	700 ppm	bd
GS08-6	Frasassi	GS1	gypsum	13-14	0.8 (0.6-1)	1400 ppm	nm
RS05-2	Frasassi	RS2	gypsum and S^o	13-14	nm	nm	nm
RS05-24	Frasassi	RS2	gypsum and S^o	13-14	16 (8-24)	>3000 ppm	nm
RS05-1	Frasassi	RS1	gypsum	13-14	nm	nm	nm
RS05-20	Frasassi	RS1	gypsum	13-14	3.4	>3000 ppm	nm
RS07-21	Frasassi	RS3	gypsum and S^o	13-14	21 (17-25)	0.2 %	bd
RS07-22	Frasassi	RS2	gypsum and S^o	13-14	14.5 (11-18)	0.2 %	bd
RS08-30	Frasassi	RS2	gypsum and S^o	13-14	11.5 (11-12)	2500 ppm	nm
RS08-31	Frasassi	RS3	gypsum and S^o	13-14	14 (12-16)	3000 ppm	nm
RS09-1	Frasassi	RS2	gypsum and S^o	13-14	8 (5-11)	0.25%	bd
RS09-10	Frasassi	RS2	gypsum and S^o	13-14	10.5 (8-13)	0.3%	bd
RS09-11	Frasassi	RS3	gypsum and S^o	13-14	21 (20-22)	0.4%	bd
PC05-1	Frasassi	PC1	gypsum	13-14	nm	nm	nm
PC05-13	Frasassi	PC1	gypsum	13-14	1.1 (0.2-2)	1200 ppm	nm
GB08-30	Frasassi	GB1	gypsum	13-14	4 (3-5)	1400 ppm	nm
AS05-5	Acquasanta	AS3	gypsum	19-21	nm	nm	nm
AS07-1	Acquasanta	AS3	gypsum	19-21	2 (1-3)	0.4 %	4.5 (2-36)
AS07-3	Acquasanta	AS4	sediment[a]	19-21	2.9 (1-4.8)	0.4 %	5 (3-7)
AS07-4	Acquasanta	AS2	sediment[a]	25-35	24 (18-30)	0.6 %	11.5 (10-13)
AS08-5	Acquasanta	AS3	gypsum	19-21	bd[c]	0.45 %	5 (4-6)
AS08-6	Acquasanta	AS2	sediment[a]	25-35	3	0.55 %	4.5 (4-5)
VL07-20	Villa Luz	VL1	gypsum	25-28	12 (6-18)	1200 ppm	bd
VL07-26	Villa Luz	VL2	gypsum	25-28	3.5 (3-4)	1100 ppm	bd
LA07-10	Luna Azufre	LA1	gypsum	28-33	3	1250 ppm	bd
LA07-13	Luna Azufre	LA2	gypsum	28-33	2	1250 ppm	bd

[a]Sediment is a mixture of gypsum, clay, and chert particles
[b]Measured by ENMET MX2100, lower detection limit (ldl) 1 ppm.
719 [c]Measured by Draeger tube, ldl 0.2 ppm.
720
721
722

