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Culture-based analysis of 'Cave Silver' biofilms on Rocks in the former Homestake mine in South Dakota, USA

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Abstract: Tunnels in a warm, humid area of the 1478 m level of the Sanford Underground Research Facility (SURF), located in a former gold mine in South Dakota, USA, host irregular, thin whitish, iridescent biofilms, which appear superficially similar to 'cave silver' biofilms described from limestone and lava tube caves, despite the higher rock temperature (32°C) and differing rock surface (phyllite) present at SURF. In this study, we investigated the diversity of cultivable bacteria constituting the cave silver by using several media: CN agar, CN gellan gum and 0.1X R2A agar. The highest colony count (CFU/g of sample) was observed on 0.1X R2A medium. The bacterial strains were grouped into 39 distinct genotypes by randomly amplified polymorphic DNA (RAPD) analysis. In addition, the bacterial strains were further characterized based on their phenotypic and biochemical properties. 16S rRNA gene sequencing classified the cave silver isolates into three major bacterial phyla: Proteobacteria, Actinobacteria and Firmicutes. Isolates included some known genera; such as *Taonella*, *Dongia*, *Mesorhizobium*, *Ralstonia*, *Pedomicrobium*, *Bauldia*, *Pseudolabrys*, *Reyrenella*, *Mizugakiibacter*, *Bradyrhizobium*, *Pseudomonas*, *Micrococcus*, *Sporichthya*, *Allokutzneria*, *Amycolatopsis*, *Pseudonocardia*, and *Paenibacillus*. Several isolates; related to *Taonella*, *Dongia* and *Variibacter*; may represent undescribed genera.

Keywords: bacteria, biofilms, underground, cultivable, 16S rRNA genes

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INTRODUCTION

Deep underground ecosystems, such as caves and mines, lack light, have low amounts of organic matter, and may have reduced inorganic molecules which can support chemolithotrophy (Barton & Jurado 2007, Tebo et al., 2015). Microbial-induced mineralization processes have been described in various cave ecosystems (Northup et al., 2003; Engel et al., 2004; Curry et al., 2009). Some caves, including limestone caves (Portillo et al., 2008; Pašić et al., 2010; Mulec et al., 2015), lava-tube caves (Northup et al., 2011) and quartzite caves (Sauro et al., 2018), contain extensive, flat, microbial biofilms, which may be whitish, golden, or tan in color, which are dominated by members of the bacterial phyla Acidobacteria, Actinobacteria, Chloroflexi, and Nitrospirae. In some cases, these biofilms condense minute droplets of water and have an iridescent appearance and have been referred to as "cave silver" (Pašić et al., 2010). The biofilms in lava-tube caves have drawn the attention of astrobiologists, who hypothesize that microbes may exist on similar

caves on the planet Mars (Northup et al., 2011). Recently, biofilms similar in appearance to "cave silver" biofilms have been discovered in a segment of the former Homestake mine in South Dakota at a depth of 1478 m below the surface.

The Homestake mine was one of the largest (592 km of tunnels) and deepest (2.4 km) gold mines in North America until it was closed in 2003 (Murdoch et al., 2012). In 2007, portions of the former mine were re-opened as the Sanford Underground Research Facility (SURF), providing an opportunity to the biologists, astrophysicists, geoscientists and engineers to explore the subsurface environment (Lesko, 2011).

SURF consists of different levels, each with its own microbial ecosystems, depending on the geochemistry of rocks, rock fracture water, and sediment, as well as the temperature, pH, humidity and composition of gasses in tunnels (Osburn, 2014). 16S rRNA gene analysis of fracture and drainage water, sediments, and biofilms sustained by fracture water flowing over rocks indicate a high diversity of chemoautotrophic bacteria such as sulfide-oxidizers, ammonia-

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oxidizers, iron-oxidizers, methane-oxidizers, and sulfate-reducers, as well as chemoheterotrophs (Rastogi et al., 2009b; Waddell et al., 2010). Little culture-based work has been done on SURF microbial communities, apart from the isolation of a few groups of microbes of industrial interest, such as cyanide-degrading *Pseudomonas* (Mudder & Whitlock, 1984); thermophilic and mesophilic cellulose-degrading bacteria of the genera *Bacillus*, *Brevibacillus*, and *Paenibacillus* (Rastogi et al., 2009a); and several species of *Clostridium* capable of fermentation of glucose, xylose, and glycerol (Rastogi et al., 2013).

