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Diversity of rock varnish bacterial communities from Black Canyon, New Mexico

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[1] Scientists vigorously debate the degree to which rock varnish is formed through the actions of microorganisms. To investigate this enigma, we utilized a three-pronged approach that combined (1) culture-independent molecular methods to characterize bacterial communities associated with varnish that coats the rhyolitic volcanic rocks of Black Canyon, New Mexico, and rocks with no visible varnish; (2) culturing of varnish in media supplemented with reduced forms of manganese and/or iron and no or low amounts of carbon to isolate bacteria capable of precipitating iron and/or manganese oxides; and (3) scanning electron microscopy (SEM) of varnish and nearby rock that lacks macroscopically visible varnish. Our culture-independent studies revealed significant differences between varnish and nonvarnish communities. *Chloroflexi* and *Ktedobacteria* dominated one varnish site, while the other varnish site was dominated by *Cyanobacteria*. The nonvarnish sites were dominated by *Actinobacteria* and, to a lesser extent, *Cyanobacteria* and were the only samples to contain *Deinococcus-Thermus* sequences. Approximately 65% of varnish cultures produced visible manganese precipitates. Most culture isolates were not closely related to known manganese oxidizers, with the exception of *Bacillus* spp. SEM revealed microbial morphologies and two types of varnish morphologies: (1) relatively smooth layers and (2) patches of botryoidal pinnacles, which were often associated with increased manganese concentrations. "Bare" rock showed evidence of incipient varnish. These results have important implications for the detection of life on extraterrestrial planets such as Mars, where putative varnish coatings have been observed, and represent some of the first culture-independent characterizations of varnish communities.

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1. Introduction

[2] Rock varnish is a natural, black to brown colored coating enriched with manganese (Mn) and iron (Fe) oxides with a typical thickness rarely exceeding 200 μm. Varnish is ubiquitous, forming on exposed rock surfaces of diverse lithology in almost every type of terrestrial weathering environment, (e.g., Antarctica [*Dorn et al.*, 1992a], Norway [*Whalley et al.*, 1990], and Hawaii [*Dorn et al.*, 1992b]), but is particularly abundant in arid and semiarid regions. Rock varnishes have attracted considerable research interest as a potential Quaternary dating tool for rock surfaces [*Liu*, 2003]; as archeological features and artifacts [e.g., *VandenDolder*, 1992]; as indicators of paleoclimatic change [e.g., *Dorn*, 1994; *Liu et al.*, 2000; *Liu and Broecker*, 2000, 2007]; as environmental monitors because of the great scavenging abilities of Mn oxides for certain heavy metals [*Dorn*, 1991; *Fleisher et al.*, 1999; *Wayne et al.*, 2006]; because of the likely role of microbes in their formation [e.g., *Perry and Adams*, 1978; *Krumbein and Jens*, 1981; *Nagy et al.*, 1991; *Gorbushina*, 2007]; as analogous environs for the search for life on other planets [*DiGregorio*, 2002; *Gorbushina et al.*, 2002; *Allen et al.*, 2004; *Edwards*, 2004]; and to assist in interpretations of remote sensing studies of varnished rock surfaces on Earth and Mars [e.g., *Israel et al.*, 1997; *Kraft and Greeley*, 2000].