723   **Table 2.** FISH cell counts for snottite communities

Sample	FISH probe abundance				
	EUBMIX	THIO1	ACM732	ARCH915	FER656
GS07-35	88.9 (±4.4)	78.1 (±2.6)	8.2 (±1.5)	9.8 (±4.7)	0.6 (±1.1)
GS08-6	95.6 (±2.7)	91.5 (±2.0)	1.7 (±1.6)	1.8 (±0.9)	nd
RS05-2	79.8 (±8.4)	68.7 (±7.9)	8.6 (±5.8)	17.7 (±16.5)	11.4 (±7.3)
RS05-24	75.3 (±8.2)	68.8 (±8.0)	5.8 (±2.0)	16.5 (±8.60	nd
RS05-1	61.9 (±4.8)	56.0 (±5.6)	3.5 (±1.7)	38.6 (±13.9)	22.5 (±4.6)
RS05-20	61.0 (±7.0)	54.4 (±7.1)	5.0 (±1.5)	34.9 (±10.2)	14.3 (±9.1)
RS07-21	66.3 (±7.7)	53.8 (±5.0)	10.7 (±3.4)	31.9 (±6.8)	4.4 (±1.4)
RS07-22	66.2 (±6.6)	57.5 (±8.9)	6.7 (±4.8)	31.2 (±6.6)	1.3 (±1.7)
RS08-30	85.5 (±7.8)	83.0 (±7.5)	0.3 (±0.4)	10.0 (±8.0)	9.3 (±4.8)
RS08-31	70.6 (±7.2)	57.2 (±8.9)	7.5 (±5.8)	22.6 (±5.8)	1.4 (±1.0)
RS09-1	63.9 (±6.3)	51.3 (±6.4)	7.0 (±2.8)	30.9 (±5.6)	4.5 (±1.6)
RS09-10	87.5 (±5.1)	81.3 (±6.6)	4.6 (±4.60	9.4 (±4.2)	nd
RS09-11	82.7 (±7.5)	68.5 (±11.5)	9.5 (±5.4)	18.6 (±4.4)	0.5 (±0.6)
PC05-1	76.3 (±7.0)	75.9 (±7.3)	nd	1.76 (±4.7)	nd
PC05-13	79.3 (±8.6)	77.8 (±8.8)	0.7 (±1.8)	0.5 (±0.8)	nd
GB08-30	86.9 (±4.7)	74.4 (±8.5)	11.4 (±6.4)	15.6 (±3.8)	0.4 (±4.8)
AS05-5	22.7 (±12.6)	22.4 (±12.0)	nd	49.0 (±11.6)	49.0 (±11.6)
AS07-1	66.9 (±11.4)	66.9 (±11.4)	nd	30.2 (±11.0)	30.2 (±11.0)
AS07-3	61.6 (±3.7)	61.6 (±3.7)	nd	38.4 (±3.7)	38.4 (±3.7)
AS07-4	91.3 (±5.9)	88.7 (±4.0)	1.5 (±1.0)	3.1 (±1.8)	nd
AS08-5	72.2 (±4.3)	72.2 (±4.3)	nd	25.8 (±4.7)	25.8 (±4.7)
AS08-6	69.4 (±4.7)	65.8 (±4.1)	2.4 (±0.8)	29.2 (±5.3)	8.7 (±1.7)
VL07-20	87.2 (±7.2)	61.9 (±7.3)	10.3 (±5.0)	10.8 (±5.1)	2.0 (±2.8)
VL07-26	90.3 (±2.7)	70.6 (±3.3)	13.7 (±2.8)	5.4 (±1.4)	0.5 (±0.5)
LA07-10	96.1 (±2.1)	79.5 (±6.3)	9.7 (±1.9)	3.1 (±3.5)	nd
LA07-13	96.8 (±0.9)	87.7 (±2.7)	6.4 (±2.2)	0.9 (±0.7)	nd

Numbers for each FISH probe represent percentage of total DAPI stained cells. Numbers in parentheses represent ± one standard deviation. nd = none detected. FISH probe specificity (Table 3) is as follows: EUMBIX = Bacteria, THIO1 = *Acidithiobacillus,* AMC732 = *Acidimicrobium,* ARCH915 = Archaea, FER656 = *Ferroplasma.*

729   **Table 3.** FISH probes used.

Probe name	Sequence (5'-3')	% formamide	specificity	label	source
EUB338	GCT GCC TCC CGT AGG AGT	0-50%	most Bacteria	FITC	Amann et al., 1990
EUB338-II	GCA GCC ACC CGT AGG TGT	0-50%	Planctomycetales	FITC	Daims et al., 1999
EUB338-III	GCT GCC ACC CGT AGG TGT	0-50%	Verrucomicrobiales	FITC	Daims et al., 1999
ARCH915	GTG CTC CCC CGC CAA TTC CT	20%	most Archaea	CY3, FITC	Stahl and Amann, 1991
THIO1	GCG CTT TCT GGG GTC TGC	35%	Acidithiobacillus spp.	CY3, CY5	Gonzalez-Toril et al. 2003
ACM732	GTA CCG GCC CAG ATC GCT G	35%	Acidimicrobium spp.	CY3	Bond et al. 2000
FER656	CGT TTA ACC TCA CCC GAT C	25%	Ferroplasma spp.	CY3	Bond et al. 2000

EUBMIX is a mixture of EUB338, EUB338-II, and EUB338-III