On the 1478 m deep level of SURF, at a site known as the 17 Ledge, warm (32°C) air, saturated with water vapor, meets cooler (25°C) air, causing condensation on the walls of the tunnel. The rocks in this region consist of phyllites of the Poorman formation. These phyllites consist mainly of sericite, biotite, graphite, ankerite, and quartz, with pyrrhotite also present (Caddey, 1991). In a zone from 8 to 60 m beyond a flowing diamond drill hole known as “*Thiothrix Falls*”, the site of an astrobiology experiment, areas of thin (less than 1 mm thick) iridescent biofilms frequently occur on the walls and ceilings of the tunnel. These nacreous white biofilms appear silvery due to the light scattered by the water droplets covering their surface. These biofilms were not characterized by previously published work on SURF microbiology cited above. These biofilms appeared similar in appearance to biofilms known as “Cave Silver” found in limestone caves in Europe and lava-tube caves. Data from high-throughput bacterial 16S rRNA gene sequencing indicated that the cave silver biofilms consisted primarily of phyla Proteobacteria and Actinobacteria, Acidobacteria, and Chloroflexi, with Bacteroidetes, Cynobacteria, Firmicutes, Chlorobi, Gemmatimonadetes, Nitrospirae, Planctomycetes, and Verrucomicrobia also present (Thompson & Bergmann, 2016). The presence of Actinobacteria is of interest because this phylum of bacteria is the source of the majority of antibacterial drugs (Suell-Silva, 2013). Hence, it is desirable to isolate bacteria from SURF cave silver, both to characterize novel taxa of bacteria and to culture Actinobacteria which might be tested for antimicrobial agents.

Although there are similarities in the appearance of the SURF cave silver biofilms with those of limestone and lava tube caves, the geology of the phyllite rock faces in SURF is very different from the limestone or solidified lava surfaces in caves. Also, the depth of the cave silver site in SURF is much greater than the limestone or lava-tube caves (near the surface vs. 1478 m), and the temperature is considerably higher (10°–19° C vs. 28–30°C). Hence, the environmental forces influencing the SURF biofilms are unique. With the exception of two studies by Mulec et al. (2015) and Velikonja et al. (2014) in limestone caves, there have been few recent culture based studies on cave silver biofilms, despite the prevalence of possible antibiotic-producing Actinobacteria in these biofilms. The aim of this project was to isolate and culture bacteria from SURF cave silver biofilms by using two different media: more nutrient rich, CN medium

(0.1% Casamino acids, 0.1% Nutrient broth with agar or GELRITE) and a more nutrient deficient medium, diluted R2A (0.1X R2A agar, Reasoner & Geldreich, 1985). We characterized phenotypic characteristics of these isolates and identified them by sequencing 16S rRNA genes. We then compared the taxa recovered by culture-based techniques with those known to be present in the biofilms based on culture-independent techniques.

MATERIALS AND METHODS

Sample collection

Cave silver biofilm samples were obtained in October 2015, at a depth of 1478 m at the 17 Ledge area of SURF, about 10–30 m beyond “*Thiothrix Falls*”. The microbial communities were primarily over 1.5 m high on the walls and ceilings of the tunnel, forming irregular, thin, silvery patches, each 25–400 cm² in area. Four samples were taken by gently scraping off the biofilms with a sterile scalpel into 15-ml Falcon tubes. After collection, the samples were transported to the lab at an ambient temperature and were then stored at 4°C until sample preparation (within 16 hours). Each sample weighed approximately 0.05 g and was homogenized in 9 mL Phosphate Buffered Saline (10 mM potassium phosphate, 0.8% NaCl, pH 7.5) and further serially diluted to 10⁻⁵ before being immediately spread-plated onto solid media.

Inoculation of media

CN agar, CN gellan gum, and 0.1X R2A agar media were selected to recover viable bacteria from the biofilm samples. For CN based medium, Casamino acids and Nutrient broth were added at a concentration of 1 g each per liter of distilled water and solidified with 1.5 agar or 1.2% gellan gum (Phytigel, Sigma-Aldrich), (Harris, 1985). A low nutrient, 0.1X R2A medium was used to recover slow-growing, oligotrophic microbes from the cave silver biofilm. 0.1X SURF R2A agar medium contained 0.1% R2A broth, 0.5% trace ATCC elements, 0.5% ATCC vitamin solution (American Type Culture Collection), 5% (W/V) cold filtered aqueous SURF sediment extract (dry sediment from tunnels where the cave silver biofilms were growing), and 1.5% agar. Cycloheximide (100 mg/mL final concentration) and nystatin (50 mg/mL final concentration) (Sigma-Aldrich) were added to the media to prevent fungal infection. 100 µL aliquots of diluted microbial suspension from each dilution were transferred to Petri plates (triplicate of each dilution) containing the sterile growth media and spread over the surface with a sterile glass spreading rod (Silva et al., 2013). The inoculated Petri plates were incubated at 28°C for 2–3 weeks and colonies counted to calculate the CFU/ml of homogenized sample (Tomasiewicz et al., 1980; Van Der Linde et al., 1999).

