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GEOMICROBIOLOGY OF BIOVERMICULATIONS FROM THE FRASASSI CAVE SYSTEM, ITALY

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Abstract: Sulfidic cave walls host abundant, rapidly-growing microbial communities that display a variety of morphologies previously described for vermiculations. Here we present molecular, microscopic, isotopic, and geochemical data describing the geomicrobiology of these biovermiculations from the Frasassi cave system, Italy. The biovermiculations are composed of densely packed prokaryotic and fungal cells in a mineral-organic matrix containing 5 to 25% organic carbon. The carbon and nitrogen isotope compositions of the biovermiculations ($\delta^{13}C = -35$ to -43‰, and $\delta^{15}N = 4$ to -27‰, respectively) indicate that within sulfidic zones, the organic matter originates from chemolithotrophic bacterial primary productivity. Based on 16S rRNA gene cloning (n=67), the biovermiculation community is extremely diverse, including 48 representative phylotypes (>98% identity) from at least 15 major bacterial lineages. Important lineages include the Betaproteobacteria (19.5% of clones), Gammaproteobacteria (18%), Acidobacteria (10.5%), Nitrospirae (7.5%), and Planctomyces (7.5%). The most abundant phylotype, comprising over 10% of the 16S rRNA gene sequences, groups in an unnamed clade within the Gammaproteobacteria. Based on phylogenetic analysis, we have identified potential sulfur- and nitrite-oxidizing bacteria, as well as both auto- and heterotrophic members of the biovermiculation community. Additionally, many of the clones are representatives of deeply branching bacterial lineages with no cultivated representatives. The geochemistry and microbial composition of the biovermiculations suggest that they play a role in acid production and carbonate dissolution, thereby contributing to cave formation.

INTRODUCTION

Sulfidic caves are limestone caves that form via the oxidation of hydrogen sulfide to sulfuric acid. Although this mechanism is not as widespread as epigenic, carbonic-acid karstification (Palmer, 1991), sulfidic processes have created many spectacular caverns, unforgettable for their massive, resplendent rooms and unusual sulfur mineral deposits. Cave formation occurs near the water table where sulfide-bearing phreatic water mixes with oxygenated vadose waters and cave air. As hydrogen sulfide is oxidized to sulfuric acid (e.g., Equation (1)), the sulfuric acid then reacts with limestone to form gypsum (Equation (2)).

$$H_2S + 2O_2 \rightarrow H_2SO_4 \quad (1)$$

$$H_2SO_4 + CaCO_3 + H_2O \rightarrow CaSO_4 2H_2O + CO_2 \quad (2)$$

Where cave streams and lakes are undersaturated with gypsum, calcium and sulfate are carried away in solution. Above the water table, however, gypsum crusts form as degassing hydrogen sulfide oxidizes in condensation droplets (Egemeier, 1981). Gypsum deposits and formations in Carlsbad Cavern and Lechuguilla Cave in the Guadalupe Mountains, New Mexico, are interpreted as evidence for past sulfidic processes (Hill, 1998). For insightful discussions of cave formation by sulfuric acid dissolution, see Egemeier (1981) and recent geomicrobiology research by Engel et al. (2004b).

The oxidation of hydrogen sulfide provides a rich energy source for chemolithotrophic microorganisms. In sulfidic caves, these microorganisms form the trophic base of aphotic ecosystems that support macroinvertebrate life, and in some cases, even vertebrates (Sarbu et al., 1996; Hose and Pisarowicz, 1999). Because these microbial communities are isolated in the subsurface and ultimately supported by energy from reduced sulfur compounds, sulfidic caves are important analogues for early Earth environments, and potentially, for sulfur-rich extraterrestrial environments (Boston et al., 2001). Microbial communities associated with sulfidic caves include viscous, highly acidic "snottites" that drip from overhanging surfaces, white microbial mats in cave streams, less conspicuous microbial communities in gypsum wall crusts and stream sediments, and biovermiculations — anastomosing, microbe-packed films of organic-rich sediment on cave ceilings and walls (Hose and Pisarowicz, 1999; Engel et al., 2004a; Hose and Macalady, 2006).

Biovermiculations resemble vermiculations common to carbonic-acid caves. Vermiculations are broadly described as discontinuous, worm-like deposits of mud and clay found on cave walls and ceilings (Hill and Forti, 1997).

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However, Hose et al. (2000) distinguished between vermiculation-like microbial formations in the sulfidic Cueva de la Villa Luz and other vermiculations because biovermiculations form more rapidly and host a rich and active microbial flora. We have adopted the term biovermiculation because the dynamic geochemical and biological processes in sulfidic caves contrast sharply with the oligotrophic conditions typical for carbonic acid caves. Although it is possible that all vermiculations are biologically mediated (Anelli and Graniti, 1967; Northup and Lavoie, 2001; Camassa and Febbroriello, 2003), this study focuses on vermiculations found in sulfidic environments.

Substantial research has focused on describing microbial communities in sulfidic caves. Stream biofilms are the most widely studied (Thompson and Olson, 1988; Hubbard et al., 1990; Sarbu et al., 1994; Angert et al., 1998; Hose et al., 2000; Engel et al., 2001; Rohwerder et al., 2003; Engel et al., 2004a; Macalady et al., 2006). Snottites from several different caves have also recently been described using molecular methods (Hose et al., 2000; Vlasceanu et al., 2000; Macalady et al., 2007). However, existing microbiological research on biovermiculations is limited to microscopy and culture-based surveys (Hose and Pisarowicz, 1999; Hose et al., 2000) and preliminary molecular work reported by Hose and Northup (2004). This report builds on observations in the Frasassi cave system made by Galdenzi (1990), and represents the first in-depth microbiological study of biovermiculations using molecular and isotopic methods.

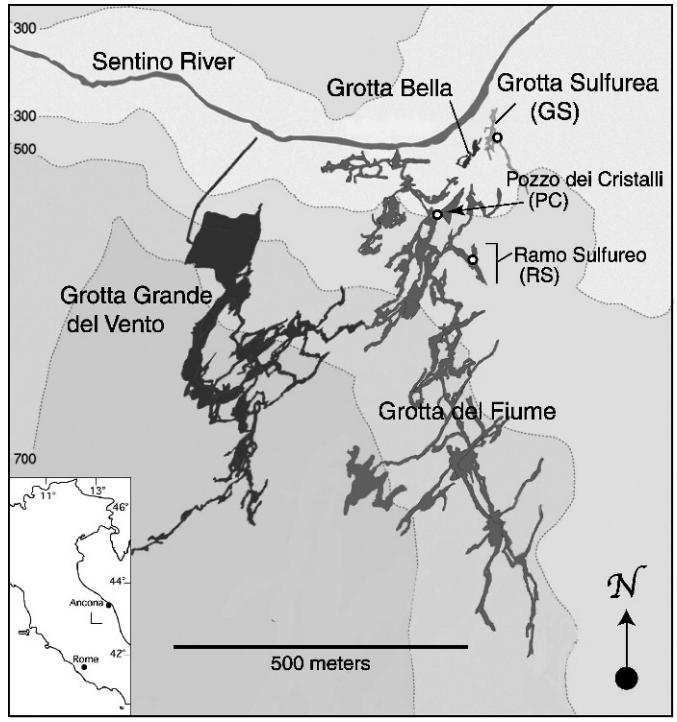

Figure 1. Map of the Grotta Grande del Vento-Grotta del Fiume (Frasassi) cave system showing sample locations (circles). Topographic lines and elevations (m) refer to surface topography above the cave. Grotta del Mezzogiorno-Grotta di Frasassi complex (not shown) is located approximately 500 m northeast of Grotta Grande del Vento. Base map courtesy of the Gruppo Speleologico CAI di Fabriano.

Methods

Field Site, Sample Collection And Geochemistry

The Grotta Grande del Vento-Grotta del Fiume (Frasassi) cave system comprises over 20 km of ramifying and irregular passage in Jurassic limestone of the Calcare Massiccio Formation (Galdenzi and Maruoka, 2003) of the Frasassi Gorge. Sulfuric acid speleogenesis is actively occurring near the water table, which is accessible via technical caving routes. For group safety, we carry an ENMET MX2100 portable gas detector with H_2S, O_2, and SO_2 sensors. We wear respirators on the rare occasions that H_2S levels exceed OSHA safety standards (10 ppm). Sampling locations are shown in Figure 1. Cave streams in Ramo Sulfureo (RS) are fast flowing and turbulent while Grotta Sulfurea (GS) and Pozzo dei Cristalli (PC) streams are characterized by slowly flowing water and stagnant pools. Dissolved sulfide levels in the cave streams are between 2–3 times higher at PC than the other two locations (Macalady et al., 2006), but hydrogen sulfide gas concentrations are highest in the cave air at RS (Macalady et al., 2007). Gypsum crusts are extensive at PC and RS, but at GS gypsum crusts are found only within two meters above the water table (unpublished observations). A vermiculation sample was collected from the older Grotta del Mezzogiorno-Grotta di Frasassi (GM-GF) cave complex, developed at higher elevation in the same geologic formation (Galdenzi and Maruoka, 2003). The Grotta del Mezzogiorno-Grotta di Frasassi system is located over 300 meters above the present day water table, and it is unclear whether this cave system originated by sulfuric acid speleogenesis (S. Galdenzi, pers. comm.). Because the GM-GF system is not currently experiencing sulfidic processes, it is not clear what, if any, biological role has been played in their formation. Therefore, we classify vermiculations from this system simply as a vermiculations, rather than biovermiculations.

