CULTURE-INDEPENDENT CHARACTERIZATION OF "CAVE SILVER" BIOFILMS FROM THE 1470 M LEVEL OF THE SANFORD UNDERGROUND RESEARCH FACILITY, LEAD, SD

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ABSTRACT

Microbiome profiling using the 16S rRNA gene was used to examine the composition and diversity of bacteria residing in thin, whitish, iridescent microbial biofilms 1470 m below the surface in the Sanford Underground Research Facility (SURF), a former gold mine in South Dakota, USA. The biofilms superficially resemble "cave silver" biofilms described from limestone caves. The communities are dominated by bacteria, although significant Thuamarchaeota are also present. The most abundant bacterial groups were Actinobacteria (mainly Pseudonocardiaceae), Acidobacteria, Proteobacteria, Chloroflexi, and Planctomycetes. The composition of the biofilms varied considerably from sample to sample. Like cave silver biofilms in Europe and other locations, Proteobacteria and Actinobacteria are abundant, but nearly all operational taxonomic units (OTUs) (at 97% similarity) are different. Apart from one *Pseudonocardia* species, most abundant OTUs observed here were not isolated by a previous culture-based study at SURF. This indicates that culture-based techniques do not accurately represent the diversity of bacterial taxa in SURF "cave silver."

Keywords

Bacteria, Archaea, mine, caves, biofilms, 16S rRNA

INTRODUCTION

Caves and other subterranean habitats have attracted the attention of microbiologists because they represent rather extreme habitats where photosynthesis cannot occur, and organic matter is sparse. Energy must be obtained either by

oxidizing the small quantities of organic molecules that are present or by chemolithotrophy (Barton & Jurado 2007). Astrobiologists have become interested in caves and deep aquifer habitats as possible models for life elsewhere in the solar system, where living organisms, if present, would be limited to subsurface habitats (Northrop et al. 2011).

One interesting kind of microbial community found in caves is the very thin microbial biofilms of various colors (often white, yellowish, or tan-colored), containing filamentous Actinobacteria. These biofilms often condense water droplets, causing them to glisten iridescent. Such biofilms are sometimes referred to as "cave silver" (Pasic et al. 2010). They were first described from limestone caves in Europe, but also have been noted in lava tube caves in North America and oceanic islands around the world. Bacteria in cave silver communities are dominated by Proteobacteria (especially Gammaproteobacteria), with considerable numbers of Actinobacteria (especially the Pseudonocardiaceae), Acidobacteria, Nitrospirae, and Chloroflexi also present (Pasic et al. 2010; Porca et al. 2012; Hathaway et al., 2014; Riquelme et al. 2015). Despite variability in their composition, Porca et al. (2012) identified three abundant operational taxonomic units (OTUs, with greater or equal to 97% similarity of 16S rRNA gene sequences) occurring in the cave silver biofilms in European caves which he considered the core constituents of the communities. Riquelme et al. (2015) noted that five of 164 Actinobacterial OTUs (at the 97% similarity level) were major constituents of microbial mat communities of lava tube caves in the Azores, Canary Islands, Hawaii, and North America. These biofilms resemble the cave silver biofilms of limestone caves in overall morphology, and may be white, yellow, or brownish. It is not unusual for individual lava tube caves, or for lava tube caves in the same region to have unique OTUs, and a large proportion of lava tube cave OTUs cannot be assigned reliably to genus (Hathaway et al. 2014; Lavoie et al. 2017)

The Sanford Underground Research Facility (SURF) is located within the former Homestake Gold mine in Lead, South Dakota. The Homestake mine was active from 1876 to 2001 and reached depths of over 2540 m (Caddey et al. 1991). From 2003 to 2007, the pumping of drainage water from the former mine ceased, and the lower levels of the mine, including the 1470 m level, filled with water. In 2008, the former mine re-opened as the SURF, pumping resumed, and portions of the 1470 m level were developed for experiments in physics and biology.

The area of SURF examined in this study, known as the 17 Ledge, lies mainly within the Poorman formation, and is composed mainly of phyllite. Deep aquifer water, warm, anaerobic, high in dissolved minerals, low in organic carbon, sometimes with dissolved methane, sulfide, and ferrous iron, intersects air in the tunnels of SURF. This creates opportunities for chemoautotrophy and methanotrophy allowing some unique, uncharacterized microbes to live in the drainage water, sediments, and biofilms of this area (Waddell et al. 2010; Rastogi et al. 2009; Osburn et al. 2014).

A unique sort of biofilm is found on the tunnel walls in one region of the 17 Ledge between two diamond drill holes, about 150 m apart, from which fracture water is flowing and forming pools on the tunnel floor (Figure 1, map). In this area, warm, humid (32 °C, 100% RH), stagnant air in the distal part of the tun-

nel cools slightly as it meets cooler (26 °C), more ventilated air. Here, in a zone about 75 m wide, areas of thin (less than 1 mm thick), whitish biofilms, mostly 25 cm^2 to 400 cm^2 in area, are abundant on the upper walls and ceiling of a mine tunnel. They are iridescent with tiny droplets of condensed water and, at least superficially, resemble the cave silver biofilms, composed primarily of Actinobacteria and Proteobacteria, found in limestone caves and lava tubes. In this study, we use high-throughput sequencing of 16S rDNA microbiome libraries to examine the composition of the "cave silver" biofilms in SURF and compare them to the "cave silver" biofilms in caves. We also examine the variability in the

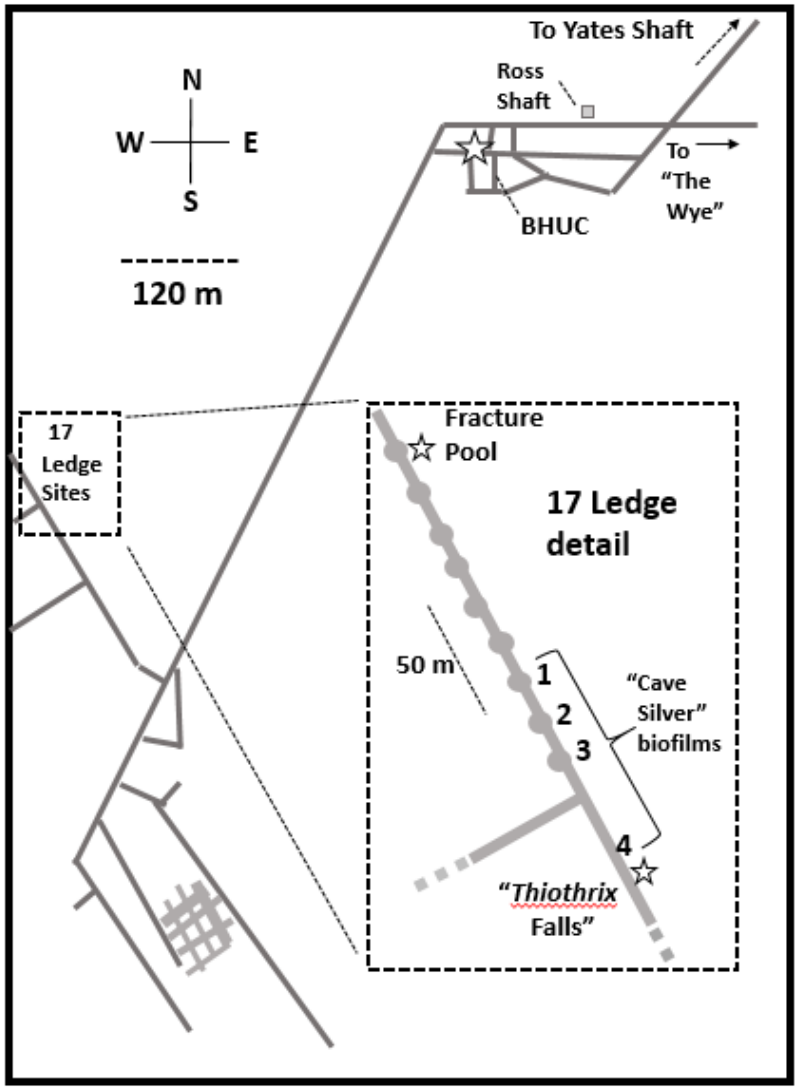

Figure 1. Map of the region of the 1470 m level of SURF where cave silver biofilms were sampled. BHUC signifies the Black Hills State University Underground laboratory.

composition of these biofilms with respect to distance from a moisture source (pools where fracture water from drill holes was collecting), gradients of air temperature and height on the tunnel walls.

METHODS

Collection of Samples—Cave silver samples were collected from the North 54 Pillar East, 17 Ledge, on the 1470 m level of SURF (Site 2 and Site 3 in Figure 1) on October 15, 2015. A total of seven samples of whitish cave silver biofilms were collected. After the initial samples were taken and the resulting 16S rRNA gene sequences were analyzed, we noticed that the composition of cave silver communities varied considerably from sample to sample. To explore possible causes for this variability, we took further samples in 2017 at sites along a transect down the length of the tunnel where the cave silver occurred from the hotter, more humid area, about 91 m from the flowing drill holes, to the somewhat cooler, less humid region, about 148 m from the flowing drill holes and only 3 m from a capped, flowing drill hole at the site known as "*Thiothrix* Falls" (Figure 1). Air temperature, rock temperature, and relative humidity were recorded at each site at a height of about 1.5 m. At each site, three cave silver samples were taken at a height of 1.6 m on the walls of the tunnel and three on the tunnel roof (about 3.0 m high). For each sample, about 10 cm^2 areas of cave silver were removed with a sterile spatula and placed in sterile 15 ml Falcon tubes, and frozen within 5 hours of collection for later DNA extraction.