A total of 164 bacterial colonies from CN agar, CN gellan gum, and 0.1X R2A agar media were selected randomly (colonies nearest to the center of a plate were selected) and were streaked onto Petri plates of 1X CN agar media or 1 R2A agar media (respectively) with 100 mg/mL cycloheximide and 50 mg/mL nystatin. Single

colonies of each isolate were then transferred to either CN agar or 1X R2A slants, respectively. Pure cultures in R2 broth were preserved in 20% glycerol and frozen at -80°C for future use (Yuan et al., 2014).

Extraction of genomic DNA from isolates and Random Amplified Polymorphic DNA (RAPD) PCR

Genomic DNA was extracted from 152 bacterial isolates using a DNeasy Blood and Tissue Kit (Qiagen Inc.), following the protocol for gram-positive bacteria. RAPD PCR was performed using the BOX-AIR primer (5'-CTA CGG CAA GGC GAC GCT GAC G-3') (Versalovic et al., 1994). The PCR reaction mixture (15 μL) for isolates contained 2 μL of template DNA, 10x BSA (Nagai et al., 1998), 10 mM BOX-AR1 primer, deoxynucleoside triphosphates (each 10 mM), 10X HotMaster Taq buffer with magnesium (5 Prime Inc.) and 0.5 U HotMaster Taq DNA polymerase. PCR was carried out under the following conditions: initial denaturation at 94°C for 2 min; 30 cycles of denaturation at 94°C for 20 s, annealing at 53°C for 1 min, and extension at 65°C for 4 min, and a final extension at 65°C for 8 min. For DNA fingerprinting of 0.1X R2A agar isolates, 5 Prime HotMasterMix (2.5X) was used for preparing the reaction mixture. The PCR products were separated by electrophoresis on a 2% agarose gel and stained with ethidium bromide as described by Suel-Silva et al. (2013). The degree of similarity of PCR amplicon sizes from the different isolates was evaluated by visual observation, and the isolates classified into operational taxonomic units (OTUs).

Phenotypic Characterization of isolates and sequencing of 16S rRNA genes

Forty-six representatives, at least one for each RAPD OTU group, were selected for phenotypic characterization. These isolates were characterized by observing colony morphology, as well as cell shape and arrangement following gram-staining, as described by Moyes et al. (2009). The ability of bacterial isolates to ferment and/or oxidize glucose was tested by using glucose phenol red broth and Hugh and Leifson's O-F medium (McFaddin, 1980). The presence of cytochrome oxidase and catalase was tested as described by McFaddin (1980). Isolates were grown in nitrate broth to test for the nitrate reductase (McFaddin, 1980).

Representative isolates from each RAPD OTU were also used for PCR amplification and sequencing of 16S rRNA gene. The 16S rRNA gene was amplified with oligonucleotide primers 27F (5'-GAGTTTGATCMTGGCTCAG-3') and 1492R (5'-GGT TAC CTT GTT ACG ACT T-3') (Weisburg et al., 1991). The reaction mixture (15 μL) contained 5 Prime HotMasterMix (2.5x), 10x BSA, 27F and 1492R primers (10 μM) and 2 μL genomic DNA. The PCR reaction started with pre-denaturation at 94°C for 2 min, followed by 32 cycles of denaturation at 94°C for 30 sec, annealing at 57°C for 15 sec and 4 min of extension at 65°C with a final extension at 65°C for 7 min. After cleaning the amplified product with ExoSAP-

IT (Affymetrix, USA) sequencing was performed with primers 27F (AGAGTTTGATCMTGGCTCAG), 338F (ACTCCTACGGGAGGCAGCAG) and 1390R (CGGTGTGTACAAGGCC) (De Lillo et al., 2006; Huse et al., 2008; Fortuna et al., 2011) using BigDye Terminator v1.1 Cycle Sequencing Kit in ABI 3130xl Genetic Analyzer Applied Biosystems, USA) at Black Hills State University (BHSU) Westcore.