Samples were collected between 2002 and 2006 in sterile tubes using sterilized metal spatulas. Snottite and stream biofilm samples in Table 1 were collected as described previously (Macalady et al., 2006; Macalady et al., 2007). Wherever possible, pH was measured with pH paper (range 5–10 and 4–7). In 2005 and 2006, cave air concentrations of H_2S, CO_2, NH_3, and N_2O were measured at each sample location using Dräeger short-duration tubes and an Accuro hand pump (Dräeger Safety Inc., Germany). Detection limits for H_2S and CO_2 are between 0.2–60 ppm and 100–6000 ppm, respectively. NH_3 and N_2O were undetectable at all sites. Lower detection limits were 0.25 for NH_3 and 0.5 for N_2O.

ELEMENTAL AND ISOTOPIC ANALYSES

Samples for elemental and isotopic analysis were stored at 4 °C immediately after collection and dried at 70 °C within 24 hours. Samples were acid-washed with HCl to remove carbonate minerals. Acid washing was performed as follows: Samples were finely ground and reacted with ACS grade 2M HCl for two hours under constant agitation. Afterwards, samples were centrifuged and the supernatant decanted. Samples were then washed several times with distilled, deionized water to remove residual chloride ions, then freeze-dried and homogenized. Elemental analysis for carbon, nitrogen, phosphorous, and sulfur were performed at the Agricultural Analytical Services Laboratory at Pennsylvania State University, where total carbon and nitrogen were determined by combustion in a Fisions NA 1500 Elemental Analyzer and phosphorous and sulfur were determined by microwave digestion (Miller, 1998). Isotopic analyses were performed via an Elemental Analyzer – Isotope Ratio Mass Spectrometer (EA-IRMS) at the Stable Isotope Biogeochemistry Laboratory at Penn State. Samples were combusted in a Costech 4010 Elemental Analyzer, and subsequently introduced by continuous flow inlet into a Thermo-Finnegan Delta Plus XP Isotope Ratio Mass Spectrometer (IRMS). Stable isotopic ratios are reported in delta (δ) notation as follows:

$$X = 10^3 \left[\left(\frac{R_{\text{sample}}}{R_{\text{standard}}} \right) - 1 \right] \quad (3)$$

where X is $\delta^{13}C$ or $\delta^{15}N$, and R denotes the abundance ratio of the heavy to light isotope, i.e., $^{13}C/^{12}C$ or $^{15}N/^{14}N$. Standards for carbon and nitrogen are Vienna PeeDee Belemnite (VPDB) and atmospheric nitrogen (AIR), respectively. Based on internal standards and replicate samples, precision of all measurements is ±0.2‰.

FLUORESCENCE MICROSCOPY AND SCANNING ELECTRON MICROSCOPY (SEM)

Samples for microscopy were stored at 4 °C and either fixed in 4% paraformaldehyde (pfa) within 24 hours of collection, or stored at −20°C in a 1:5 dilution of RNAlater (Ambion, U.S.A.) and later fixed in 4% pfa. Fixation was performed as in Macalady et al. (2007). The fluorescent dye 4′,6′-diamidino-2-phenylindole (DAPI) was used to label cells for microscopy. Fixed samples and control cells were applied to multiwell, Teflon-coated glass slides, air-dried, and dehydrated with ethanol. Ten µL of DAPI (1 µg/mL) was applied to each well, incubated for 5 minutes, rinsed with distilled water, dried, and mounted with Vectashield (Vectashield Laboratories, USA). Fluorescence *in situ* hybridization (FISH) analyses were performed using methods and probes exactly as described in Macalady et al. (2007). Slides were viewed on a Nikon E800 epifluorescence microscope. Air-dried and gold-coated samples were also examined with a Hitachi S-3000N SEM. Secondary electron images were taken under high vacuum mode at an accelerating voltage of 10 KeV.

CLONE LIBRARY CONSTRUCTION AND DNA SEQUENCING

A clone library of bacterial 16S rRNA genes was constructed from biovermiculation sample GS03-5, collected in Grotta Sulfurea in 2003 at the same location as samples for geochemical and isotopic analyses (Table 2). DNA was extracted at the Osservatorio Geologico di Coldigioco Geomicrobiology Lab within 24 hours of collection using the MoBio Soil DNA extraction kit (MoBio Laboratories, Inc., USA) according to the manufacturer's instructions. Small subunit rRNA genes were amplified and cloned as in Macalady et al. (2007) using bacterial forward primer 27f (5′-AGAGTTTGATCCTGGCTCAG-3′) and universal reverse primer 1492r (5′-GGTTACCTTGTTACGACTT-3′). Clones were sequenced at the Penn State University Biotechnology Center using T3 and T7 plasmid-specific primers. Partial sequences were assembled with Phred base calling using CodonCode Aligner v.1.2.4 (CodonCode Corp., USA) and manually checked. Assembled sequences were submitted to the online analyses CHIMERA_CHECK v.2.7 (Cole et al., 2003) and Bellerophon 3 (Huber et al., 2004). Putative chimeras were excluded from subsequent analyses.

PHYLOGENETIC AND DIVERSITY ANALYSES

The nearly full-length gene sequences were compared against sequences in public databases using BLAST (Altschul et al., 1990) and the online workbench greengenes (http://greengenes.lbl.gov/cgi-bin/nph-index.cgi; DeSantis et al., 2006). Sequences were aligned using the NAST aligner available at the greengenes web site. NAST-aligned sequences were added to a phylogenetic tree containing >150,000 bacterial sequences using the ARB_parsimony tool (Ludwig et al., 2004). Further phylogenetic analyses were generated using maximum parsimony. Maximum parsimony phylograms (heuristic search) and bootstrap consensus trees (heuristic search, 500 replicates) were computed using PAUP* 4.0b10 (Swofford, 2000). Analyses included closest BLAST matches of environmental sequences and cultivated representatives. Phylogenetic relationships of the clones are referenced to the Hugenholtz taxonomy (Desantis et al., 2006; available at http://greengenes.lbl.gov).

Clones in the 16S rRNA library were grouped into operational taxonomic units (OTUs) based on >98% nucleotide identity. Rarefaction analyses were computed using EstimateS 7.5.0 (Colwell, 2005).

Representative 16S rRNA gene sequences for each phylotype determined in this study have GenBank accession numbers DQ499275 to DQ499330 and EF530677 to EF530681.

RESULTS

DISTRIBUTION AND MORPHOLOGY OF BIOVERMICULATIONS

Biovermiculations occur throughout the Frasassi cave system and are found immediately above the water table to over 40 meters above it where no sulfide is detectable by

Table 1. BLAST summary of 16S rRNA clone library.