Microscopy—Representative samples of cave silver were chosen for Scanning Electron Microscopy (SEM) at Site 2 and Site 3 in 2015. All were glistening, thin, whitish biofilms growing about 1.5 m above the tunnel floor. Approximately 1-4 cm diameter, thin pieces of phyllite with the cave silver were pried from the tunnel walls with a sterile spatula and transported to lab in sterile 50 mL Falcon tubes. Samples were placed in 100 mM potassium phosphate buffer pH 7.5 with 2.5% gluteraldehyde and stored at room temperature for 14 days. The buffer was removed and replaced with increasing ethanol concentrations (15 to 100%) for at least two hours. Samples in ethanol were dehydrated using liquid carbon dioxide as a replacement fluid in a Critical point dryer (Structure Probe, Inc., West Chester, PA) and coated with colloidal gold in an 18930 EffaCoater sputter coater (E.F. Fullam Co., Lantham, NY). Samples were viewed in a JEOL 5600 LV scanning electron microscope (SEM) using a secondary electron detector under high vacuum conditions.

Genomic DNA and 16S rDNA Library Preparation—For DNA extraction, a PowerLyzer® PowerSoil® DNA Isolation kit (MoBio) was used. DNA samples were cleaned and concentrated with Zymogen Research DNA Clean & Concentrator™-5. Bacterial microbiome 16S rDNA libraries for NextGen sequencing were prepared. The Qubit™ 2.0 Fluorometer (Invitrogen, Inc.) was utilized to determine DNA concentration, and libraries were prepared with an Illumina Nextera™ kit. Library preparation was done according to Illumina "16S Metagenomic Sequencing Library Preparation," Part # 15044223 Rev. B. (Qiagen, Inc.) using the 16S rRNA gene PCR primers and conditions described below.

In 2015, to prepare libraries of the V3 and V4 regions of bacterial 16S rRNA genes, we used the PCR primers (5'-TCG-TCG-GCA-GCG-TCA-GAT-GTG-TAT-AAG-AGA-CAG-CCT-ACG-GGN-GGC-WGC-AG-3') and 16S Reverse (5'-GTC-TCG-TGG-GCT-CGG-AGA-TGT-GTA-TAA-GAG-ACA-GGA-CTA-CHV-GGG-TAT-CTA-ATC-C-3') (Klindworth et al. 2013). The PCR reactions included about 5-10 ng of environmental DNA, 1X KAPA HiFi HotStart ReadyMix (KAPA Biosystems), 3 pmoles each of forward and reverse primers, in a total volume of 15 uL. The following protocol was used for PCR on an Applied Biosystems 9700 thermal cycler: 95 °C (3 min), followed by 25 cycles of 95 °C (30 s), 55 °C (30 s), 72 °C (30 s) and a final five minute extension step of 72 °C. Five microliters of PCR product on 1.5% agarose gel confirmed template amplification.

In 2017, the following primers from the Earth Microbiome project (http://www.earthmicrobiome.org/protocols-and-standards/16s/) developed by Parada et al. (2016) were used to amplify the V4 region of the 16S rRNA genes of both Bacteria and Archaea: forward (515F) 5'-GTG-YCA-GCM-GCC-GCG-GTA-A-3' and reverse (806R) 5'-GGA-CTA-CHV-GGG-TWT-CTA-AT-3'). The concentrations of components of PCR reactions were the same as before. The following protocol was used for PCR on an Applied Biosystems 9700 thermal cycler: initial denaturation of 94 °C (3 min), followed by 35 cycles of denaturation at 94 °C (45 s), annealing at 50 °C (60 s), and extension 72 °C (90 s) and a final 10 minute extension step of 72 °C.

PCR products were centrifuged and purified with AMPure XP magnetic beads, as directed by the manufacturer (Agencourt). Five microliters of each PCR amplicon was transferred to a new PCR plate and arranged according to the TruSeq Index Plate Fixture. Nextera XT Index Primers N7xx and S5xx, 2x KAPA HiFi HotStart ReadyMix, and PCR grade water were added, mixed, and centrifuged. Thermal cycler protocol for the indexing PCR reaction was: 95 °C (3 min), followed by 8 cycles of 95 °C (30 s), 55 °C (30 s), 72 °C (30 s) and a final 5 minute step at 72°C.

PCR products were again purified with AMPure XP magnetic beads. The supernatant contained the final library for quantification and sequencing. Library quantity was determined on Qubit™ 2.0 Fluorometer (Invitrogen, Inc.). Samples were multiplexed and sequenced on a MiSeq (Illumina, Inc.) instrument by the WestCore DNA Core Facility at Black Hills State University. The resulting paired-end 300-bp reads, analyzed for run quality and adapters, were trimmed using Illumina™ BaseSpace

Analysis of SURF Cave Silver V3-V4 16S rRNA Gene Sequence Data—Quality checked and adapter trimmed sequencing reads from 2015 Samples 2, 4, 6, 7, 10, 11, and 12 were downloaded from Illumina BaseSpace in.fastq format and imported into the CLC Genomics v10 Workbench (https://digitalinsights.qiagen.com) as paired reads. Paired sequences were merged with mismatch costs at 1, minimum score of 40, gap cost of 4, and maximum unaligned end mismatches of 5. Sequences were trimmed to a uniform length of 440 BP OTU clustering using 97% SILVA v132 database with the following parameters: 97% for OTU clustering, 80% similarity percentage for OTU matching to database;

2 minimum occurrences; a chimera crossover cost of 3; Kmer size of 6; and with "Find Best Match" chosen. OTUs with less than ten combined occurrences were removed from the dataset. The 16S rDNA gene sequence *Thermococcus pacificus* (accession number Y16227) was used as an outgroup for phylogenetic tree construction. Sequences were aligned with MUSCLE (Edgar 2004), and the 100 most abundant OTUs from the dataset were used for a comparison between cave silver biofilms from SURF and from published datasets from caves (Porca et al. 2012; Hathaway et al. 2014).

A maximum likelihood phylogenetic tree was constructed with the matched OTUs of the SURF samples. Sequences were aligned with known Operational Taxonomic Units. "Find Diagonals" was chosen with the following parameters: 1,000 maximum hours; 1,000 Mb maximum memory; 16 maximum iterations; 10 minimum combined abundances; 0.0% minimum combination percentage; 100 maximum number of sequences. The resulting alignment was used in making a maximum likelihood phylogeny. The phylogeny used the Neighbor-Joining method with the Jukes-Cantor (1969) nucleotide substitution method and bootstrap analysis with 1,000 replicates.

Alpha diversity was evaluated on the resulting tree and its given OTU Clustering Table. Diversity measures chosen were the number of OTUs, corrected Chao1 (Chao 1984), Simpson's Index (Simpson 1949), and Shannon (1948) entropy. Rarefaction Analysis used 1 Minimum Depth to Sample, 100,000 Maximum Depth to Sample, 20 Number of Depths to be Sampled, and 100 Replicates at Each Depth.

Beta diversity was conducted on the resulting tree and its given OTU Clustering Table. The Bray-Curtis method was chosen as the beta-diversity measure**.** Diversity measures were unweighted UniFrac, weighted UniFrac, D_0.5 UniFrac. Because CLC Bio did not assign some OTUs to taxa in the Silva database, these unassigned OTU sequences were exported from CLC Genomics Workbench as FASTA files and used to query the Ribosomal Database (RDB) using the Classifier program (Wang et al. 2007) to assign taxa. Taxonomic assignments were then downloaded from RDB as an Excel file, edited, and added to the OTU.

Comparison with Published Cave Silver Biofilm 16S rDNA Data—To compare composition of the 2015 V3-V4 16S rRNA gene SURF cave silver biofilm data to 16S rRNA gene libraries from morphologically similar cave biofilms, we chose two earlier studies: Porca et al. (2012) of limestone caves in Europe and Hathaway et al. (2014) of lava tube caves in the Azores and Hawai'i (Accession numbers HM445833–HM445541, HM545238; FJ535064–FJ535113, AY960218–AY960221, AY960224, AY960225, AY960228–AY960231, AY960233, AY960234, AY960236, AY960248–AY960250, AY960253, AY255254, AY960256–AY960258, AY960269 and AY960272). Biofilm accessions from these studies were downloaded from NCBI and divided into categories based on their collection location. Sequences were imported into CLC Bio Genomics workshop V9. These sequences consisted of the entire 16S rRNA gene and were trimmed to include only the V3 to V4 regions by using the "Trim Sequences" tool. The adapters used were the forward and reverse primers in Illumina's 16S rRNA MiSeq library preparation protocol. The parameters of the

primers were: mismatch of 3, gap cost of 2, internal matches minimum score of 5, and allowed end matches minimum of 10. All sequences were trimmed accordingly. Sequences were further trimmed using the Fixed Length Trim tool. The sequences were clustered into OTUs using the OTU Clustering tool following the same parameters as the SURF protocol. OTUs were filtered to the top 100 most abundant OTUs. The resulting OTUs were aligned using MUSCLE in the same parameters as the SURF protocol. The resulting alignments were used for a comparison between SURF and cave biofilms. Alpha and Beta diversity measures were not conducted on either Porca et al. (2012) samples or Hathaway et al. (2014) samples. The three top 100 OTU alignments were combined using the Join Alignments tool, creating an alignment 300 OTUs large. A maximum likelihood phylogeny was created using the same parameters as in the SURF protocol.