The 16SrRNA gene sequences were checked for the presence of chimeras using the Bellerophon server (<http://foo.maths.uq.edu.au/~huber/bellerophon.pl>) and the closest matches among cultured isolates were found using the BLASTN program on the NCBI database (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome). The sequences with greater than 97% identity to the top BLAST hit were considered resolved at species level and 95-97% at genus level (Rhoads et al. 2012). The 16S rRNA gene sequences were also classified using the Classifier program (Wang et al. 2007) at the Ribosomal Database Project website (<https://rdp.cme.msu.edu/classifier/classifier.jsp>). After aligning the sequences using MUSCLE (Edgar, 2004), a phylogenetic tree was constructed using the Jukes and Cantor (1969) model for the neighbor-joining method in the CLC Bio software, Version 11 (Qiagen, Inc.). 16S rRNA gene sequences from isolates were deposited in the GenBank database under accession numbers MH669510-MH669553.

RESULTS

For this study, two types of high-nutrient CN based media were compared; CN agar and CN gellan gum, both of which contain Casamino acids and beef extract but differed in the type of solidifying agent used. This was done to determine the effect of gelling agent (agar or gellan gum) for culturing of cave silver biofilms. We also tested a low nutrient medium, 0.1X R2A, an agar-based medium. Numbers of colony forming units per g of homogenized biofilm (CFU/g) were for slightly higher for the same samples for CN gellan gum than for CN agar media, but the highest counts were obtained for a sample plated on low nutrient 0.1X R2A medium (Fig. 1).

Fig. 1. Colony forming units (CFU) per gram of SURF cave silver samples. Samples A, B, and C were plated on CN agar and CN gellan gum, while sample D was plated on 0.1 X R2A. Standard errors of the mean are indicated by the vertical bars.

A total of 164 bacterial isolates were obtained; 118 from CN agar/CN gellan gum and 46 from 0.1X R2A agar media. Some CN and 0.1X R2A based isolates were lost during the sub-culturing process due to fungal contamination, while seven isolates could not be separated as pure cultures.

Of the 118 bacterial isolates recovered from CN based media, 46.6% had white, circular, raised and mucoid colonies of mostly gram-negative cocci; 17.7% formed white, powdery, wrinkled colonies with well-developed aerial mycelia; 8.4% formed some round, pink colored colonies of gram-negative coccobacilli; 5.1% formed smooth, yellow colored colonies (which were later confirmed by 16S rRNA analysis to be Actinobacteria); and the remaining 22.0% of isolates included white or transparent colonies.

Of the 46 0.1X R2A isolates, 32.6% consisted of white, convex colonies forming an aerial mycelium. These colonies produced a pink colored diffusible pigment, turning the media completely black after 4-5 days of incubation. The 16S rRNA analysis revealed that they were Actinobacteria belonging to the genus *Amycolatopsis*. The second most common (19.6%) group included whitish, mucoid colonies which were later found to be *Bradyrhizobium*.

Random Amplified Polymorphic DNA (RAPD) was used to classify isolates into OTUs. Isolates from 1X CN media and 0.1X R2A media were classified into 25 and 14 RAPD groups, respectively (Fig. 2, Table 1). Largely dissimilar RAPD groups were observed for isolates from the 1X CN agar/ gellan gum and the 0.1X R2A agar media.

Fig. 2. 2.0% agarose gel showing an example of RAPD amplicons from 37 SURF cave silver isolates from CN media. On the basis of amplicon sizes, the isolates were assigned to RAPD genotype groups (A, B, C, etc.). The 100 BP ladder has ten DNA fragments, differing in size by 100 BP, from 100 to 1000 BP, and also a fragment 1500 BP.

Table 1. Genotypic characteristics of isolates from SURF cave silver biofilms. Isolates ending in "C" are from CN media, while those ending in "R" are from 0.1X R2A media. Letters designate RAPD groups with a common pattern of amplicons. The closest match to the sequence of the 16S rRNA gene in the cultured isolates in the NCBI database is listed, along with the percent identity of the match. Isolates from CN (a) and R2A media (b).