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[3] The first scientific descriptions of rock varnish and its possible biogenic origin date as far back in the literature as 1852, demonstrating that the deposition of Mn and Fe coatings on rocks has occupied the minds of many generations of scientists [cf. *Krumbein and Jens*, 1981]. Rock varnish is mostly composed of clay minerals that are cemented to rock by Mn and Fe oxides in a laminated structure resembling the botryoidal morphology that is occasionally seen in stromatolites [*Perry and Adams*, 1978]. Mn and Fe concentrations can vary widely, but in most cases, Mn is highly enriched over Fe as compared to their natural distribution [*Engel and Sharp*, 1958; *Krumbein*, 1968, 1971; *Potter and Rossman*, 1979; *Knauss and Ku*, 1980], Mn oxides usually accounting for 20% or more of the total oxides. It is widely accepted that the Mn and Fe of rock varnish come from a variety of sources including the atmosphere, meteoric water, dust, and from the surrounding soils [*Allen*, 1978; *Engel and Sharp*, 1958; *Scheffer et al.*, 1963; *Krumbein and Jens*, 1981; *Potter and Rossman*, 1979; *Hooke et al.*, 1969; *Linck*, 1901; *Bao et al.*, 2001; *Thiagarajan and Lee*, 2004]. Due to its distinct chemical composition from that of the substrate rock, varnish is usually considered to be of sedimentary origin [*Hooke et al.*, 1969; *Perry and Adams*, 1978]. The question remains as to whether processes concentrating Mn and Fe in varnish deposits are microbially or inorganically mediated. Evidence of biogenicity from the literature includes the detection of amino acids in varnish including β-alanine and γ-amino butyric acid, which are formed by enzymatic carboxylation, thereby indicating possible organismal activity [*Perry et al.*, 2003].

[4] There is considerable evidence suggesting that microbes can directly or indirectly control Mn precipitation [*Nealson*, 1983; *Tebo et al.*, 2004] and biomineralization of manganese has been suggested in a variety of environments including, marine nodules [cf. *Ehrlich*, 2000], hot springs [*Chafetz et al.*, 1998; *Ferris et al.*, 1987; *Mita et al.*, 1994], freshwater sediments [*Maki et al.*, 1987], soils [*Ghiorse*, 1988; *Stiles et al.*, 2001], and caves [*Spilde et al.*, 2005]. Evidence for a biological origin for rock varnishes has been mounting based on microscopic and culture-based results suggesting that bacteria are intimately associated with varnish coatings [*Dorn and Oberlander*, 1981; *Jones*, 1991; *Hungate et al.*, 1987; *Krumbein and Jens*, 1981; *Palmer et al.*, 1986; *Perry et al.*, 2003; *Raymond et al.*, 1992; *Taylor-George et al.*, 1983]. However, it can be argued that we still do not have sufficient evidence to state a biogenic origin of varnish emphatically.

[5] It is widely accepted that only ~1% of microbes are cultured in the laboratory using standard techniques [*Hugenholtz et al.*, 1998]. With the advent of culture-independent detection techniques, particularly rRNA-based techniques, we are able to characterize microbial communities more fully, revealing significantly broader diversity than previously recognized. Nevertheless, to date our knowledge of bacterial communities associated with rock varnish is due mostly to culture-based studies, with the exception of *Kuhlman et al.* [2006, 2008] and *Schelble et al.* [2005]. While molecular methods are valuable tools in characterizing microbial communities, simply demonstrating the presence of an organism is not enough to prove involvement in the process of interest; we must correlate presence with function.

[6] The purpose of this research was to characterize the bacterial communities associated with rock varnish from a semiarid environment in New Mexico using 16S rDNA clone library analysis, to compare these communities to those found on rock bare of macroscopically visible varnish, and to identify members of these communities able to precipitate Mn oxides based on enrichment culture studies. These results represent some of the first published molecular studies of rock varnish microbial communities. In addition, we utilize scanning electron microscopy to relate the organisms present to their chemical and mineral products.

2. Site Description

[7] Samples were obtained from a rock varnish site located several kilometers southwest of Socorro in central New Mexico, USA, which is characterized as semiarid Chihuahuan desert, which receives approximately 245 mm of precipitation annually. The study site is on the south slope of Black Canyon, an east–west canyon that crosscuts part of the Socorro Cauldron, a dissected volcanic caldera, where volcanic activity has been dated from 33 MA to around 7 MA [*Eggleston et al.*, 1983]. The rocks in the area consist of hydrothermally altered rhyolite, and at the Luis Lopez mining district, just to the east of the sample site, a manganese mining operation exploited crytomelane and hollandite from hydrothermal veins in the volcanic rock through World War II. Black rock varnish is common on the reddish brown rocks throughout the area. This site was chosen for two key characteristics. First, unlike the thick, layered varnish of places such as the Mojave Desert in California [cf. *Broecker and Liu*, 2001], the varnish here is discontinuous, often thin, and concentrated into micropits and pockets in the rock surface. This may help preserve primary structures and microbial remains that are destroyed in harsher, more arid environments. Second, this site is unique because the region contains elevated manganese levels that may provide a more substantial source of manganese than most other rock varnish sites, although rock varnish composition seems to be independent of the underlying rock composition [*Potter and Rossman*, 1977, 1979; *Dorn and Oberlander*, 1981]. Manganese leached from the surrounding rock by weakly acidic rainwater may provide a source of Mn(II). With elevated manganese levels available in the local country rock, the rock varnish may grow more actively than other areas.