Comparison of 16S rRNA Gene Sequences from 2015 SURF Cave Silver DNA with Sequences from Cultured Bacterial Isolates from SURF—16S rRNA gene sequences from bacteria isolated from SURF cave silver by Brar and Bergmann (2019, Genbank accession numbers MH669510-MH669553) were trimmed to the V3-4 regions using a text editor and imported into CLC Bio. OTU sequences from the 2015 SURF cave silver V3-V4 16S rRNA gene library were aligned to those from those 16S rRNA gene sequences from bacterial isolates predicted to be from the same taxon (family and genus) using MUSCLE, as described earlier. Phylogenetic trees of were prepared using the Neighbor-Joining method as described earlier.

RESULTS

Ultrastructure of SURF Cave Silver Biofilms—Various microbial cell shapes, including mycelia, bacilli in chains or clusters, and cocci were observed in SEM images of cave silver (Figure 2). Some of the larger chains of bacilli (each about 1 x 4 mm) have short projections on their surfaces and may represent the spores of *Pseudonocardia* species, which may be formed by acropetal budding (Goodfellow et al. 2012).

Analysis of SURF Cave Silver 16S rDNA Sequences with V3-V4 Primers, 2015—Imported SURF paired sequences were merged with 508,118 to 588,430 merged sequences per sample (84.24% to 88.69% of total sequences per sample). Sequences were trimmed to 440 base pairs with 98.84% to 99.83% of sequences successfully trimmed. All samples passed filtering based on number of reads. A total of 1,065 OTUs were predicted from all 7 samples combined with 525,165 reads from all 7 samples. 4,064 chimeric reads were identified and discarded. 350 of the most abundant OTUs were used for MUSCLE alignment. The maximum likelihood phylogeny was used in alpha diversity measures.

Alpha diversity was measured with replacement (Table 1). Chao1 estimates of OTUs ranged from 176 (Sample 10) to 336 (Sample 6). Shannon-Weiner diversity ranged from 3.80 (Sample 10) to 6.13 (Sample 6). Simpson diversity ranged from 0.88 (Sample 10) to 0.97 (Sample 11).

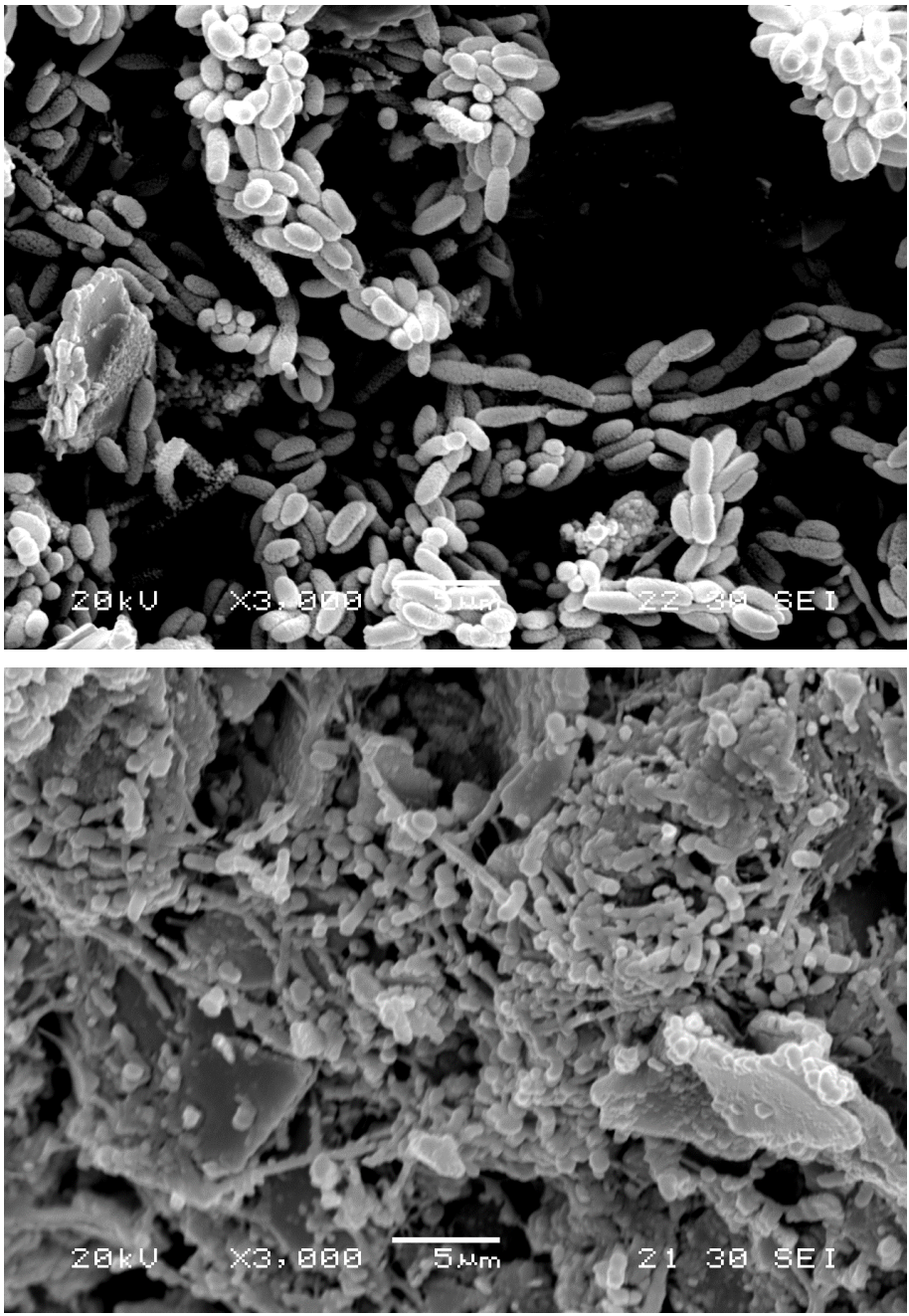

Figure 2. Scanning electron micrographs (3000X) of cave silver biofilms from SURF. The bars are 5.0 μm long.

A range of OTUs were matched (at least 80% similarity) to known taxa at the using the SILVA v.132 97% database, with sample 10 having the lowest percentage and sample 7 having the highest percentage. The most abundant phyla (>1.0% of sequences) were Acidobacteria (15.1%), Actinobacteria (21.0%), Chloroflexi (23.7%), Planctomycetes (6.7%), Proteobacteria (23.7% {Alphaproteobacteria 12.3%, Deltaproteobacteria 5.8%, and Gammaproteobacteria 5.6%}) and Firmicutes (2.3%) (Figure 3). Acetothermia, Armatimonadetes, Bacteroidetes, Chlamydiae, Cyanobacteria, Dadbacteria, Dependentae, FCPU426, Firmicutes, GAL15, Gemmatimonadetes, Nitrospirae, Patescibacteria, Rokubacteria, and Verrucomicrobia were each present in less than 2% of sequences. Despite all the samples being collected within 60 m of each other, the proportions of phyla represented in the samples varied considerably (Figure 4).

Only 30.8% of SURF V3-V4 16S rRNA gene sequences were identified to genus, indicating that a large proportion of uncharacterized taxa are present in SURF cave silver (Table 2). The most abundant genera were *Crossiella* and *Pseudonocardia* (both Actinobacteria), as well as *Gemmata, Pedomicrobia, Methylovirgula*, and *Mizugakiibacter*. A total of nine OTUs (putative species) from the SURF V3-V4 16S rRNA gene library appear to correspond to the 16S rRNA gene sequences from SURF cave silver bacterial cultures isolated by Brar and Bergmann (2019) (Table 3). Most of these OTUs represented less than 0.1% of 16S rRNA gene sequences, but one OTU for a *Pseudonocardia* species represented 7.01% of sequences.

Comparison of SURF Cave Silver Community with those of Limestone and Lava Tube Caves—CLC Genomics Workbench was used to compare the SURF cave silver V3-V4 regions of 16S rRNA gene sequences with the V3-V4 16S rRNA gene sequences previously published by Porca et al. (2012, Figure 5) and Hathaway et al. (2014, Figure 6). Like SURF, the limestone cave biofilms had large proportions of Proteobacteria, Actinobacteria (especially Pseudonocardiacieae), and Acidobacteria. The lava tube biofilms were dominated by Gammaproteobacteria and Acidobacteria, with a smaller proportion of Actinobacteria (but still largely Pseudonocardiaceae). SURF biofilms had a much larger proportion of Chloroflexi than cave biofilms, and a much lower proportion of Nitrospirae. Also, the cave biofilms had a much higher proportion of autotrophic ammonia oxidizing bacteria (*Nitrosococcus* and *Nitrosospira*) than SURF cave silver biofilms.

Table 1. Alpha diversity estimates (Corrected Chao 1, Simpson's diversity, and Shannon-Wiener diversity) for SURF cave silver samples collected in 2015, using V3-V4 16S rRNA gene PCR primers.

Sample	2	4	7	6	10	11	12
Shannon	5	5.07	4.9	6.13	3.8	6.06	3.88
Simpson	0.92	0.94	0.91	0.96	0.88	0.97	0.89
Chao 1	261	201	251	336	176	331	191

Table 2. Percentage of 2015 SURF cave silver Operational Taxonomic Units (OTUs) identified to known genera by CLC Bio, using V3-V4 16S rRNA gene primers. The classification of these genera (phylum, class, order, family, and genus) is also shown.