(a)

Isolate	RAPD OTU	16S rDNA closest match	Family	Percent Identity	Habitat
HS001C	A	<i>Taonella mepensis</i> H1	Rhodospirillaceae	90%	activated sludge
HS009C	A	<i>Taonella mepensis</i> H1	Rhodospirillaceae	91%	activated sludge
HS014C	B	<i>Taonella mepensis</i> H1	Rhodospirillaceae	90%	activated sludge
HS039C	B	<i>Taonella mepensis</i> H1	Rhodospirillaceae	91%	activated sludge
HS017C	C	<i>Taonella mepensis</i> H1	Rhodospirillaceae	91%	activated sludge
HS125C	H	<i>Taonella mepensis</i> H1	Rhodospirillaceae	91%	activated sludge
HS062C	Q	<i>Taonella mepensis</i> H1	Rhodospirillaceae	99%	activated sludge
HS150C	W	<i>Taonella mepensis</i> H1	Rhodospirillaceae	92%	activated sludge
HS025C	E	<i>Dongi rigui</i> 04SU4-P	Rhodospirillaceae	91%	freshwater wetland
HS153C	V	<i>Dongi rigui</i> 04SU4-P	Rhodospirillaceae	91%	freshwater wetland
HS063C	Y	<i>Dongi rigui</i> 04SU4-P	Rhodospirillaceae	91%	freshwater wetland
HS101C	F	<i>Dongia mobilis</i> LM22	Rhodospirillaceae	95%	malachite green activated sludge

HS130C	T	<i>Dongia mobilis</i> LM22	Rhodospirillaceae	95%	malachite green activated sludge
HS112C	I	<i>Bradyrhizobium japonicum</i> DSM30131	Bradyrhizobiaceae	99%	soybean root nodules
HS022C	I	<i>Bradyrhizobium japonicum</i> DSM30131	Bradyrhizobiaceae	99%	soybean root nodules
HS081C	G	<i>Mesorhizobium acaciae</i> RITF471	Phyllobacteriaceae	99%	<i>Acacia melanoxylon</i> root nodules
HS129C	J	<i>Micrococcus aloverae</i> AE-6	Micrococcaceae	99%	<i>Aloe barbadensis</i> leaves
HS035C	J	<i>Micrococcus yunnanensis</i> YM65004	Micrococcaceae	99%	endophytic, <i>Catharanthus roseus</i>
HS092C	K	<i>Ralstonia picketti</i> NBRC102503	Burkholderiaceae	100%	soil, water, clinical samples
HS067C	K	<i>Ralstonia picketti</i> NBRC102503	Burkholderiaceae	100%	soil, water, clinical samples
HS067C	L	<i>Allokutzneria albata</i> R761-7	Pseudonocardiaceae	99%	soil
HS117C	N	<i>Pseudonocardia spinospora</i> LM141	Pseudonocardiaceae	98%	gold mine sediment
HS050C	M	<i>Pseudonocardia eucalypti</i> EUM374	Pseudonocardiaceae	96%	<i>Eucalyptus microcarpa</i> root
HS107C	S	<i>Paenibacillus favisporus</i> GMP01	Paenibacillaceae	99%	cow feces
HS103C	D	<i>Sporichthya polymorpha</i> DSM43042	Sporichthyaceae	100%	soil
HS103C	X	<i>Variibacter gotjawalensis</i> GI-W30	Bradyrhizobiaceae	94%	soil
HS138C	U	<i>Pedomicrobium manganicum</i> ATCC 33121	Hyphomicrobiaceae	95%	soil, water

(b)