Representative clone[a]	# clones	Top BLAST match	Accession	% identity
Gammaproteobacteria	12			
Piscirickettsiaceae	1			
CV92	1	Gas hydrate associated sediment clone Hyd89-87	AJ535246	93
Nevskiaceae	1			
CV109	1	polychlorinated biphenyl-polluted soil clone WD280	AJ292674	95
Acidithiobacillaceae	9			
CV44	2	Guanting Reservoir (China) sediment clone 69-25	DQ833501	98
CV45	7	Acid mine drainage sediment clone H6	DQ328618	94
Unclassified	1			
CV77	1	Uranium contaminated aquifer clone 1013-28-CG33	AY532574	99
Betaproteobacteria	13			
CV10	3	Frasassi stream biofilm WM93	DQ415787	98
CV11	3	Green Bay ferromanganous micronodule clone MND1	AF293006	98
CV21	3	Uranium contamination clone AKAU3480	DQ125519	93
CV30	1	siliceous sedimentary rock clone MIZ05	AB179496	95
CV41	2	African subsurface water clone EV221H2111601SAH33	DQ223206	95
CV71	1	Manganese-oxidizing soil clone JH-GY05	DQ351927	97
Alphaproteobacteria	4			
CV17	1	farm soil clone AKYH1192	AY921758	98
CV25	1	farm soil clone AKYH1192	AY921758	97
CV43	1	freshwater sponge *Spongilla lacustris* clone SS31	AY598790	97
CV81	1	denitrifying quinoline-removal bioreactor clone SS-57	AY945873	94
Deltaproteobacteria	3			
CV1	1	Mangrove soil clone MSB-5C5	DQ811828	93
CV34	1	Arabian sea picoplankton clone A714011	AY907798	89
CV90	1	Soil clone WHEATSIP-CL55	DQ822247	95
Cytophaga-Flexibacter-Bacteroides	3			
CV24	1	Guanting Reservoir (China) sediment clone 69-27	DQ833502	98
CV70	2	flooded poplar tree microcosm soil clone 21BSF16	AJ863255	92
Nitrospira	5			
CV22	2	Chinese deep sea sediment nodule clone E75	AJ966604	96
CV64	1	Green Bay ferromanganous micronodule clone MNC2	AF293010	98
CV82	2	U contaminated aquifer clone 1013-28-CG51	AY532586	95
Verrucomicrobia	2			
CV35	1	coastal marine sediment clone Y99	AB116472	95
CV80	1	Freshwater clone PRD01a004B	AF289152	93
Planctomycetes	5			
CV14	1	Broad-leaf forest soil clone AS47	AY963411	89
CV47	1	Lake Kauhako Hawaii 30 m clone K2-30-19	AY344412	90
CV73	1	marine Pirellula clone 5H12	AF029076	94
CV97	1	Soil bacteria CWT SM02_H07	DQ129094	94
CV104	1	Anaerobic ammonia oxidizing community clone LT100PlB4	DQ444387	93
Acidobacteria	7			
CV12	1	Bor Khlueng hot spring (Thailand) clone PK-85	AY555795	93
CV18	2	drinking water biofilm clone Biofilm_1093d_c1	DQ058683	97
CV68	1	Lake Washington sediment clone pLW-64	DQ066994	97
CV76	1	Hamelin Pool stromatolite clone HPDOMI1G01	AY851769	91
CV79	1	PCB polluted soil clone Lhad8	DQ648907	98
CV94	1	Amazon soil clone 1267-1	AY326533	97
Actinobacteria	2			
CV54	2	San Antonio urban aerosol clone AKIW984	DQ129354	95

Table 1. Continued.

Representative clone[a]	# clones	Top BLAST match	Accession	% identity
Chloroflexi	1			
CV37	1	Lost City Hydrothermal Field clone LC1537B-77	DQ272585	94
OP11	2			
CV31	1	deep groundwater clone KNA6-NB23	AB179676	92
CV46	1	Massachusetts river clone PRD01a009B	AF289157	96
RCP2-18	1			
CV51	1	Deep sea cold seep sediment clone BJS81-120	AB239028	90
SPAM	1			
CV52	1	Lake Washington sediment clone pLW-68	DQ067010	97
TM7	2			
CV63	1	batch reactor clone SBR2013	AF269000	94
CV78	1	Sulfur-oxidizing community clone IC-42	AB255066	89
WS3	2			
CV39	1	Chinese deep sea sediment nodule clone MBAE32	AJ567590	91
CV106	1	antarctic sediment clone MERTZ_2CM_290	AF424308	91
Unclassified	2			
CV40	1	3.5 Ma oceanic crust clone CTD005-79B-02	AY704386	91
CV60	1	siliceous sedimentary rock clone MIZ45	AB179536	94

[a] Taxonomic groupings based on Hugenholtz taxonomy (Desantis et al., 2006). Representative clones correspond to OTUs.

portable gas sampling equipment (0.2 ppm detection limit). Hydrogen sulfide concentrations at sample collection sites ranged from undetectable to 4.3 ppm (Table 2), and in general, gas concentrations in Frasassi are seasonally variable (Galdenzi et al., 2007). Biovermiculations have only been observed on limestone surfaces where no gypsum crust is present. Several attempts to identify biovermiculations or similar structures beneath gypsum crusts on walls immediately adjacent to biovermiculations have been unsuccessful. We also noted the strict absence of biovermiculations on the surface of chert nodules, even when adjacent limestone walls were thickly colonized (Fig. 2c).

Frasassi biovermiculations exhibit several of the morphologies described by Parenzan (1961): bubble-like spots, elongated spots, leopard spots, and tiger skin. In some places, we observed biovermiculations so dense that they formed continuous wall-covering sheets. Sample PC06-106 (Fig. 2e), sampled at the high water mark one meter above the stream level in Pozzo dei Cristalli, was very light brown to grey. Other biovermiculations were dark brown or occasionally black. In contrast, vermiculations sampled from GM-GF (GM05-1, Fig. 2f) displayed dendritic morphology and were surrounded by an obvious light-colored halo. The GM-GF vermiculations were light grey in color, and covered a small area of the limestone walls relative to sample sites in sulfidic zones.

GEOCHEMICAL ANALYSES

Geochemical data for biovermiculation samples are listed in Table 2. Vermiculations from GF-GM (sample GM05-1) are listed separately because this cave has not experienced sulfidic processes in over 200,000 years, if ever. We also provide data from stream biofilm and snottite samples to enable comparison with other microbial communities within the same cave system. In addition to the values presented in Table 2, we measured the pH of numerous biovermiculations in sulfidic zones over the past five years and all measurements were between 5.5 and 6.5 (data not shown). Replicate gas measurements made in a given location on the same day were within 20%.

Previously measured carbon and nitrogen isotopic ratios of invertebrates and microbial communities within sulfidic zones indicate a chemolithotrophic energy base for the cave ecosystem (Galdenzi and Sarbu, 2000). Biovermiculation $\delta^{13}C$ and $\delta^{15}N$ values measured in this study (Fig. 3) fall within or near the range of sulfidic zone samples analyzed by Galdenzi and Sarbu (2000), Sarbu et al. (2000), and Vlasceanu et al. (2000). In contrast, the GM-GF (GM05-1) sample falls within the isotopic range of materials collected from the surface and near cave entrances (Fig. 3).

FLUORESCENCE MICROSCOPY AND SEM

We used the fluorescent DNA stain DAPI to examine several biovermiculation samples. Characteristic photomicrographs of DAPI-stained samples are shown in Figure 4. A range of cell morphologies and sizes can be observed, although small coccoid-shaped cells (<1 μm) are the most common. Short rod-shaped (1 to 3 μm) cells are also abundant and sometimes form filaments (Fig. 4b and c). Larger, undifferentiated filaments with multiple nuclei are common and likely represent fungal hyphae. Attempts to analyze biovermiculation samples using fluorescence *in situ* hybridization (FISH) were largely unsuccessful because of high autofluorescence in the biovermiculation matrix. However, cells binding to bacteria-specific probes were

Table 2. Summary of elemental and isotopic analysis of biovermiculation samples.

Parameter	Sample GS04-58	Sample GS06-31	Sample PC02-24	Sample PC06-106	Sample RS03-18	Sample GM05-1	Sample GS06-15	Sample RS06-120
Sample Collection Info.	Biovermiculations					Epigenic Vermiculation	Beggiatoa Stream Mat	Snottite
Sample Type								
Location[a]	GS	GS	PC	PC	RS	GM	GS	RS
Date Collected	7/22/04	8/16/06	12/8/02	8/21/06	10/28/03	8/15/05	8/16/06	8/23/06
Height Above Water table	2 m	2 m	20 m	1 m	10 m	>200 m	0 m	2 m
pH	nm	6	6.0–6.5	6	nm	nm	7.1	0–1
Elemental Analysis								
Percent Carbon	6.67	5.72	19.57	24.96	19.63	27.82	12.98	5.83
Percent Nitrogen	0.76	0.62	2.47	3.12	2.59	4.92	2.11	0.31
Percent Phosphorous	0.2	0.16	nm	nm	nm	nm	0.15	0.04
Percent Sulfur	1.27	0.49	nm	nm	nm	nm	8.25	21.08
C:N	8.8:1	9.2:1	9.2:1	9.3:1	8.8:1	6.6:1	6:1	19:1
C:N:P[b]	33:4:1	35:4:1	nm	nm	nm	nm	85:14:1	139:7:1
Isotopes								
$\delta^{13}C$	−37.3	−36.4	−35.1	−36.9	−42.7	−20.5	−37.1	nm
$\delta^{15}N$	1.2	2.5	2.7	−26.5	4.2	16.1	−9.9	nm
Air Chemistry[c]								
$[H_2S]$ ppm	<0.2	<0.2	<0.2	2–4.3	0.4	nm	nm	17–25
$[CO_2]$ ppm	1000	1000	1300	1600	>3000	nm	nm	3300

[a] Sampling locations are given in Figure 1. GS = Grotta Sulfurea, PC = Pozzo dei Cristalli, RS = Sulfureo, GM = Grotta Madonna.
[b] Rounded to nearest whole number.
[c] Based on gas measurements at sample site in 2005–2006.
nm = Not measured.

detected in a few regions with low autofluorescence, indicating at least some active microbial populations (results not shown). Scanning electron microscopy (SEM) (Fig. 4a) revealed the presence of clay minerals and corroded calcite grains. We also observed abundant prokaryotes and noted the presence of larger eukaryotic cells. In some regions, amorphous and filamentous microbial biofilm material coated mineral grains (Fig. 4a).