Phylum	Class	Order	Family	Genus	Percent
Acidobacteria	Acidobacteriia	Solibacterales	Solibacteriaceae	*Bryobacter*	0.124
				can. Solibacter	0.354
Actinobacteria	Actinobacteria	Frankiales	Acidothermaceae	*Acidothermus*	1.512
		Micromonosporales	Micromonosporaceae	*Rhizocola*	0.413
		Pseudonocardiales	Pseudonocardiaceae	*Crossiella*	6.59
				Pseudonocardia	8.523
	Thermoleophilia	Gaiellales	Gaiellaceae	*Gaiella*	0.101
		Solirubrobacterales	Solirubrobacteraceae	*Connexibacter*	0.207
Dependentiae	Babeliae	Babeliales	Babeliaceae	*Babella*	0.611
Firmicutes	Bacilli	Bacillales	Alicyclobacillaceae	*Acidibacillus*	0.134
				Alicyclobacillus	0.655
				Effusibacillus	0.157
Nitrospirae	Nitrospira	Nitrospirales	Nitrospiraceae	*Nitrospira*	0.494
Planctomycetes	Planctomycetacia	Gemmatales	Gemmataceae	*Gemmata*	1.773
			Schlesneriaceae	*Planctopirus*	0.319
Proteobacteria	Alphaproteobacteria	Reyranellales	Reyranellaceae	*Reyranella*	0.258
				Methylovirgula	1.16
		Rhizobiales	Hyphomicrobiaceae	*Hyphomicrobium*	0.119
				Pedomicrobium	2.579
				Bauldia	0.15
	Deltaproteobacteria			*Haliangium*	0.361
	Betaproteobacteria			*Thiobacillus*	0.147
	Gammaproteobacteria			*Mizugakiibacter*	1.177

Some cave silver OTUs found in SURF were also observed in limestone caves (Porca et al., 2012), and lava tube caves (Hathaway et al., 2014). One SURF OTU (AB257641.1.1513), an uncultured species of *Crossiella*, which represented 0.014% of SURF 16S rDNA reads, also occurred in the limestone caves studied by Porca et al. (2012). Four SURF OTUs were also noted from the lava tube caves studied by Hathaway: 1) JN615675.1.1355, which constituted 1.143% of SURF reads, an uncultured member of the Acidobacteria; 2) EF019706.1.1344, which comprised 1.044% of SURF reads, an uncultured *Pedomicrobium* species; 3) EU133546.1.1332, comprising 0.169% of SURF reads, an uncultured member of the Methyloligellaceae; and 4) EU133546.1.1332, comprising 0.350% of SURF reads, an uncultured member of the Gammaproteobacteria.

Table 3. OTUs from the 2015 V3-V4 library of SURF cave silver16S rRNA gene sequences which correspond to 16S rRNA sequences from cultured bacterial SURF cave silver isolates. The percentage of sequences in the 2015 16S rRNA gene library belonging to each OTU is indicated.

16S rRNA Library OTU	Percent of Sequences	Bacterial Genus	Bacterial Isolates Represented
AB30184.1.1517	7.01	*Pseudonocardia*	HS050C, HS062R
MO1316:22:et01	0.02	*Amycolatopsis*	HS035R, HS033R
JQ306.1.11521	0.025	*Allokutzneria*	HS067C
AJ249206.1.1512	0.05	*Pseudonocardia*	HS117C
JQ923554.1.1457	0.08	*Dongia*	HS130C, HS101C
HN7800881.1.1464	0.018	*Reyranella*	HS110R
AB839882.1.1453	0.014	*Reyranella*	HS095R, HS071R
KY053158.1.1407	0.086	*Pseudolabrys*	HS067R, HS070R
AF509582.1.1449	0.01	*Bradyrhizhobium*	HS111R, HS028R, HS022C, HS108C, HS099R, HS029R

Analysis of SURF Cave Silver along a Transect using V4 16S rRNA Primers- The four sites where cave silver was sampled in 2017 varied in air temperature and relative humidity, both increasing with distance from the capped borehole at site at "*Thiothrix* Falls" (Table 4). Rock temperature also increased slightly with distance. Relative humidity and temperature were higher near the roof of the tunnel (about 2.5 m high) than in the middle of the walls (about 1.8 m).

Alpha diversity estimates for the 2017 samples are presented in Table 5. Corrected Chao1 estimated diversity ranged from 289 OTUs (sample T4C3) to 573 (1C2); Simpson diversity from 0.9 (4W3) to 0.97 (3W3, 1C1, and 1C2); and Shannon-Weaver diversity from 4.96 (4W2) to 6.27 (1C2). A significant positive correlation $R^2 = 0.545 (P < 0.05)$ was found between distance from "*Thiothrix* Falls" (d) and Shannon-Weiner diversity (s); $s = (1.311 \times 10^{-3}) \bullet d + 0.897$. No other significant correlations between diversity measurements and sample variables were noted.

Combined OTU abundance data from the 2017 V4 16S rRNA sequences indicated that, as was observed with the V3-V4 primers, Proteobacteria (32.2% of sequences, mainly Alphaproteobacteria), Actinobacteria (14.3%, largely Pseudonocardiaceae), Acidobacteria (8.2%) Chloroflexi (7.9%) and Planctomycetes (7.2%) were abundant phyla (Figure 7) . However, the V4 primers detected large numbers of sequences from Thaumarchaeota (7.5%) and Dadabacteria (9.7%), not previously observed by the V3-V4 primers.

There was considerable variation among samples in terms of the abundance of taxa in the 2017 samples, observed with the V4 primers, even at the phylum level (Figure 8). Principal Coordinate Analysis (PCoA) was used to examine possible patterns in abundance of taxa among samples. PCoA Axis 1 explained 38%

of variance, and PCoA Axis 2 explained 19% of variance. However, there is no discernible pattern in the distribution of samples on the PCoA axes (Figure 9), and there were no significant correlations ($P \geq 0.05$) between PCoA axis scores of samples and environmental variables.

To compare the diversity results obtained with V3-V4 PCR primers with the V4 primers, we prepared a library of 16S rRNA genes for Illumina sequencing from one of the cave silver DNA samples from 2015, HS7. A library of 16S rRNA genes from HS7, prepared using primers V3-V4, had previously been sequenced. With the V4 primers, sample HS7 had Chao1 diversity of 379 (higher than V3-V4 primers), Simpson diversity of 0.83 (lower than V3-V4 primers), and Shannon-Weiner diversity of 4.46 (lower than V3-V4). Although most of the same common phyla and genera were recovered from sample HS7 using both primer sets (Figure 10 and Figure 11, Table 6), the proportion of Acidobacteria was much higher using V4 primers, perhaps reducing the evenness of proportions of taxa and, hence, lower diversity. Also, the V4 primers did not detect some of the less common phyla and genera shown by the V3-V4 primers.

Table 4. Environmental characteristics of SURF cave silver samples collected in 2017, including distance from "Thiothrix Falls", air and rock temperature, relative humidity, and height above the floor of the tunnel. There were four sampling sites (1-4), each of which had one to three replicate samples from about 1.7 m (W) or 2.7 m (C) high. Only samples used in 16S rRNA gene libraries are shown.

Site/ Sample	Distance (m)	Humidity (%)	Rock Temp.	Air Temp.
1_C1	60.96	90	33	33
1_C2	60.96	90	33	33
1_W2	60.96	99	33	33
1_W3	60.96	99	33	33
2_W1	45.72	87	32	33
2_W3	45.72	87	32	32
3_C1	30.48	80	31	33
3_C2	30.48	80	31	33
3_W1	30.48	78	31	32
3_W3	30.48	78	31	32
4_C1	9.13	82	30	32
4_C3	9.13	82	30	32
4_W2	9.13	78	30	28
4_W3	9.13	78	30	28

Table 5. Alpha diversity estimates (Corrected Chao 1, Simpson's diversity, and Shannon-Wiener diversity) for SURF cave silver samples collected in 2017, using V4 16S rRNA gene PCR primers.

Site	Chao1	Simpson	Shannon-Weaver
1_C1	380	0.97	6.07
1_C2	573	0.97	6.24
1_W2	472	0.96	5.81
1_W3	407	0.94	5.32
2_W1	407	0.94	5.32
2_W3	555	0.95	5.89
3_C1	344	0.94	5.31
3_C2	441	0.94	5.55
3_W1	472	0.95	5.72
3_W3	486	0.97	6.2
4_C1	427	0.93	5.2
4_C3	289	0.92	5.04
4_W2	306	0.9	4.96
4_W3	531	0.93	5.36

DISCUSSION

Possible Metabolism of SURF Cave Silver Microbes—Some microbes present in SURF cave silver microbes, especially Actinobacteria such as *Pseudonocardia*, some Alphaproteobacteria such as *Bauldia* and *Rhizobium*, and Firmicutes such as *Paenibacillus* are capable of chemoheterotrophic growth because they were isolated previously on complex media with a variety of carbon sources (Brar and Bergmann, 2019). Some major groups of SURF microbes are not readily isolated on complex media, hence their metabolism is uncertain. Cultured members of the Acidobacteria are chemoheterotrophs, and utilize a variety of carbohydrates for growth (Kielak et al. 2016). The class Ktedonobacteria is abundant in SURF cave silver, and, although at least one species, *Ktedonobacter racemifer*, is chemoheterotrophic (Caveletti et al. 2006), other species appear to be capable of chemoautotrophic growth using unknown electron sources (Fullerton & Moyer 2016). The Planctomycetes in SURF cave silver, which are primarily Pirellulaceae, may include chemoheterotrophic members, which utilize carbohydrates and sulfur for respiration (Elshahed et al. 2007). Chemoautotrophic ammonia oxidation is suggested by the abundance of Thaumarchaeota related to *Nitrosopumilus* as well as the presence of less abundant members of the Nitrosomonadaceae. *Nitrospira* in SURF cave silver is a chemoautotrophic nitrite oxidizer.