Isolate	RAPD OTU	16S rDNA closest match	Family	Percent Identity	Habitat
HS013R	h'	<i>Bradyrhizobium japonicum</i> DSM 30131	Bradyrhizobiaceae	99%	soybean root nodules
HS099R	h'	<i>Bradyrhizobium japonicum</i> DSM 30131	Bradyrhizobiaceae	99%	soybean root nodules
HS111R	h'	<i>Bradyrhizobium japonicum</i> DSM 30131	Bradyrhizobiaceae	99%	soybean root nodules
HS022R	f'	<i>Amycolatopsis helveola</i> TT 00-43	Pseudonocardiaceae	95%	soil
HS088R	f'	<i>Amycolatopsis helveola</i> TT 00-43	Pseudonocardiaceae	95%	soil
HS094R	f'	<i>Amycolatopsis helveola</i> TT 00-43	Pseudonocardiaceae	95%	soil
HS071R	b'	<i>Raeynella massillensis</i> 521	Rhodospirillaceae	99%	freshwater
HS058R	e'	<i>Dongia rigui</i> 04SU4-P	Rhodospirillaceae	92%	freshwater wetland
HS083R	k'	<i>Pseudomonas aeruginosa</i> DSM50071	Pseudomonadaceae	99%	soil, water, clinical samples
HS029R	l'	<i>Pseudolabrys taiwanensis</i> CC-BB4	Xanthomonadaceae	99%	soil
HS067R	c'	<i>Pseudolabrys taiwanensis</i> CC-BB4	Xanthomonadaceae	99%	soil
HS096R	c'	<i>Pseudolabrys taiwanensis</i> CC-BB4	Xanthomonadaceae	99%	soil
HS070R	l'	<i>Pseudolabrys taiwanensis</i> CC-BB4	Xanthomonadaceae	99%	soil
HS003R	d'	<i>Pseudonocardia yunnanensis</i> YIM75926	Pseudonocardiaceae	98%	soil
HS001R	a'	<i>Pseudonocardia eucalypti</i> EUM374	Pseudonocardiaceae	96%	<i>Eucalyptus microcarpa</i> root
HS057R	g'	<i>Paenibacillus lautus</i> AB236D	Paenibacillaceae	99%	clinical sample
HS097R	m'	<i>Mizugakiibacter sediminis</i> skMP5	Xanthomonadaceae	99%	thermophilic lake sediment
HS068R	j'	<i>Bauldia consociata</i> 11	unclassified Rhizobiales	96%	soil, water
HS063R	n'	<i>Bauldia consociata</i> 11	unclassified Rhizobiales	96%	soil, water

Forty-six isolates, one or more from each RAPD OTU group, were selected for phenotypic characterization. Most isolates had cells which were gram-negative coccobacilli or bacilli (later found to be Proteobacteria), or were gram-positive bacilli in chains or mycelia (later found to be Actinobacteria) (Supplementary Table 1). Most isolates were catalase and oxidase positive, with strictly respiratory metabolism (Supplementary Table 1).

The same 46 representatives of RAPD OTU groups were subjected to Sanger sequencing of nearly full-length 16S rRNA gene to classify them according to bacterial taxa in the Ribosomal Database (Table 1). From the relative abundances of each RAPD OTU group among the isolates from CN and R2A media the relative abundance of these taxa among the isolates was estimated (Table 2 and Fig. 3). For all media combined (CN agar, CN GELRITE and 0.1X R2A agar), Proteobacteria was found to be the most abundant phylum (65.5% of isolates) followed by Actinobacteria

(31.1%) and Firmicutes (3.31%). The CN based media were more successful in isolating Alphaproteobacteria, constituting 67% of 106 isolates. The low nutrient (0.1X R2A agar) media favored Actinobacteria and Alphaproteobacteria nearly equally, comprising 47% and 42% of cultures respectively (Fig. 3). The low recovery of Firmicutes on both media is likely related to their low proportions (2.6%) in the original biofilm, as determined by the culture independent study (Thompson & Bergmann, 2016).

Based on sequence similarity of isolates to the sequences in GenBank, 80.1% and 43.7% were identified at genus (95% identity or greater) and species 97% identity or greater level. Nearly 20% of the 16S rRNA gene sequences from CN and 0.1XR2A derived isolates may represent novel genera; with closest similarities (less than 95% identity) to *Taonella*, *Dongia*, and *Variibacter* (Fig. 4) (Schlaberg et al., 2012). A total of 17 different genera, 11 from

Table 2. The abundance of each bacterial genus (number of isolates / percentage of isolates) from two kinds of media; CN agar/ gellan gum and 0.1X R2A agar.

Bacterial Groups	CN agar/GELRITE (number of isolates/sequence similarity)	0.1X R2A agar (number of isolates/sequence similarity)
Alphaproteobacteria	<i>Dongia</i> (27/ 95%) <i>Brayrhizobium</i> (10/ 99%) <i>Taonella</i> (1/ 99%) <i>Variibacter</i> (3/ 94%) <i>Pedomicrobium</i> (2/ 95%) <i>Mesorhizobium</i> (2/ 99%) <i>Related to Taonella</i> (24/ 90-91%) <i>Related to Dongia</i> (3/ 91%)	<i>Bradyrhizobium</i> (9/ 99%) <i>Reynella</i> (1/ 99%) <i>Pseudolabrys</i> (6/ 99%) <i>Bauldia</i> (2/ 96%) <i>Related to Dongia</i> (1/ 92%)
Betaproteobacteria	<i>Ralstonia</i> (5/ 100%)	–
Gammaproteobacteria	–	<i>Mizugakiibacter</i> (3/ 99%) <i>Pseudomonas</i> (1/ 99%)
Actinobacteria	<i>Pseudonocardia</i> (9/ 96-98%) <i>Micrococcus</i> (6/ 99%) <i>Sporichthya</i> (1/ 100%) <i>Allokutzneria</i> (10/ 99%)	<i>Pseudonocardia</i> (6/ 96%) <i>Amycolatopsis</i> (15/ 95%)
Bacilli	<i>Paenibacillus</i> (4/ 99%)	<i>Paenibacillus</i> (4/ 99%)