16S rRNA Clone Library

We created a clone library of bacterial 16S rRNA genes from biovermiculation sample GS03-5, collected in Grotta Sulfurea in 2003. This sample had pH 5.6, and was collected from the same location as the samples for geochemical and isotopic analysis (Table 2). The 67 non-chimeric 16S rRNA sequences were analyzed by adding NAST-aligned sequences to a phylogenetic tree containing >150,000 bacterial sequences using the ARB_parsimony tool (Ludwig et al., 2004). The resulting 48 phylotypes (defined as monophyletic clades with >98% sequence identity) were consistent with taxonomy inferred based on BLAST similarities. Taxonomic affiliations and closest BLAST matches (Altschul et al., 1990) of the clones are summarized in Table 1. Clones grouped within 15 major bacterial lineages including the Gamma(beta)-, Alpha-, and Deltaproteobacteria, Acidobacteria, Planctomyces, Cytophaga-Flexibacter-Bacteroides, Verrucomicrobia, Nitrospirae, Actinobacteria, and Chloroflexi (Fig. 5a). More than 10% of the clones belong to deeply-branching, phylum-level clades with no cultivated representatives, including TM7, OP11, RCP2-18, WS3, and SPAM. Further phylogenetic analyses were conducted for phylotypes which fell within clades with a consistent metabolic strategy, or which had close relationships with cultivated species. These analyses are shown in Figure 6 through 9 and are discussed in the text below.

Discussion

Biovermiculation Communities

Rarefaction analysis of the CV clone library (Fig. 5b) indicates that biovermiculations are among the most diverse microbial communities analyzed thus far from the Frasassi cave complex, and the shape of the rarefaction curve indicates that additional diversity in the biovermiculations remains to be described in future efforts. The majority of 16S rRNA gene sequences retrieved from the biovermiculation sample are distant (<90% identity) from cultivated isolates, and many are distant from environmental sequences as well (Table 1). This makes it difficult to hypothesize about potential metabolisms for most of the biovermiculation clones. However, inferences about the physiology of certain clones can be made on the basis of geochemistry and phylogenetic relationships.

We performed a maximum parsimony analysis targeting CV45, the most abundant phylotype in the clone library. CV45 represents seven clones, comprising over 10% of the library, which suggests it is an important member of the biovermiculation community. Phylogenetic analysis (Fig. 6) shows that CV45 groups in an unnamed clade within the Gammaproteobacteria. Figure 6 also includes phylotype CV44 (2 clones) and the iron-oxidizing acidophilic isolate m-1, which was originally misclassified as *Acidithiobacillus ferrooxidans* (Johnson et al., 2001). The closest BLAST match and phylogenetic neighbor to CV45 is clone H6, sequenced from acid mine drainage (Hao et al., 2007), an environment in which the primary electron donors are reduced iron and sulfur. Numerous other sequences from acid mine drainage are also found in the clade as shown in Figure 6. These phylogenetic relationships suggest that CV45 may be a sulfur or iron oxidizer. Analysis of another phylotype, CV10, suggests that it is a sulfur-oxidizer (Fig. 7). CV10 is closely related to *Thiobacillus* species within the Betaproteobacteria. Named *Thiobacillus* species in Figure 8 are sulfur-oxidizing autotrophs (Drobner et al., 1992; Beller et al., 2006), including *Thiobacillus thioparus*, which is important in the sulfidic Movile Cave, Romania (Vlasceanu et al., 1997). Figure 7 also includes the heterotrophic, sulfur-oxidizing genus *Spirillum*, (Podkopaeva et al., 2006), several other sulfur-oxidizing isolates, and Frasassi stream biofilm clones sequenced by Macalady et al. (2006). In addition to the phylogenetic evidence for sulfur-oxidation in biovermiculations presented here, Hose et al. (2000) observed growth in both thiosulfate and sulfide enrichment cultures inoculated with biovermiculations from sulfidic Cueva de Villa Luz, Mexico. For other sequences in the clone library (Table 1), distant relationships to cultivated species do not allow further speculation about their metabolisms. Work in progress by our group will attempt to enrich microbes from sulfidic cave biovermiculations to further explore important metabolisms.

We also performed a maximum parsimony analysis on the genus *Nitrospira*, which includes five biovermiculation clones represented by phylotypes CV22, CV64, and CV82 (Fig. 8). The genus *Nitrospira* includes named species *N. moscoviensis* and *N. marina*, both characterized by autotrophic nitrite reduction (Watson et al., 1986; Ehrich et al., 1995; Altmann et al., 2003). Many of the other environmental clones in the *Nitrospira* were sequenced from nitrifying environments. The presence of this group suggests that oxidation of reduced nitrogen compounds may be an important type of microbial energy generation in biovermiculation communities.

Organotrophy is another potential means for energy production in biovermiculations. Phylogenetic analysis of CV109 (Fig. 9) shows that it groups in the Nevskiaceae family, related to the organotrophic genera *Nevskia* and *Hydrocarboniphaga* (Glöckner et al., 1998; Palleroni et al., 2004) as well as other hydrocarbon-degrading isolates.

In addition to the phylotypes discussed above, the clone library contained diverse members of the phyla Acidobacteria and Actinobacteria. Members of these two lineages were

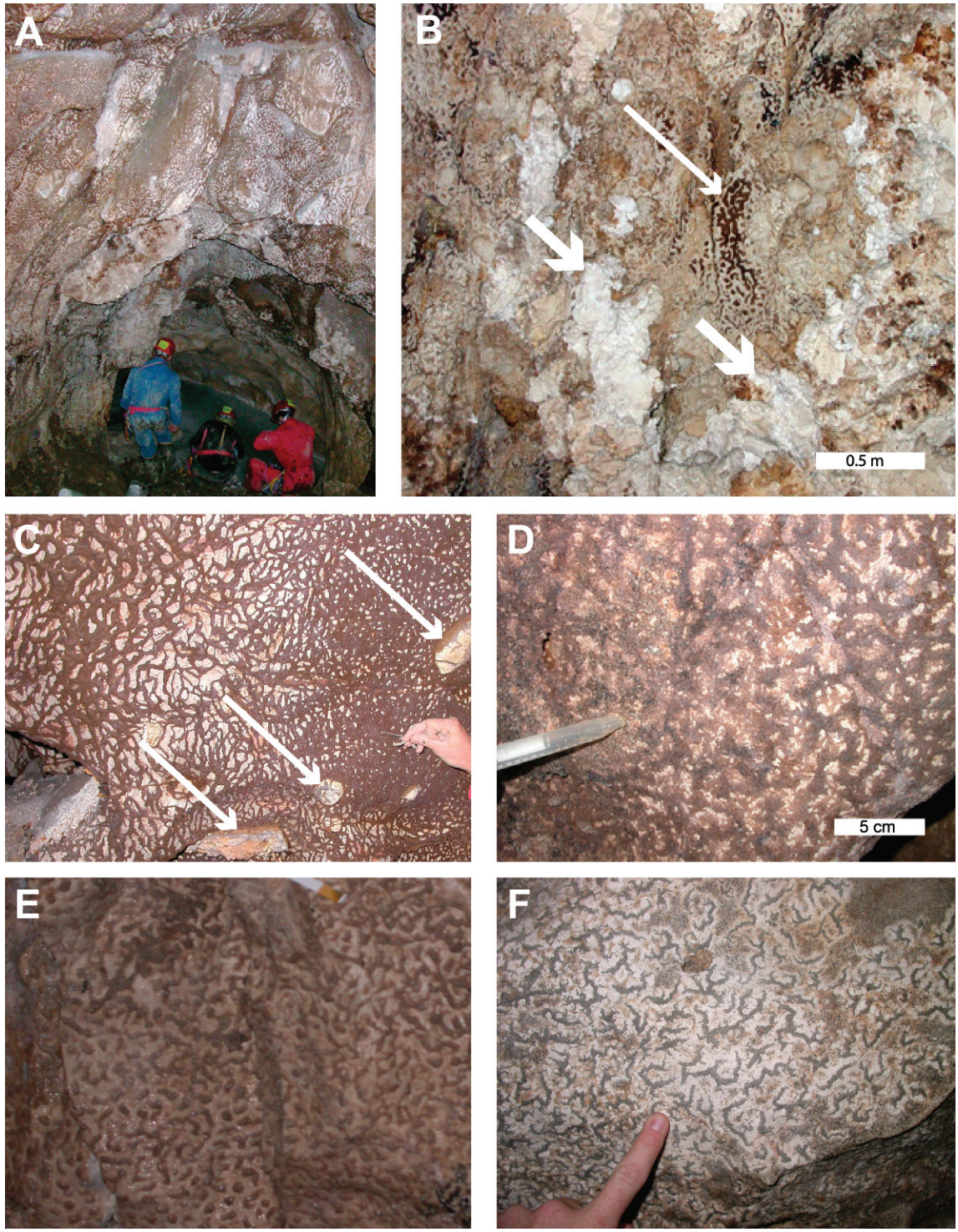

Figure 2. Biovermiculations from the Frasassi cave system. (A) Biovermiculations covering cave walls in Grotta Sulfurea. (B) Biovermiculations covering an isolated patch of limestone surrounded by thick gypsum wall crust, in Pozzo dei Cristalli. Long arrow indicates biovermiculations and fat arrows indicate white gypsum crust. (C) Biovermiculations from Grotta Sulfurea. Note the absence of biovermiculations on chert nodules (white arrows). (D) Biovermiculations from Ramo Sulfureo. (E) Sample PC06-106, from Pozzo dei Cristalli. This sample was forming below the high water mark within 50 cm above the cave stream surface. (F) Sample GM05-1. Vermiculations from the Grotta del Mezzogiorno-Grotta di Frasassi complex are over 300 meters above the current water table.