Table 6. Percentage of 2017 SURF cave silver Operational Taxonomic Units (OTUs) identified to known genera by CLC Bio, using V4 16S rRNA gene primers. The classification of these genera (phylum, class, order, family, and genus) is also shown.

Phylum	Class	Order	Family	Genus	Percent
Acidobacteria	Acidobacteriia	Solibacterales	Solibacteriaceae	*Bryoba +E2:E46cter*	0.124
				Candidatus Solibacter	0.353671
Actinobacteria	Actinobacteria	Frankiales	Acidothermaceae	*Acidothermus*	1.512769
		Micromonosporales	Micromonosporaceae	*Rhizocola*	0.413
		Pseudonocardiales	Pseudonocardiaceae	*Allokutzneria*	0.247
				Crossiella	6.598869
				Pseudonocardia	8.523443
	Thermoleophilia	Gaiellales	Gaiellaceae	*Gaiella*	0.101
		Solirubrobacterales	Solirubrobacteraceae	*Conexibacter*	0.207
Dependentiae	Babeliae	Babeliales	Babeliaceae	*Candidatus Babela*	0.611
Firmicutes	Bacilli	Bacillales	Alicyclobacillaceae	*Acidibacillus*	0.134
				Alicyclobacillus	0.655354
				Effusibacillus	0.157
Nitrospirae	Nitrospira	Nitrospirales	Nitrospiraceae	*Nitrospira*	0.4935
Planctomycetes	Planctomycetacia	Gemmatales	Gemmataceae	*Gemmata*	1.77
		Planctomycetales	Schlesneriaceae	*Planctopirus*	0.319
Proteobacteria	Alphaproteobacteria	Reyranellales	Reyranellaceae	*Reyranella*	0.258
		Rhizobiales	Beijerinckiaceae	*Methylovirgula*	1.16
			Hyphomicrobiaceae	*Hyphomicrobium*	0.119
				Pedomicrobium	2.578904
			Rhizobiales Incertae Sedis	*Bauldia*	0.15
				Nordella	0.57
	Deltaproteobacteria	Myxococcales	Haliangiaceae	*Haliangium*	0.361098
	Gammaproteobacteria	Acidiferrobacterales	Acidiferrobacteraceae	*Sulfurifustis*	0.503744
		Betaproteobacteriales	Hydrogenophilaceae	*Thiobacillus*	0.147
		Xanthomonadales	Rhodanobacteraceae	*Mizugakiibacter*	1.177

Table 7. Number of 2015 SURF cave silver Sample 7 OTUs identified to known genera by CLC Bio, using V3-V4 and V4 16S rRNA gene PCR primers.

Genus (Aggregated)	V3-V4	V4	Genus	V3-V4	V4
Acidothermus	21	0	*Legionella*	107	25
Actinoallomurus	23	0	metagenome-01	14	0
Apfia	0	12	*Methylocaldum*	9	0
Alicyclobacillus	2	0	*Methylosinus*	3	0
Allokutzneria	23	0	*Methylovirgula*	667	173
Amycolatopsis	21	0	*Mizugakiibacter*	35	0
Aquisphaera	21	0	*Nitrospira*	47	0
Azovibrio	6	0	*Nordella*	108	14
Bacillus	336	77	*oc32*	1	0
Bauldia	141	0	*Paenibacillus*	116	0
Bradyrhizobium	29	0	*Pajaroellobacter*	12	0
Brevibacillus	72	0	*Pedomicrobium*	1673	426
Bryobacter	272	0	*Pir4 lineage*	22	0
C1-B045	77	0	*Planctopirus*	490	12
Candidatus Babela	1206	0	*Pseudolabrys*	215	21
Candidatus Berkiella	37	0	*Pseudonocardia*	1387	629
Candidatus Solibacter	288	0	*RB41*	7	0
Crossiella	4186	2347	*Reyranella*	499	45
Delftia	6	0	*Rhizocola*	35	0
denitrifying enrichment	317	0	*Rhodopirellula*	2	0
Desulfobacca	24	0	*Rhodoplanes*	201	17
Effusibacillus	264	12	*Ruminiclostridium 1*	8	0
Ellin6055	921	355	*Saccharopolyspora*	3	0
FCPS473	199	0	*Salimesophilobacter*	2	0
Fontimonas	4	0	*Sandaracinus*	261	70
Gaiella	11	0	*Singulisphaera*	23	0
Gemmata	2	0	*Sorangium*	2	0
Geothermomicrobium	1	0	*Stenotrophomonas*	6	0
Hyphomicrobium	280	169	*Sulfurifustis*	8	14
Ignavibacterium	7	0	*Sulfurirhabdus*	31	0
Immundisolibacter	12	0	*Thauera*	2	0
Kineosporia	5	0	*Thermincola*	28	0
Lacunisphaera	95	0	*Thiobacillus*	15	0

Comparison of Culture-independent and Culture-dependent Techniques for Assessing the Diversity of SURF Cave Silver Microbial Communities—By using culture-independent techniques, we were able to detect three abundant phyla in SURF cave silver: Acidobacteria, Chloroflexi, and Planctomycetes. These had not been found in a previous culture-based study by Brar and Bergmann (2019). These phyla are also abundant in the cave silver biofilms in limestone caves in Europe (Porca et al. 2012), but, as with our experience with the cave silver biofilms at SURF, the phyla were not be isolated on diluted, complex organic media (Velikonja et al. 2014).

We found several genera in our culture-independent investigation of cave silver biofilms in SURF which had been isolated previously from the same biofilms using low-nutrient media by Brar and Bergmann (2019). These genera were *Pseudonocardia, Amycolatopsis, Paenibacillus, Bradyrhizobium, Pseudolabrys, Bauldia, Pedomicrobium, Reyranella*, and some uncharacterized genera of Rhodospirllaceae.

Surprisingly, Brar and Bergmann (2019) isolated several genera from SURF cave silver which we did not observe in our 16S rRNA gene libraries: *Allokutneria, Sporoichthya, Micrococcus, Pseudomonas*, and *Ralstonia*. It is possible that these bacteria were transients brought into the tunnels by air currents but not actively growing in the cave silver. Perhaps, when these transient bacterial cells were plated on dilute complex media, they resumed growth. In addition, several abundant phyla observed in our 16S rRNA gene libraries were not isolated on low-nutrient media at all, including Acidobacteria, Chloroflexi, and Planctomycetes.

Several OTUs noted in the 2015 V3-V4 16S rRNA gene library are likely to represent bacteria isolated by Brar and Bergmann (2019) from SURF cave silver at the same site. However, most were not abundant in the original cave silver microbial community, with the exception of a *Pseudonocardia* species, which was both abundant in the original cave silver community and was isolated in culture as well. This indicates that even some media with relatively low concentration of organic compounds still select strongly for groups of microbes which were not abundant in the original cave silver community and may not recover many abundant groups (even most chemohetroptrophs).

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LITERATURE CITED

Barton, H.A., and V. Jurado. 2007. What's up down there? Microbial diversity in caves. Microbe 2**:**132–138. https://www.asm.org/ccLibraryFiles/FILENAME/000000002921/znw00307000132.pdf

Brar, A., and D.J. Bergmann. 2019. Culture-based analysis of 'cave silver' biofilms on rocks in the former Homestake gold mine in South Dakota, USA. International Journal of Speleology 48:145-154. doi: 10.5038/1827-806X.48.2.2226.

Caddey, S., Bachman, R., Campbell, T., Reid, R., and R. Otto. 1991. The Homestake Gold Mine, an Early Proterozoic Iron-Formation-Hosted Gold Deposit, Lawrence County, South Dakota. U.S. Geological Survey Bulletin 1857-J. https://pubs.usgs.gov/bul/1857j/report.pdf.

Cavaletti, L., P. Monciardini, R. Bamonte, P. Schumann, M. Rohde, M. Sosio, and S. Donadio. 2006. New lineage of filamentous, spore-forming, gram-positive bacteria from soil. Applied Environmental Microbiology 72:4360-4369. doi:10.1128/AEM.00132-06.

Chao, A. 1984. Non-parametric estimation of the number of classes in a population. Scandinavian Journal of Statistics 11:265–270.

Edgar, R.C. 2004. MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Research 32:1792-97.

Elshahed, M., N.H. Youssef, Q Luo, F.Z. Najar, B.A. Roe, T.M. Sisk, S.I Bühring, K.U. Hinrichs, and L.R. Krumholz. 2007. Phylogenetic and metabolic diversity of Planctomycetes from anaerobic, sulfide- and sulfur-rich Zodletone Spring, Oklahoma. Applied Environmental Microbiology 73:4707-16. doi: 10.1128/AEM.00591-07.

Fullerton, H., and C.L. Moyer. 2016. Comparative single-cell genomics of Chloroflexi from the Okinawa Trough Deep-Subsurface Biosphere. Applied Environmental Microbiology 82:3000–3008. doi:10.1128/AEM.00624-16.

Goodfellow, M., P. Kämpfer, H.J. Busse, M.E. Trujillo, K. Suzuki, W Ludwig, and W.B. Whitman, editors. 2012. Bergey's Manual of Systematic Bacteriology, vol 5, The Actinobacteria, parts A and B. Springer, New York, NY.

Hathaway, J.J.M., M.G. Garcia, M.M. Balasch, M.N. Spilde, F.D. Stone, M.N.E. Dapkevicius, I.R. Amorim, R. Gabriel, P.A.V. Borges, and D.E. Northup. 2014. Comparison of bacterial diversity in Azorean and Hawai'ian lava cave microbial mats. Geomicrobiology Journal 31:205-220. doi:10.1080/01490451.2013.777491.