3. Methods

3.1. Sampling

[8] A total of five sites were sampled at Black Canyon, three containing rock varnish and two with no macroscopically visible varnish. Varnish sample sites 1 and 2 were collected from a dry, south facing ledge with scattered patches of black rock varnish, and were considered together as one sample (site 1-2) for molecular analyses. Site 3 was a bare rock surface several meters from site 1-2, but on the same rock outcrop. Site 4, approximately 10 m uphill from the first three sites, was also a bare rock surface, a road cut blasted approximately 60 years ago. Sites 3 and 4 were "bare" rock surfaces with no macroscopically visible varnish that were to serve as control samples for the varnish sam-

ples. Site 5 possesses a large amount of black varnish due to its location in an ephemeral watercourse on a vertical rock face that drains several hundred square meters of rock and scattered soil above it during rain and snowmelt events. Several small rock chips were aseptically cleaved from the surface of each sample site for culturing and DNA analysis. After the aseptic sampling, additional fragments were cleaved, nonaseptically, for scanning electron microscope (SEM) study and for processing into polished mounts for electron microprobe (EMP) analysis. Larger hand samples were collected from the immediate outcrop area around site 1-2 for bulk chemical and X-ray diffraction analysis.

[9] Samples of rainwater and standing water were taken from the Black Canyon site during and after a rainstorm to test for dissolved metals (e.g., Mn, Fe and Ni) in the water that may be the source for metals in the rock varnish, particularly reduced metals in solution that could be taken up by metal oxidizing bacteria. A sample of dripping water was collected from the ephemeral watercourse at site 5. The standing water sample was collected from a small pothole in the rock surface above the watercourse. Additional rainwater samples and dry deposition samples were analyzed from the nearest Sevilleta LTER site, Deep Well Met Station 40, approximately 51.5 km to the northeast. Samples of rainwater and dust from this site are part of an archive maintained by the Sevilleta LTER.

3.2. Manganese Enrichment Cultures

[10] To ascertain which groups of organisms are capable of oxidizing reduced manganese, enrichment cultures were initiated using aseptically collected chips of the desert varnish from the Black Canyon site. Samples were inoculated in the field and transported to the laboratory for incubation. Approximately 45 different cultures that showed initial growth were inoculated into replicate series of manganese complex challenge media developed previously [*Spilde et al.*, 2005]. Based on our extensive experience with these types of rock-inhabiting communities of organisms, we have observed that the ability to oxidize metals is frequently a consortium property rather than the capability of an individual strain [*Boston et al.*, 2009]. Because of this, we work to develop a minimum microbial consortium (MMC), which represents what appear to be the organisms that are functioning together to produce the oxides and subsequent crystallization that we observe in the cultures. Attempts to isolate individual strains in pure culture often do not result in mineral precipitation that matches the minerals we observe in nature [*Spilde et al.*, 2005]. In addition, these consortia tend to be very slow growing, particularly when we challenge them with media containing no or low organic carbon. Even when we provide moderate levels of organic carbon, they can be exceedingly slow to produce visible growth and oxides and subsequent crystallization can take months or years to appear. Because of this, we have developed simple methods of keeping the growth media hydrated using parafilm sealing of Petri plates or tubes and we incubate in closed growth chambers where adequate humidity levels can be maintained.