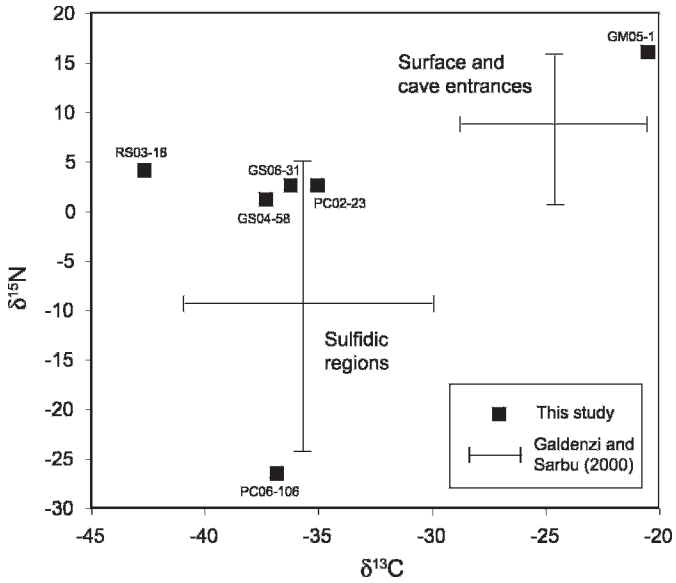

Figure 3. Carbon and nitrogen stable isotopic ratios of organic matter from sulfidic cave zones and outside environments. Biovermiculations analyzed in this study are marked by black squares, and values associated with sample names are given in Table 2. Bars indicate the range of values reported by Galdenzi and Sarbu (2000) for other sample types (n = 18 for surface samples, n = 32 for sulfidic samples). Note the clear separation between values from sulfidic regions compared to surface and cave entrances.

reported in biovermiculations from sulfidic Cueva de Villa Luz (Hose and Northup, 2004). In our clone library, Acidobacteria and Actinobacteria represent 10.5% and 3% of clones, respectively (Table 1; Fig. 5a). In addition to the prokaryotic community in biovermiculations, we also observed larger nucleated cells with morphologies typical for protists and fungal hyphae. We also noted several types of macroinvertebrates living on the cave walls in close proximity to biovermiculations, including spiders (*Nesticus sp.*), isopods (*Androniscus sp.*), springtails (Order Collembola), annelid worms (Class Oligochaeta), and nematodes (Phylum Nematoda) (Sharmishtha Dattagupta, unpublished results).

ELEMENTAL AND ISOTOPIC COMPOSITION

Based on elemental analysis, Frasassi biovermiculations have an organic carbon content between 5.7 to 25% percent dry weight (Table 2), in the upper range of values reported for soil "A" horizons and as high as values for some peat soils (Singer and Munns 2002). Carbon to nitrogen (C/N) ratios are similar across all samples, roughly 9:1, and carbon to nitrogen to phosphorous (C/N/P) ratios are roughly 34:4:1. Engel et al. (2001) measured C/N ratios of organics from Cesspool Cave, VA, and report ratios of 13.5 and 16.06 for stream and wall biofilms, respectively. Low C/N ratios are indicative of a high quality food source for detrital feeders (Taylor and Roff, 1984); Engel et al. used these ratios along with measurements of autotrophic versus heterotrophic production to conclude that organics from Cesspool Cave have low nutritional quality. Compared to Cesspool Cave, stream biofilms from Frasassi have been considered a nutritious energy source for heterotrophic organisms (Galdenzi and Sarbu, 2000; Engel et al., 2001). Although biovermiculation C/N ratios are not as low as those of stream biofilms (Table 2), 9:1 is a low C/N ratio compared with many detrital organics (e.g., Taylor and Roff, 1984; Janssen, 1996). Additionally, C/N/P ratios indicate phosphorous is abundant in biovermiculations. These results suggest that biovermiculations could represent an important food resource for terrestrial invertebrates on the cave walls. Further research is currently underway by our group to explore trophic relationships between biovermiculations and cave wall invertebrates.

Nitrogen isotopic ratios indicate that there are two different nitrogen sources for the biovermiculation communities. Biological nitrogen fixation has little fractionation associated with it (+0.7‰, Sharp, 2007). Four of the samples have $\delta^{15}N$ values grouped near zero (Fig. 3), suggesting that N_2 fixation is the dominant mechanism for nitrogen supply to the communities. In sharp contrast, sample PC06-106 has a $\delta^{15}N$ value of -26.5‰. This is in the range of nitrogen isotopic ratios from extremely acidic cave wall biofilms reported by Galdenzi and Sarbu (2000) and Vlasceanu et al. (2000). Stern et al. (2003) hypothesized that depleted $\delta^{15}N$ values on sulfidic cave walls could result from volatilization of NH_3 gas from streams, with subsequent assimilation by subaerial microbial communities. If so, biovermiculations near cave streams may assimilate isotopically depleted ammonium, while those forming farther away or near more diluted waters primarily fix N_2 from the cave air. Our data support this hypothesis. Near-zero $\delta^{15}N$ values for Grotta Sulfurea biovermiculations sampled just above the water table are consistent with low cave air H_2S concentrations compared to sample PC06-106 (Fig. 3, Table 2). Work in progress will test the relationship between stream degassing and the $\delta^{15}N$ of wall biofilms including biovermiculations.

INFLUENCES ON CAVE FORMATION

Egemeier (1981) theorized that sulfidic cave formation occurs primarily by replacement-solution, in which hydrogen sulfide is oxidized to sulfuric acid in condensation droplets on cave walls and ceilings. Sulfuric acid reacts with limestone to produce gypsum, which builds up as thick crusts that eventually detach and accumulate on the cave floor or dissolve in cave streams. Subsequently Engel et al. (2003) suggested that the buildup of gypsum crusts may protect limestone walls from sulfide vapors, and that subaerial corrosion is fastest at exposed limestone surfaces. This hypothesis suggests an important role for biovermiculation microorganisms in subaerial sulfuric acid speleogenesis. In sulfidic zones of the cave system, nearly all exposed limestone surfaces are covered with biovermicula-