Jukes, T.H., and C.R. Cantor. 1969. Evolution of Protein Molecules. Academic Press, New York, NY.

Kielak, A.M., C. Barreto, G.A. Kowalchuk, J.A. van Veen, and E.E. Kuramae. 2016. The ecology of Acidobacteria: moving beyond genes and genomes. Frontiers in Microbiology. 7:744. doi:10.3389/fmicb.2016.00744.

Klindworth, A, E. Pruesse, T. Schweer, J. Peplies, C. Quast, M. Horn, and F.O. Glockner, 2013. Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. Nucleic Acids Research 41:e1. doi: 10.1093/nar/gks808.

Lavoie, K.H., A.S. Winter K.J.H. Read, M. Hughes, M.N. Spilde, and D. Northup. 2017. Comparison of bacterial communities from lava cave microbial mats to overlying surface soils from Lava Beds National Monument, USA. PLOS ONE. DOI:10.1371/journal.pone.0169339.

Northup, D.E., L.A. Melim, M.N. Spilde, J.J.M. Hathaway, M.G. Garcia, M. Moya, D. Stone, P.J. Boston, M.N.E. Dapkevicius, and C. Riquelme. 2011. Lava cave microbial communities within mats and secondary mineral deposits: implications for life detection on other planets. Astrobiology 11:1-18. DOI: 10.1089/ast.2010.0562.

Osburn, M.R., D.E. La Rowe, L.M. Momper, and J.P. Amand. 2014. Chemolithotrophy in the continental deep subsurface: Sanford Underground Research Facility (SURF) USA. Frontiers in Microbiology 5:1-14. https://dx.doi.org/10.3389%2Ffmicb.2014.00610.

Parada, A.E., D.M. Needham, and J.A. Fuhrman. 2016. Every base matters: assessing small subunit rRNA primers for marine microbiomes with mock communities, time series and global field samples. Environmental. Microbiology 18:1403–1414. http://doi.org/10.1111/1462-2920.13023.

Pašic, L., B. Kove, B. Sket, and B. Herzog-Velikonja. 2010. Diversity of microbial communities colonizing the walls of a Karstic cave in Slovenia. FEMS Microbiology Ecology 71:50–60. https://doi.org/10.1111/j.1574-6941.2009.00789.x.

Porca, E., V. Jurado, D. Žgur-Bertok, C. Saiz-Jimenez, C., and L. Pašić. 2012. Comparative analysis of yellow microbial communities growing on the walls of geographically distinct caves indicates a common core of microorganisms involved in their formation. FEMS Microbiology Ecology 81:255–266. https://academic.oup.com/femsec/article/81/1/255/567644.

Rastogi, G., L.D. Stetler, B.M. Peyton, and R.K. Sani. 2009. Molecular analysis of prokaryotic diversity in the deep subsurface of the former Homestake gold mine, South Dakota, USA. Journal of Microbiology 47**:**371–384. https://doi.org/10.1007/s12275-008-0249-1.

Riquelme, C, J.J.M. Hathaway, M. Enes Dapkevicius, A.Z. Miller, A. Kooser, D.E. Northrup, V. Jurado, O. Fernandez, C. Saitz-Jimenez, and N. Cheeptham. 2015. Actinobacterial diversity in volcanic caves and associated geomicrobiological interactions. Frontiers in Microbiology. 6:1-16. https://doi.org/10.3389/fmicb.2015.01342.

Shannon, C. 1948. A mathematical theory of communication. The Bell System Technical Journal. 27:379–423 and 623–656.

Simpson, E.H. 1949. Measurement of diversity. Nature 163:688. doi:10.1038/163688a0

Velikonja, B.H., R. Tkavc, and L. Pasic. 2014. Diversity of cultivable bacteria involved in the formation of macroscopic colonies (cave silver) on the walls of a cave in Slovenia. International Journal of Speleology 43:45-56. http://dx.doi.org/10.5038/1827-806X.43.1.5

Waddell, E.J., T.J. Elliott, R.K. Sani, J.M. Vahrenkamp, W.M. Roggenthen, C.M. Anderson, and S.S. Bang. 2010. Phylogenetic evidence of noteworthy microflora from the subsurface of the former Homestake gold mine, Lead, South Dakota. Environmental Technology 31:979–991. https://doi.org/10.1080/09593331003789511.

Wang, Q., G.M. Garrity, J.M. Tiedje, and J.R. Cole. 2007. Naïve Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. Applied Environmental Microbiology 73:5261-7. doi: 10.1128/AEM.00062-07.

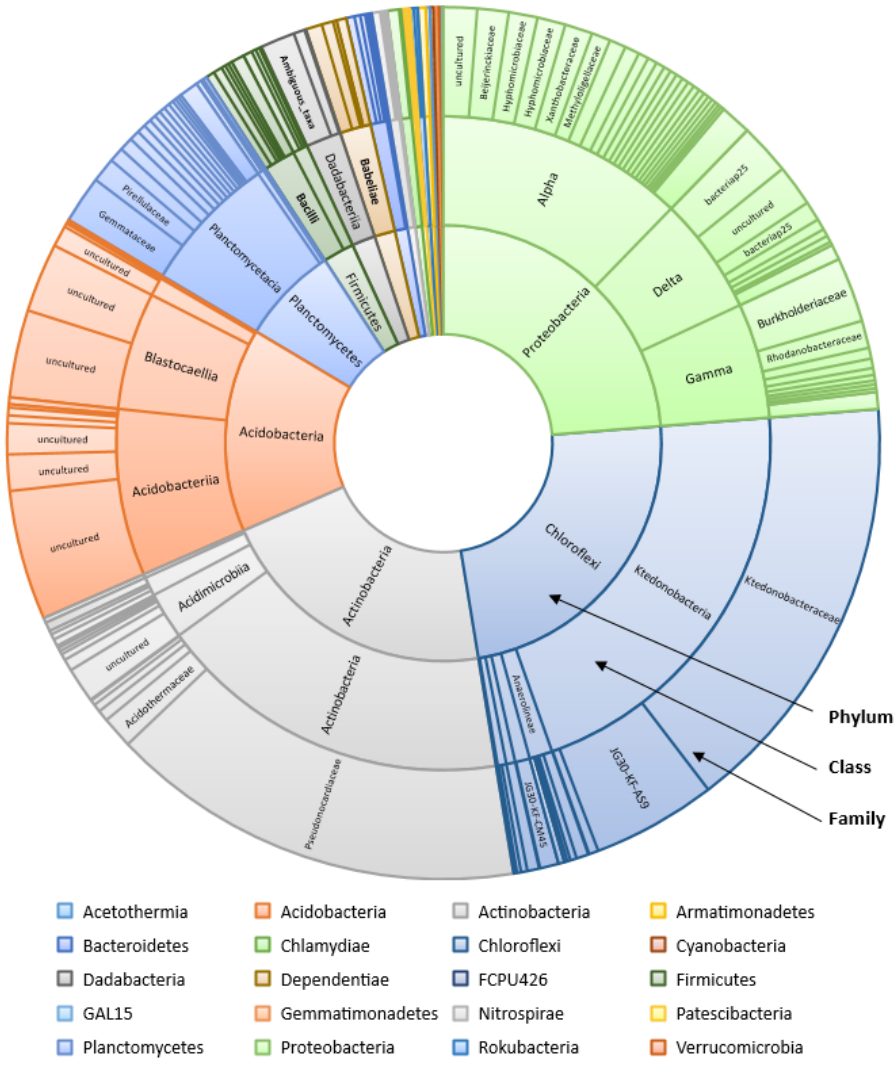

Figure 3. Proportion of bacterial phyla, classes, and families present in combined SURF cave silver samples from 2015, using V3-V4 16S rRNA gene PCR primers.

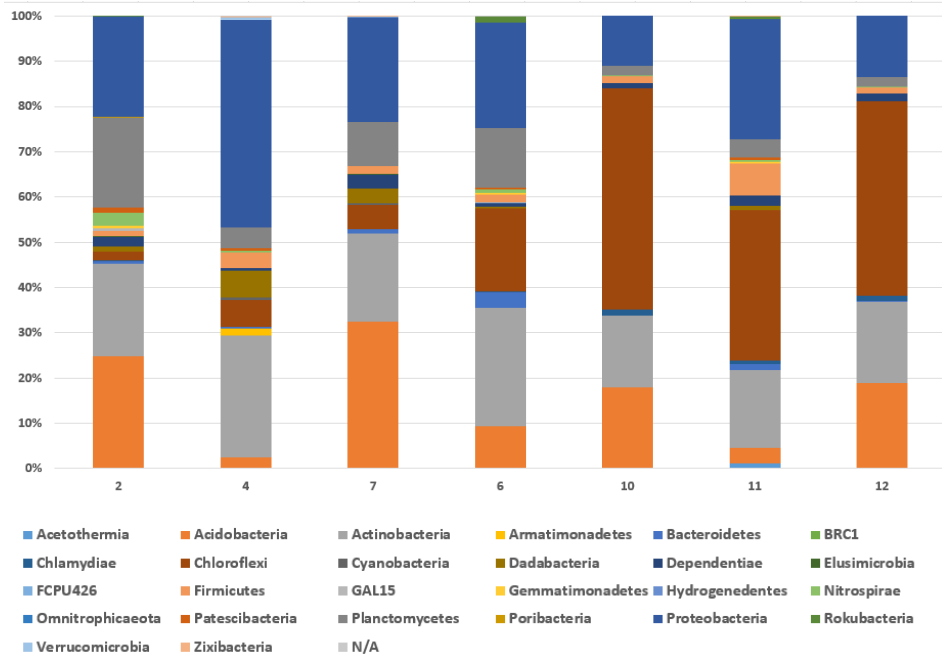

Figure 4. Proportion of bacterial phyla in individual SURF cave silver samples from 2015, using V3-V4 16S rRNA gene PCR primers.