[11] The media used in this study were enriched with 0.1 mmol concentrations of one of four forms of reduced manganese ($MnCO_3$, Mn_2O_3, $Mn(NO_3)_2 \cdot 4H_2O$, and $MnCl_2 \cdot 4H_2O$). A fifth medium contained 0.1 mmol concentration of both $MnCO_3$ and $FeCO_3$. Each of these media was prepared in two forms: (1) without any organic carbon or (2) with low concentrations (0.1% w/v) of both acetate and glucose (Figure 1). Identical replicates of each culture variant are inoculated to provide the ability to conduct killed controls at periodic intervals, using 2.5% glutaraldehyde solution to flood the cultures, in order to follow the process of mineralization. Uninoculated media, serving as negative controls, were incubated under the same conditions for the same period of time. All cultures were incubated at 25°C for up to 2.5 years and were subcultured further onto media described above as colonies and mineral precipitates of interest occurred (Figure 1).

3.3. Molecular Phylogeny

3.3.1. Extraction of Nucleic Acids

[12] Genomic DNA was extracted from enrichment cultures using the method described by *Marmur* [1961]. DNA was purified using the UltraClean™ Microbial DNA Isolation Kit (MoBio Laboratories, Inc.) reagents and protocol. Genomic DNA was extracted from rock chips with varnish stored in sucrose lysis buffer using the Power Soil DNA Extraction kit (MoBio Laboratories, Inc.).

3.3.2. The 16S rDNA PCR Amplification and Clone Library Construction

[13] The 16S rRNA gene was amplified from both enrichment culture and environmental DNA by PCR with universal primers, p46 forward (5′-GCYTAAYACATGCAAGTCG-3′) and p1409 reverse (5′-GTGACGGGCRGTGTGTRCAA-3′, provided by C. Takacs-Vesbach, unpublished data, 2004) and AmpliTaq LD (Applied Biosystems) with an MJ thermal cycler as follows: 4 min denaturation at 94°C, followed by 35 cycles of 45 s annealing at 55°C, 2 min at 72°C (extension), and 30 s at 94°C (denaturation), with a final 45 s 55°C annealing and 20 min 72°C extension step after cycling was complete. Amplification products were cloned using the TOPO TA Cloning kit (Invitrogen), and plated on LB/ampicillin agar plates [*Sambrook et al.*, 1989].

3.3.3. RFLP

[14] To assist in determining which clones to sequence, the 16S ribosomal DNA of 173 (site 1-2) and 234 (site 5) varnish clones and 138 (site 3) and 140 (site 4) nonvarnish clones was digested with enzymes to produce restriction fragment length polymorphisms (RFLPs). The restriction enzymes *HhaI* and *RsaI* were used in double digestions of the whole cell PCR amplicons of clone DNA as follows: 8 μl of PCR amplicon DNA, 1 μl of NE React Buffer 4, 0.4 μl of double distilled water, and 0.2 μl of *HhaI* and 0.4 μl of *RsaI* enzymes. RFLP patterns were visualized using a 4% Metaphor (FMC Rockland, Maine) electrophoresis gel in TAE, stained with 2 μl of ethidium bromide, visualized on a UV transilluminator, and compared visually by the authors.

3.3.4. The 16S rDNA Sequencing

[15] Representative clones of each RFLP pattern were grown overnight in LB broth containing 100 μg/ml ampicillin and were purified with a QIAprep plasmid miniprep kit (Qiagen Inc., Chatsworth, Calif.). 125–300 ng of purified DNA was used as a template in cycle sequencing reactions with the ABI PRISM dye terminator cycle sequencing kit (Perkin-Elmer-Applied Biosystems). Primers used for sequencing were T7, T3 and the internal primers (533F, 907R, and 765F) of the 16S rRNA gene. Some sequencing