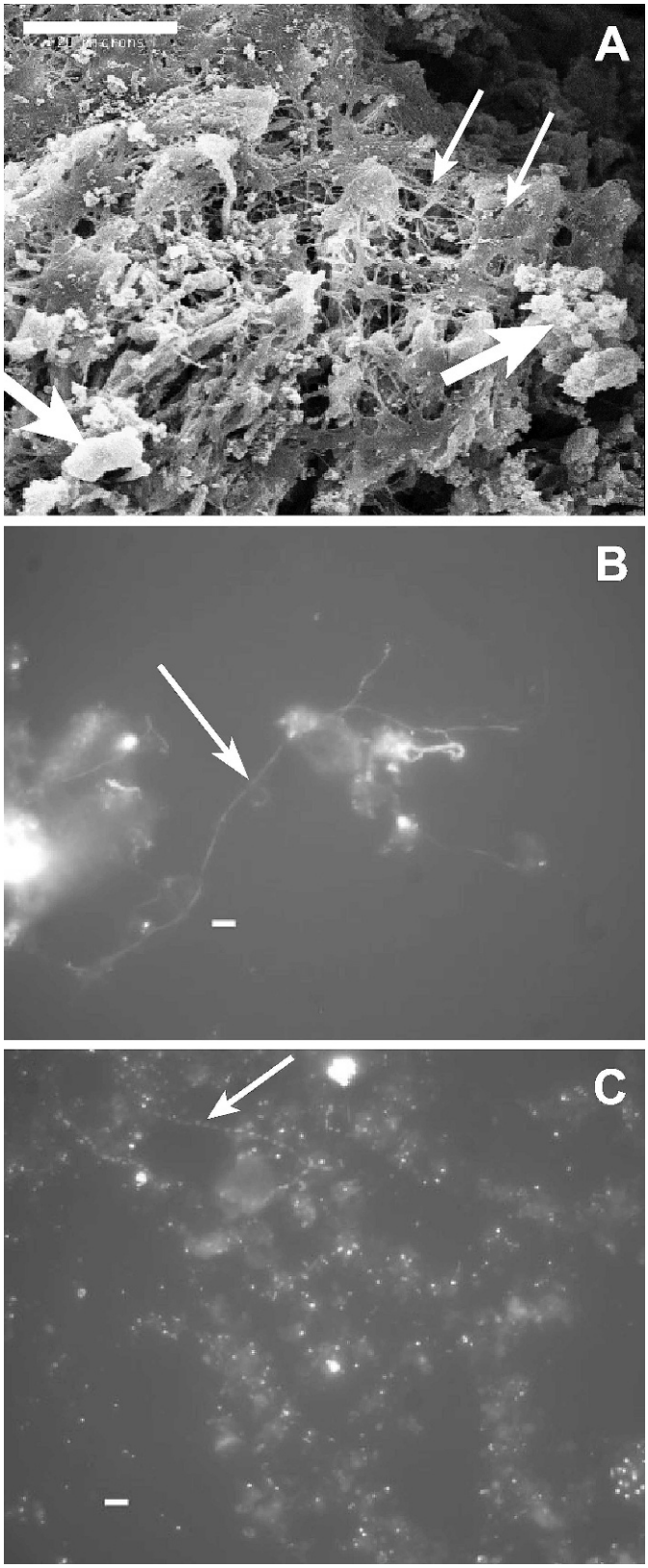

Figure 4. (A) SEM image of biovermiculation matrix. Small arrows indicate putative amorphous extracellular organic material, and large arrows indicate mineral grains. Scale bar is 20 μm (B and C) Representative fluorescence photomicrographs showing the range of DAPI-stained cell morphologies observed in Frasassi biovermiculations. Arrows indicate cells linked into filaments. Scale bars are 5 μm.

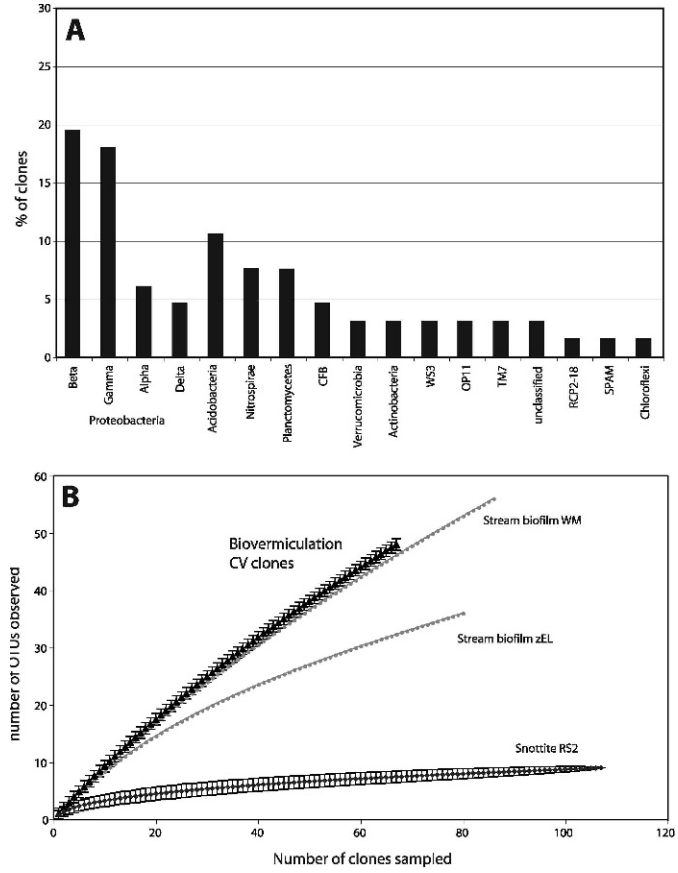

Figure 5. Bacterial diversity of biovermiculation sample GS03-5, based on 16S rRNA gene sequencing. (A) Percentages of biovermiculation clones in major bacterial lineages. (B) Rarefaction curves depicting observed microbial richness of Frasassi microbial communities. Operational taxonomic units (OTUs) are defined by >98% sequence identity. Biovermiculation library CV (n=67, 48 OTUs), this study; stream biofilms WM (n=86, 56 OTUs) and zEL (n=78, 36 OTUs), Macalady et al. (2006); snottite sample RS2 (n=107, 9 OTUs), Macalady et al. (2007). Error bars represent 95% confidence intervals based on variance of OTUs drawn (y-axis) by clone library size (x-axis), from 100 randomizations/sample.

tions, even isolated patches completely surrounded by gypsum crust (Fig. 2b). Sulfur-oxidizing microorganisms in stream biofilms have been shown to accelerate cave formation (Engel et al., 2004b; Jones et al., 2006), suggesting that acid-producing microorganisms in biovermiculations could play a similar role. Biovermiculation clones retrieved in this study include close relatives of

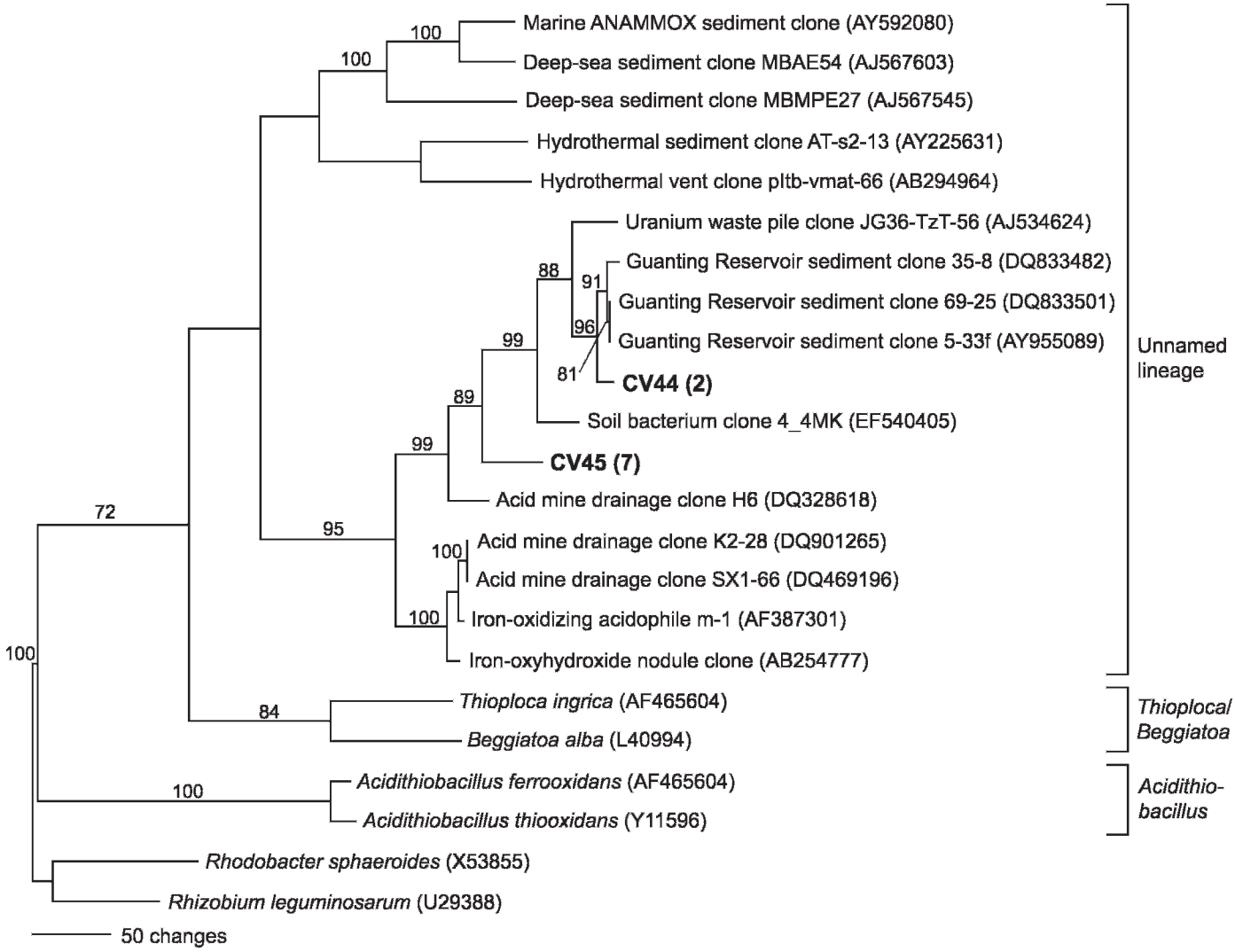

Figure 6. Maximum parsimony phylogram of 16S rRNA gene sequences in an unnamed lineage within the Gammaproteobacteria. GenBank accession numbers are listed with the sequence names. Maximum parsimony bootstrap values >70% are shown. Number of clones represented by each phylotype is given in parentheses.

sulfur- and nitrogen-oxidizing species that would be expected to produce strong acids (Table 1). In addition, many organoheterotrophic microorganisms, including fungi, produce organic acids as a by-product of their oxidative or fermentative metabolism. Because biovermiculations occur directly on limestone walls, any organic or inorganic acids produced by microorganisms would interact directly with the carbonate wall surface and contribute to speleogenesis. Significantly, subaerial limestone dissolution in Frasassi has been shown to be occurring at roughly the same rate as subaqueous limestone dissolution, at least near the water table where walls are exposed to sulfide degassing (Galdenzi et al., 1997).