Figure 5. Proportion of bacterial phyla, classes, and families present in combined cave silver biofilms sampled from limestone caves by Porca et al. (2012). 16S rRNA gene sequences were trimmed to the V3-V4 prior to analysis with CLC Bio software.

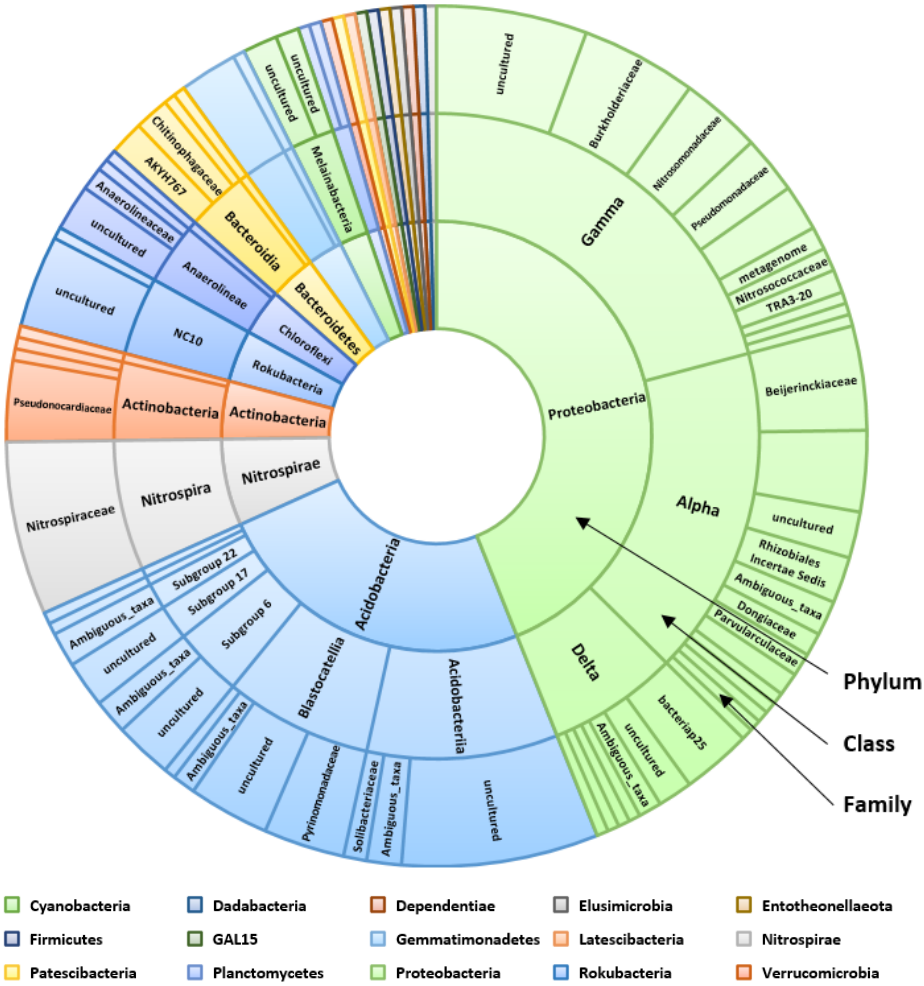

Figure 6. Proportion of bacterial phyla, classes, and families present in combined cave silver biofilms sampled from lava tube caves by Hathaway et al. (2013). 16S rRNA gene sequences were trimmed to the V3-V4 prior to analysis with CLC Bio software.

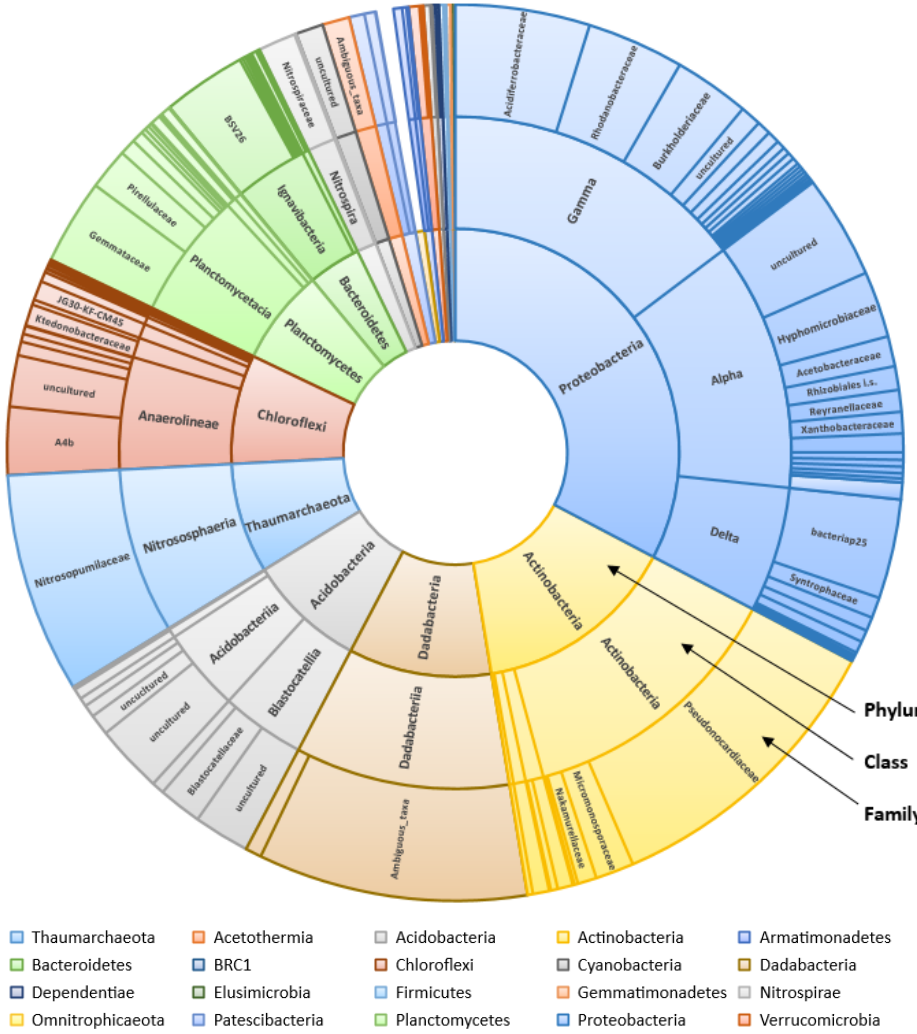

Figure 7. Proportion of phyla, classes, and families present in combined SURF cave silver samples from 2017, using V4 16S rRNA gene PCR primers.

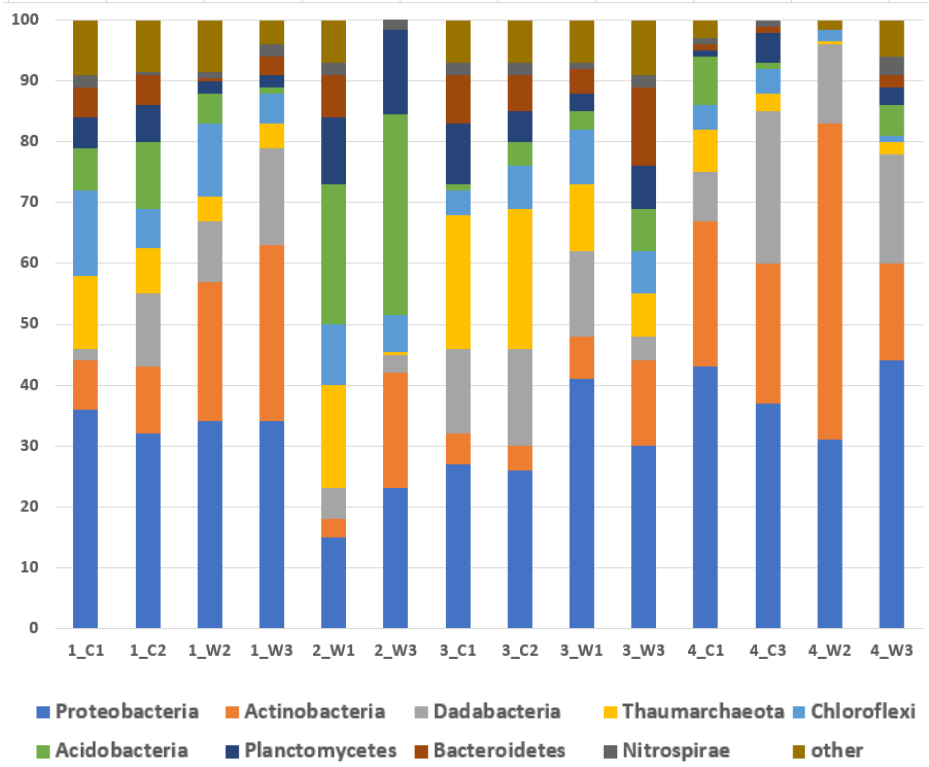

Figure 8. Proportion of prokaryotic phyla in individual SURF cave silver samples from 2017, using V4 16S rRNA gene PCR primers. There were four sampling sites (1-4), each of which had one to three replicate samples from about 1.7 m (W) or 2.7 m (C) high. Only samples used in 16S rRNA gene libraries are shown.