Proteobacteria, five from Actinobacteria and one from Firmicutes were identified, including

Pseudolabrys, *Reynella*, *Mizugakiibacter*, *Bradyrhizobium*, *Pseudomonas*, *Micrococcus*, *Sporichthya*, *Allokutzneria*, *Amycolatopsis*, *Pseudonocardia*, and *Paenibacillus* (Table 2 and Fig. 4). CN and R2A based media supported mainly different groups of microbial taxa, except the members of genera *Bradyrhizobium*, *Pseudonocardia*, and an unclassified group related to genera *Dongia*, which were present in both CN and R2A based media (Table 2). The 16S rRNA gene sequence of these isolates from CN and R2A based media align with 100% sequence identity. Nearly 81% of CN and 73% of R2A derived bacterial strains belongs to different taxonomic groups.

DISCUSSION

The two media tested in this study, high-nutrient CN agar and low nutrient 0.1X R2A, were both dominated by isolates from the Alphaproteobacteria and the Actinobacteria. However, the abundance of most genera of bacteria was very different from the two media. Hence, to isolate the most diverse assemblage of bacteria from an environmental sample, several media should be used (Velikonja et al., 2014).

The abundance of bacterial genera isolated from cave silver in SURF differed considerably from those isolated from cave silver in European caves. While Alphaproteobacteria such as *Bradyrhizobium* and uncharacterized Rhodospirillaceae, as well as Actinobacteria such as *Pseudonocardia* and *Amycolatopsis* were abundant in cave silver from SURF, Actinobacteria such as *Streptomyces*, *Micrococcus*, *Rhodococcus*, and *Agrobacterium* were most abundant in isolates from cave silver from Slovenia (Velikonja et al., 2014). A study by Mulec et al. (2014) used both high- and low-nutrient media to isolate microbes from yellowish biofilms in limestone caves in Slovenia recovered primarily *Pseudomonas* and *Flavobacterium*, as well as *Streptomyces*, *Paenibacillus*, *Bacillus*, and *Staphylococcus*.

Some of the major groups of bacteria noted by Thompson and Bergmann (2016) in cave silver in SURF

using high-throughput sequencing of 16S rRNA gene; such as *Pseudonocardia* and other Pseudonocardiales, and Alphaproteobacteria, including *Bradyrhizobium*, *Bauldia*, and uncultured Rhodospirillaceae; were also represented among isolates from SURF cave

Fig. 3. Proportion of isolates in different bacterial classes on CN (a) and 0.1X R2A (b) media.

Four genera of Actinobacteria; *Pseudonocardia*, *Amycolatopsis*, *Allokutzneria*, and *Micrococcus*; were abundant among isolates from SURF cave silver. *Amycolatopsis* and *Allokutzneria* species are

chemoheterotrophs which can oxidize a variety of organic molecules, including sugars. The former genus is the source of numerous antibiotics, including vancomycin and rifamycin, while the latter is the source of anti-viral cycloviracins (Tomita et al., 1993; Tamura et al., 2010; Labeda & Goodfellow, 2012).

Pseudonocardia includes species with diverse metabolism, mostly chemoheterotrophs which can utilize many sources of organic molecules, including hydrocarbons, some chemoautotrophs, and one which can use carbon monoxide as its sole carbon source. (Labeda & Goodfellow, 2012). Some species, which have mutualistic associations with ants, produce antimicrobial quinones (Carr et al., 2012).

The abundance of *Bradyrhizobium* among SURF cave silver isolates is interesting. Although often *Bradyrhizobium* species are often known for their ability to fix nitrogen and form mutualistic associations with legumes, other species of *Bradyrhizobium* are free-living in the soil, and lack nitrogenase (VanInsberghe et al., 2015). Hence, it is uncertain if nitrogen fixation occurs in SURF cave silver.