BIOVERMICULATION FORMATION

The generally accepted model for vermiculation formation in epigenic caves was described by Bini et al. (1978), who proposed that they form during the drying of wet, sediment-covered cave walls as the sediments shrink and flocculate. Different types occur depending on the initial water to sediment ratio, although other factors may influence pattern morphology (Hill and Forti, 1997). In the Bini et al. model, many materials can form vermiculations. Sources include residual silicate mineral grains remaining after corrosion of the parent limestone and allochthonous material transported to the cave interior by air or water. Based on our observations, we hypothesize that biological processes are very important in the formation of biovermiculations in sulfidic zones of the cave system. We have identified two types of potentially important biological effects, (1) microorganisms may directly enrich the biovermiculation matrix by supplying organic material, and (2) micro- and macroorganisms may play an active role in trapping and binding sediment particles.

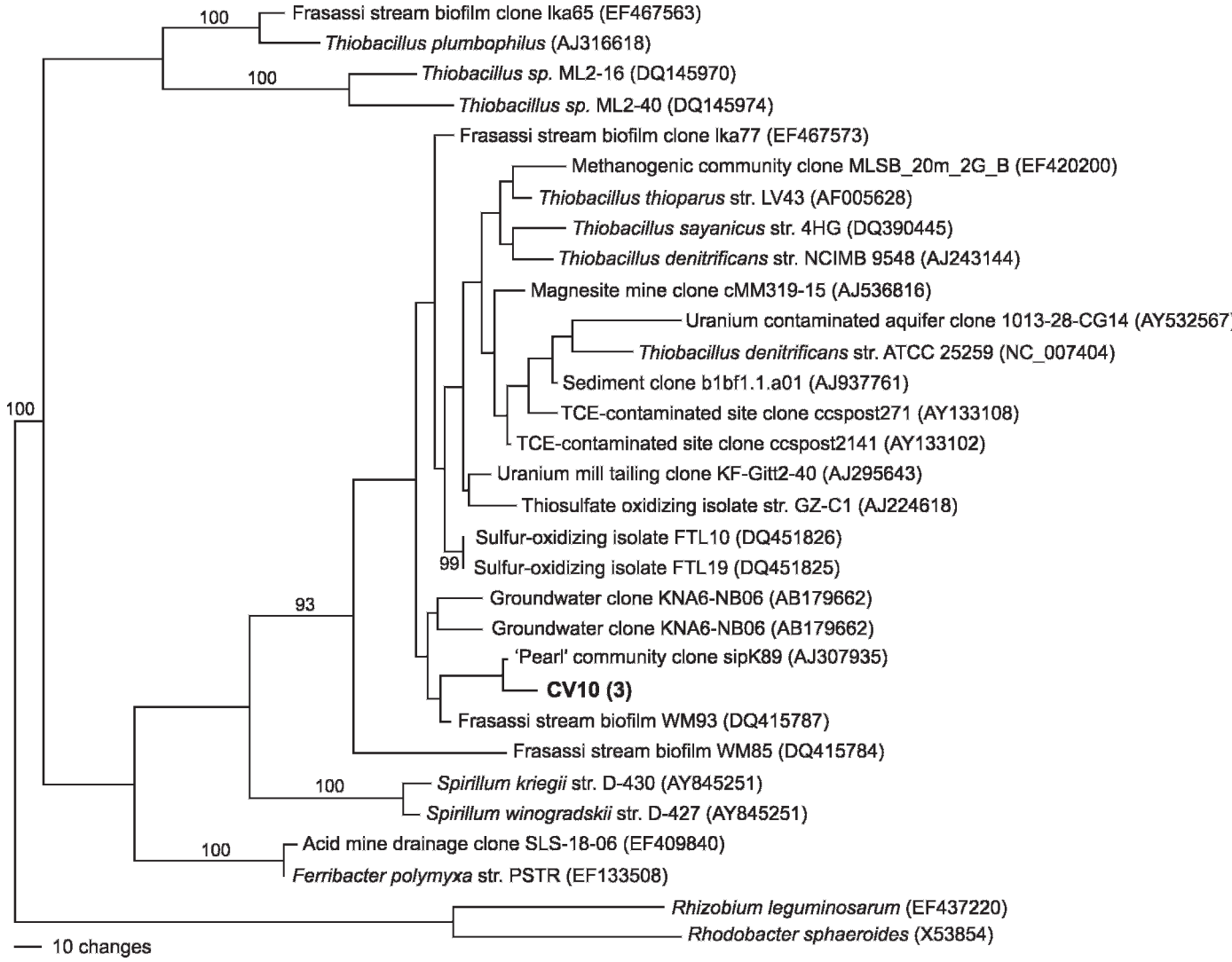

Figure 7. Maximum parsimony phylogram of 16S rRNA gene sequences related to the genus *Thiobacillus* in the Betaproteobacteria. GenBank accession numbers are listed with the sequence names. Maximum parsimony bootstrap values >70% are shown. Number of clones represented by each phylotype is given in parentheses.

The biovermiculations we sampled have a high organic matter content (between 5–25% organic carbon). Furthermore, biovermiculation carbon and nitrogen in sulfidic zones is isotopically distinct from sources outside of the cave system (Fig. 3), indicating that the organic matter is produced *in situ*. Hose and Northup (2004) suggested that biovermiculations might form via microbial trapping of sediments in a manner analogous to stromatolite formation. Fluorescence microscopy and SEM imaging of biovermiculations in this study revealed filamentous microorganisms and amorphous extracellular organic matter entwining and binding mineral grains in the biovermiculation matrix (Fig. 4a). Nematodes in the biovermiculations may also play an important role because many species of nematodes are known to agglutinate sediments (Riemann and Helmke, 2002). Whereas vermiculations have been previously observed on a variety of different surfaces (Hedges, 1993), the biovermiculations we observed occur on limestone surfaces but not on chert (Fig. 2c and d). This implies either a requirement for buffering offered by the carbonate rock or a difference in the mineral surface properties that affect adhesion of the biovermiculations.

If biological activity were important for biovermiculation formation, we would expect to see more abundant biovermiculations in regions of higher biological productivity. If this hypothesis is correct, the richest development of biovermiculations should be closest to the sulfidic water table. Such a relationship is broadly consistent with observations we have made throughout the Frasassi cave system. However, two factors may impose limitations on this pattern. In the areas with the highest sulfide gas concentrations, biovermiculation formation is in competition with gypsum crust formation. Therefore, growth of