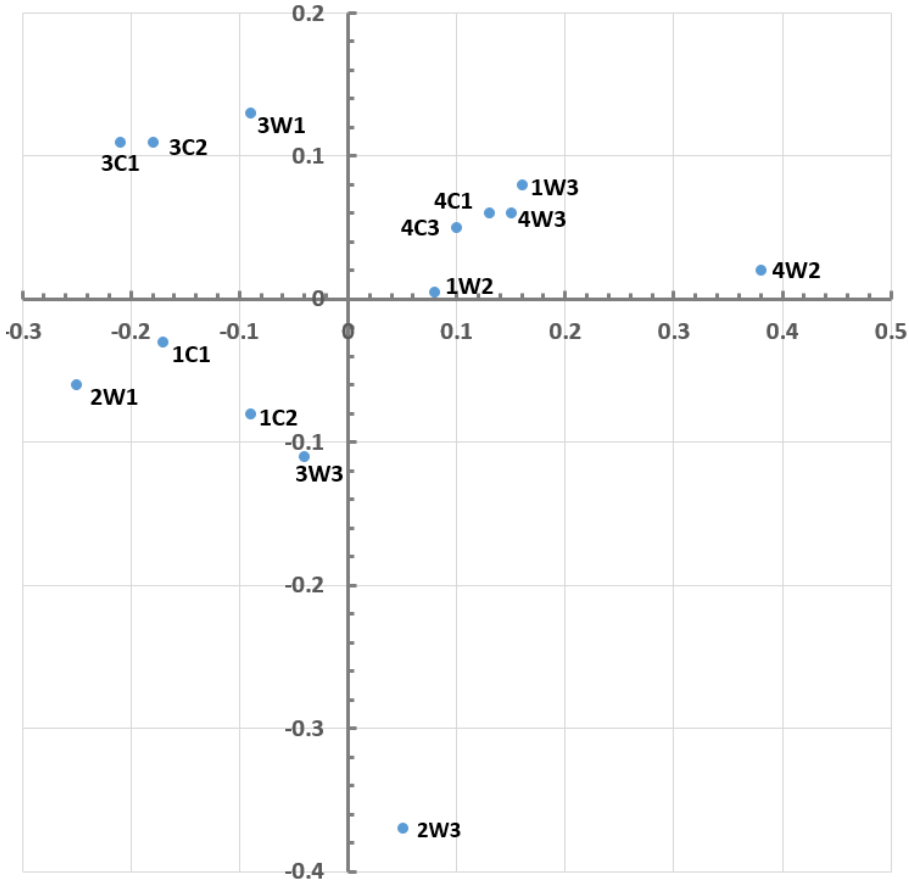

Figure 9. Principal Coordinate Analysis (PCoA) of SURF 2017 cave silver samples according the abundance of microbial taxa, using V4 16S rRNA gene PCR primers. The scores of the samples on the first two axes of PCoA ordination are shown.

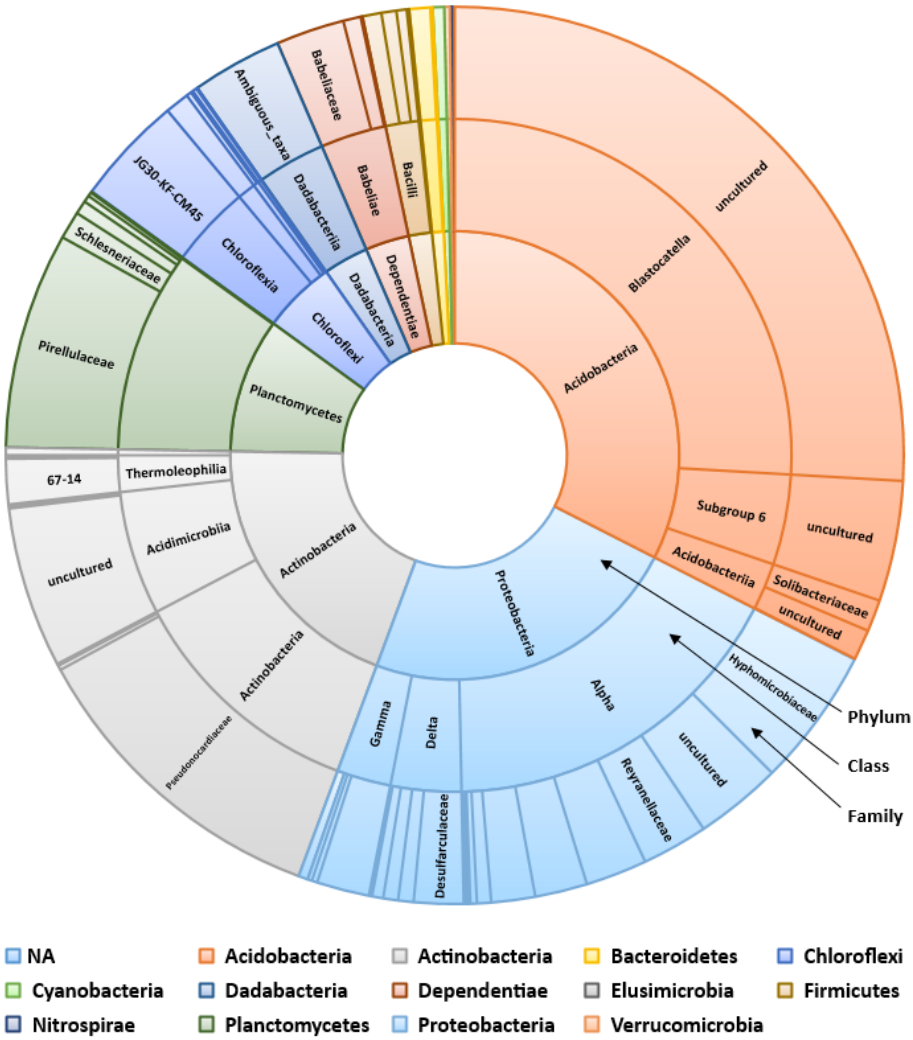

Figure 10. Taxa present (phylum, class, and family) in 2015 SURF cave silver Sample 7, using 16S rRNA gene PCR primers V3-V4.

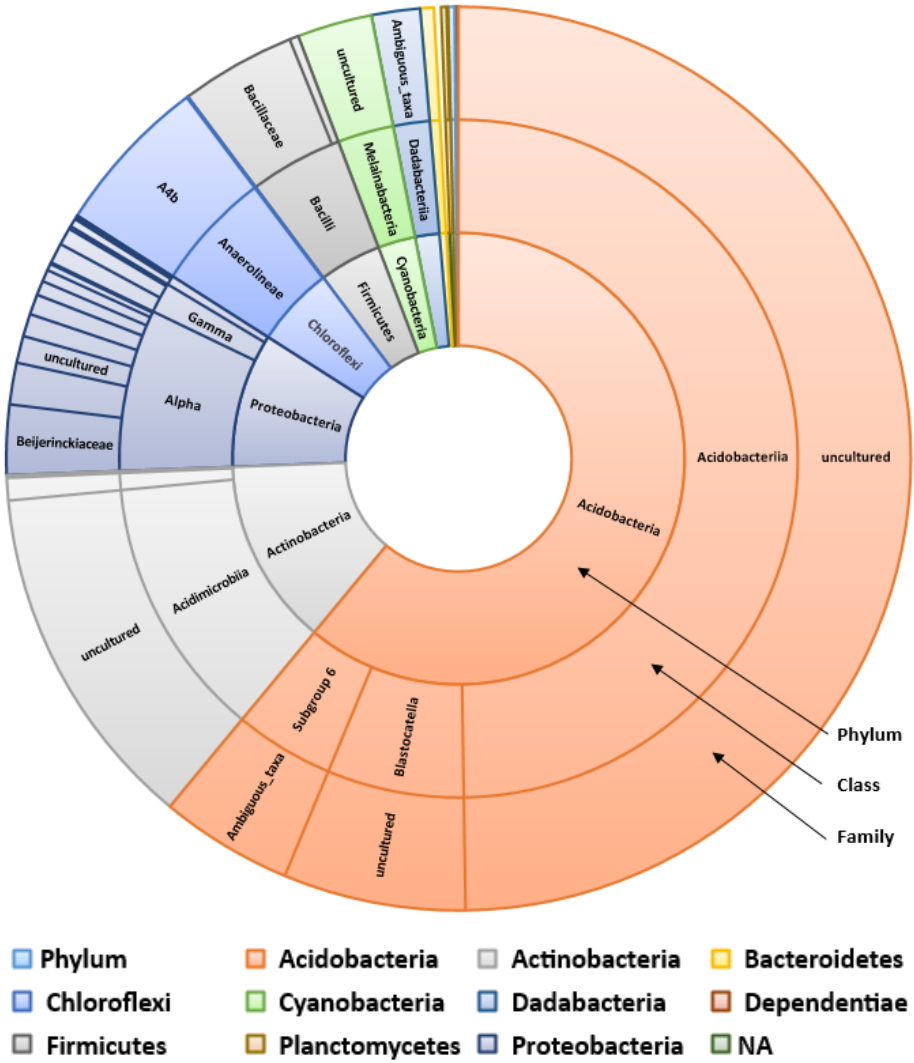

Figure 11. Taxa present (phylum, class, and family) in 2015 SURF cave silver Sample 7, using 16S rRNA gene V4 PCR primers.