It appears that most of the genera isolated from cave silver at SURF in this study, as well detected by 16S rRNA gene sequencing (Thompson & Bergmann, 2016) are likely to be chemoheterotrophic or mixotrophic. However, the source of organic molecules utilized by cells in cave silver is not obvious, because the cave silver biofilms in SURF are not exposed to fracture water, but instead condense water on their surface. It is possible that volatile organic molecules, produced by extensive microbial biofilms where fracture water emerges 100 m down the tunnel, may be utilized by the cave silver.

A survey of cave silver biofilms using Illumina 16S rRNA gene sequencing by Thompson and Bergmann (2016) indicated that Actinobacteria (especially Pseudonocardiaceae), Chloroflexi, Acidobacteria, Proteobacteria (especially Alphaproteobacteria) and Planctomycetes were the most abundant bacterial groups, with a total of 552 OTUs identified at a 97% level of similarity. We were not able to isolate any members of the Acidobacteria, Chloroflexi, or Planctomycetes in this study. We were able to isolate members of genera *Dongia*, *Reynella*, *Bradyrhizobium*, *Variibacter*, *Pseudolabrys*, *Bauldia*, *Mesorhizobium*, *Micrococcus*, *Sporichthya*, *Allokutnzeria* and *Pseudonocardia*. However, we estimate that the groups we isolated represent only 4.7% of the total 16S rRNA gene sequences obtained from Illumina sequencing. Despite the fact that the isolates were obtained represent only a small fraction of the diversity of bacteria present in SURF cave silver biofilms, a number of isolates appear to represent previously uncultured species and genera in the Rhodospirillaceae. We hope that these may be characterized more fully, to provide inferences about their role in the cave silver community in SURF.

It is striking that the genera of cultivable bacteria from cave silver biofilms in limestone caves in Slovenia by Velikonja et al., (2014) were largely different from those isolated from cave silver in SURF. Although 16S rRNA gene analysis of community DNA

indicated that both the Slovenian (Pasic et al., 2010) and SURF (Thompson & Bergmann, 2016) cave silver communities had an abundance of Actinobacteria of the family Pseudonocardiaceae, Velikonja et al. (2014) isolated primarily *Streptomyces*, *Rhodococcus*, and *Micrococcus*; and did not isolate the genera *Pseudonocardia*, *Amycolatopsis*, and *Allokutneria* abundant in SURF isolates. *Micrococcus* was found in both Slovenian and SURF cave silver, however, and 16S rRNA gene sequence (an approximately 1360 BP long region) suggests that 13 Slovenian isolates (accession number KC978879) may represent the same species as SURF isolates 27R and 35R; this was the only cultured species shared between these sites. This may partly reflect the different media used for each study, but it may also reflect that there are substantial differences in cultivable microbes in cave silver from SURF and from limestone caves.

It is likely that some of the bacteria in SURF cave silver may be derived from bacteria living in terrestrial habitats. Half of the bacterial isolates from SURF cave silver have greater than 98% 16S rRNA gene identity (over their entire length) to bacterial isolates from surface habitats (Fig. 1). Even some SURF isolates which have less than 97% 16S rRNA gene identity with surface isolates still show greater than 97% 16S rRNA gene identity with environmental 16S rRNA gene libraries from surface habitats. For example, SURF isolates HS025C, HS063C, HS130C, and HS153C had 97.8% identity with a 16S rRNA gene (accession number LN567343) from a refuse pile made by leaf-cutter ants; isolates HS063R and HS068R had 99.7% identity with a 16S rRNA gene (AF234728) from activated sludge; and isolate HS138C had 98.8% identity with a 16S rRNA gene (JF776953) from the rhizosphere of eggplant.

In general, our experience attempting to use culture-based techniques to recover bacteria from cave silver in SURF is similar to other recent attempts to recover bacteria from cave rocks using low-nutrient media (Barton, 2015; Ortiz et al., 2015; and Vellikonja et al., 2014) in that certain abundant cave phyla, such as Acidobacteria, Chloroflexi, and Planctomycetes are not recovered, while various species of Proteobacteria and Actinobacteria, which are not the predominant members of the community, predominate in cultured isolates. In all cases, the species isolated were highly dependent on the media used. Nonetheless, culture-based techniques may be still useful, not so much to isolate the main members of cave silver communities, but to isolate and characterize new species of microbes, because both cave rock surfaces and SURF cave silver communities contain previously unknown species and even genera.

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