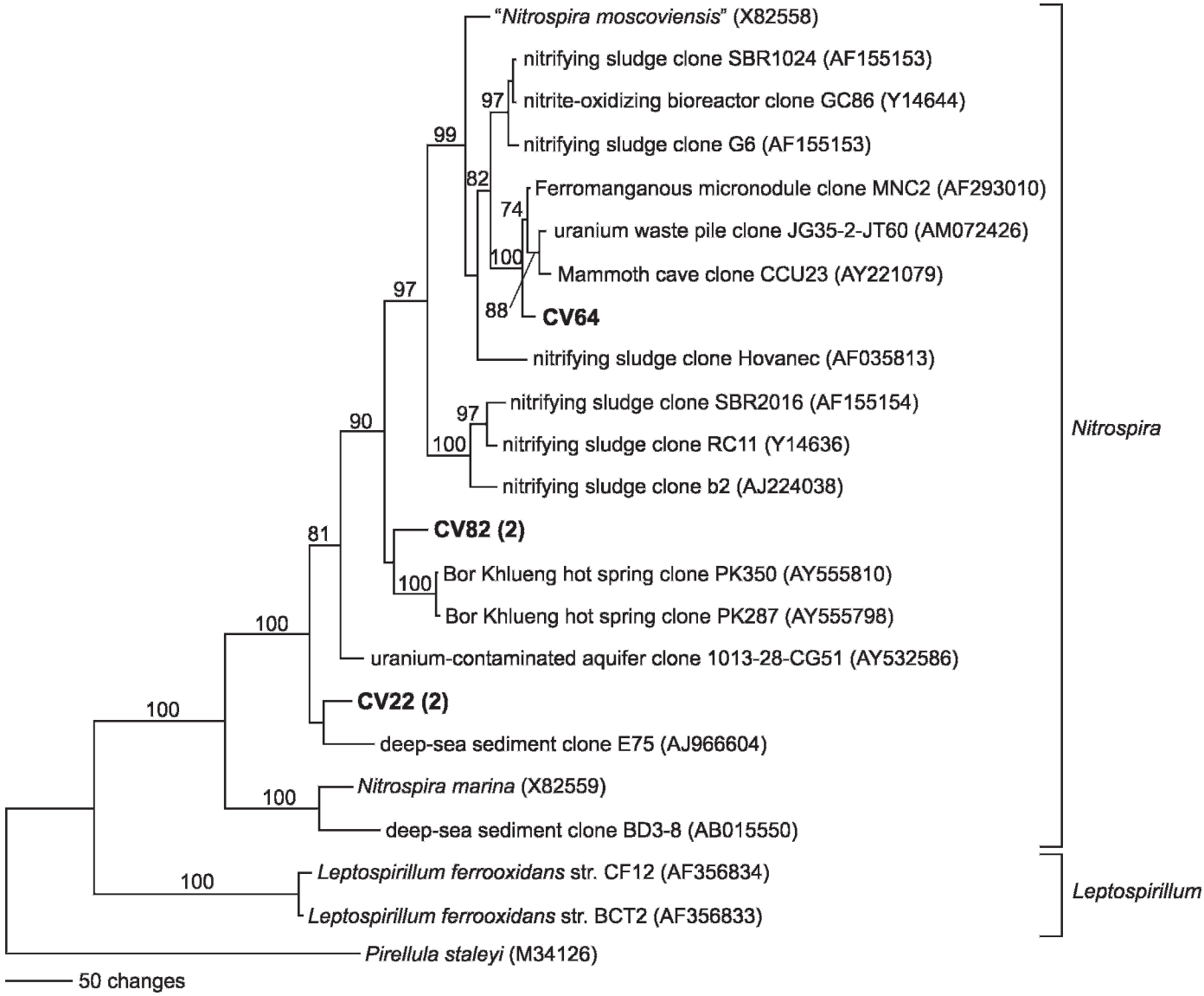

Figure 8. Maximum parsimony phylogram of 16S rRNA gene sequences within the Nitrospirae. GenBank accession numbers are listed with the sequence names. Maximum parsimony bootstrap values >70% are shown. Number of clones represented by each phylotype is given in parentheses.

biovermiculations might be outstripped by rapid gypsum accumulation in some locations (e.g., Fig. 2b). Additionally, seasonal and decadal high water events may wash away biovermiculations forming within several meters of the average stream level. Nonetheless, we often observed biovermiculations beginning to form below high water marks (for example, sample PC06-106, Fig. 2e). This observation is consistent with high biological productivity and rapid formation of biovermiculations in the presence of high sulfide gas concentrations, and suggests that there are conditions under which biovermiculations form more rapidly than gypsum crusts. Hose and Northup (2004) cleared biovermiculations from a patch of cave wall in sulfidic Cueva de Villa Luz and noted that new biovermiculations began to grow into the clear area after only a few weeks.

Previous authors have described microorganisms in vermiculations from non-sulfidic caves and speculated about biological influences on their formation (Anelli and Graniti, 1967; Camassa and Febbroriello, 2003). The work presented here offers evidence that microbial activity is important for creating biovermiculations in sulfidic caves. However, the origin of the geometric patterns that make biovermiculations so visually striking remains to be established. Our study does not exclude the possibility that the physical morphology of biovermiculations is influenced by drying processes described by Bini et al. (1978). Our work does suggest that enhanced biological

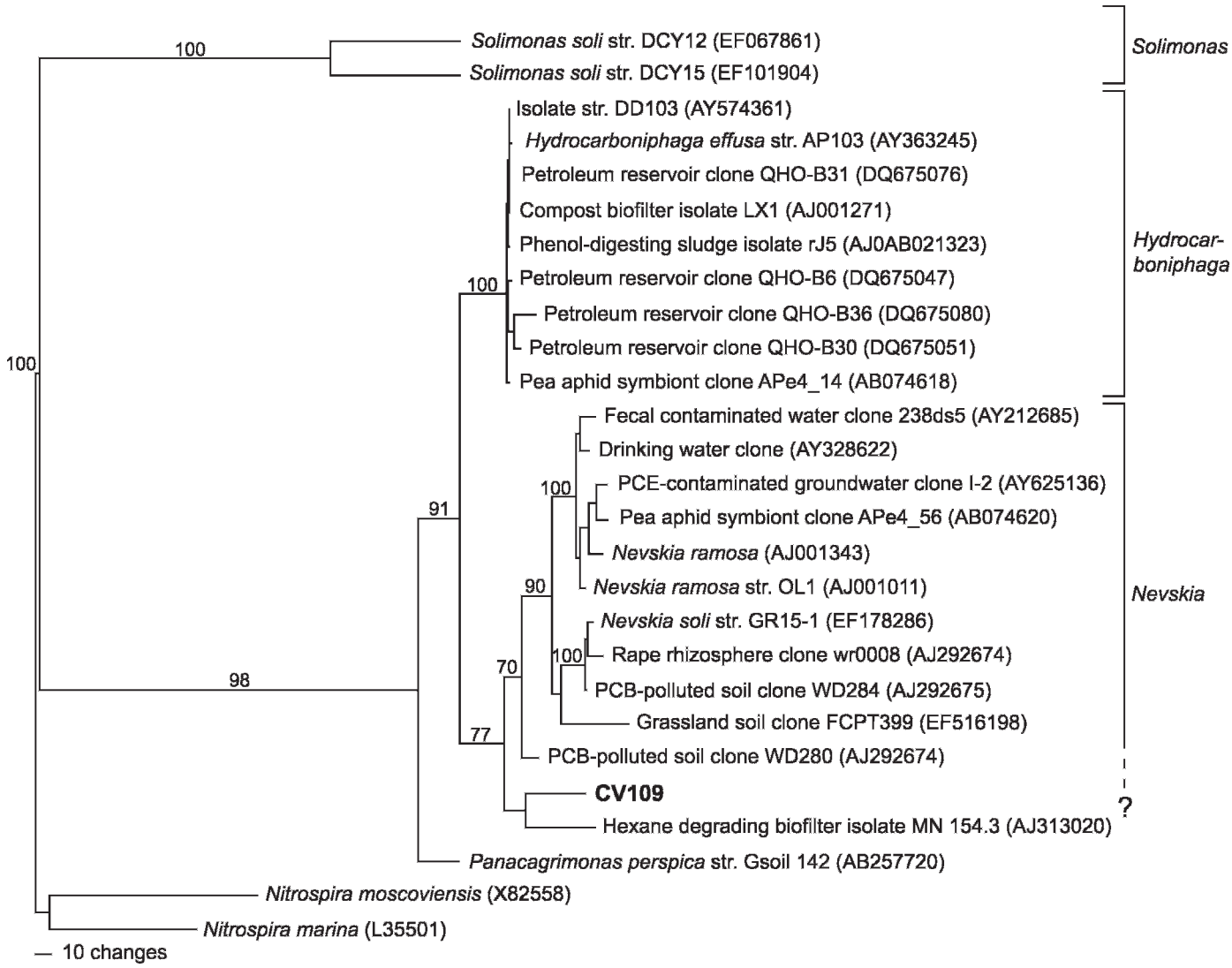

Figure 9. Maximum parsimony phylogram of 16S rRNA gene sequences related to the genera *Nevskia* and *Hydrocarboniphaga* within the Gammaproteobacteria. GenBank accession numbers are listed with the sequence names. Maximum parsimony bootstrap values >70% are shown.

activity in sulfidic caves enables biovermiculation formation on a time scale of years to decades, and that a significant fraction of their dry mass is organic matter produced *in situ*. Thus, in sulfidic cave environments, biovermiculation morphology could also be influenced by microbial growth patterns.

Conclusions and Future Directions

Carbon and nitrogen isotope values indicate that within sulfidic zones, the abundant organic matter in biovermiculations originates from *in situ* chemolithotrophic bacterial productivity rather than from sources outside the cave system. The high density of microbial cells and the likely presence of sulfur and nitrite oxidizing bacteria suggests that biovermiculations may play a role in acid production and carbonate dissolution, thereby contributing to cave formation. The 16S rRNA gene sequences analyzed to date indicate that biovermiculation communities can be extremely diverse, and that they contain representatives of deeply branching, phylum-level lineages with no cultivated representatives.

Further work is required to elucidate the microbial role in biovermiculation formation and limestone dissolution in sulfidic caves. Biovermiculations collected in different areas of the cave system and in other sulfidic caves should be compared to determine whether they have a characteristic assemblage of microbial species. Mineralogical analyses are needed to better describe the composition and provenance of inorganic constituents of biovermiculations such as clay minerals. Future research should also include comparisons with vermiculations sampled in non-sulfidic

environments to determine whether similar biogeochemical processes, including sulfur cycling, are responsible for their formation in both sulfidic and non-sulfidic karst systems.

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