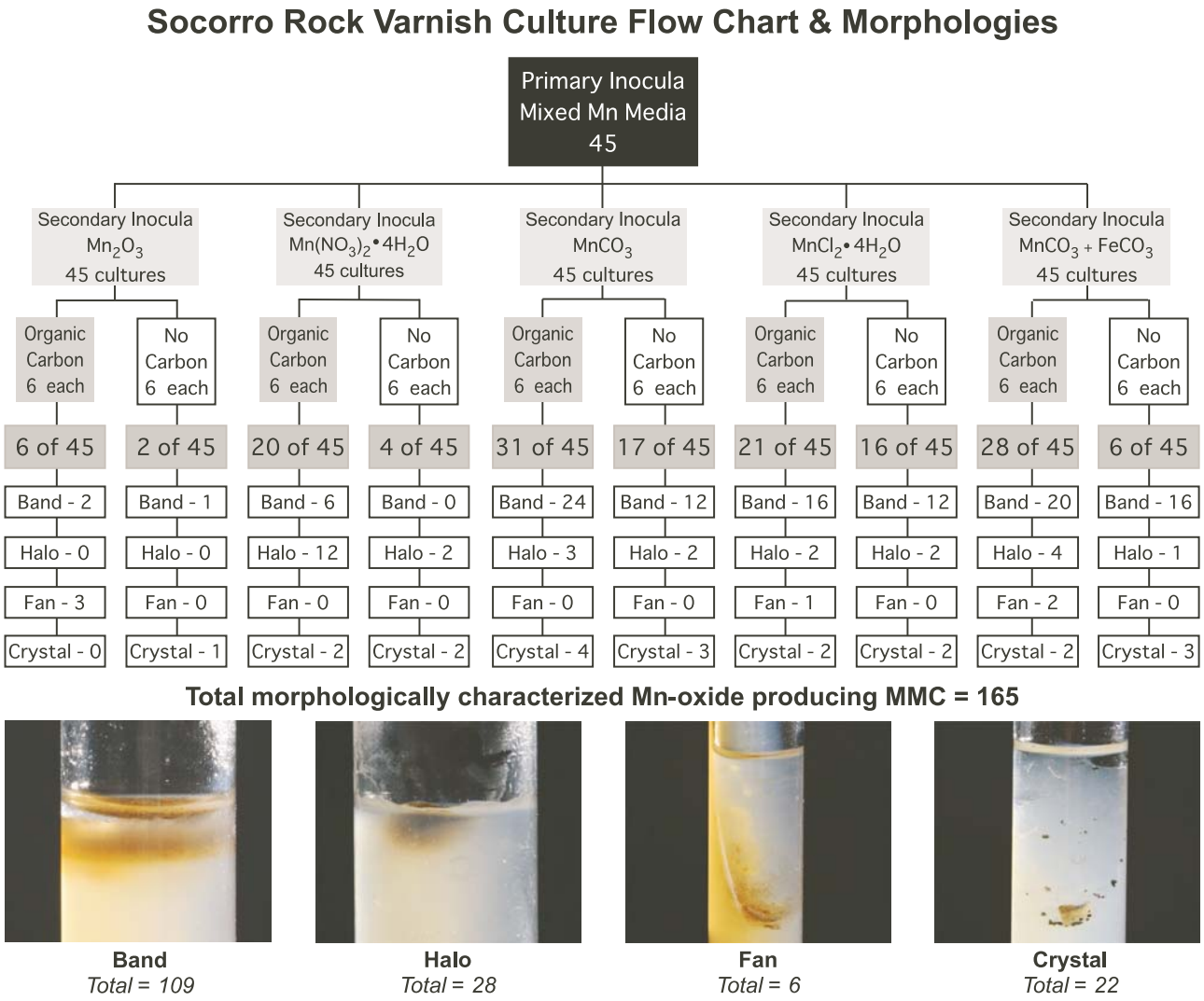

Figure 1. Logical schematic of the culturing experiments. An initial 45 cultures directly isolated from the varnish sites were subcultured in five different media types and each of those were subcultured in organic carbon and no-carbon versions. Six replicates of each medium were inoculated from the 45 primary inocula to enable the potential to serially kill cultures at different development points to follow the sequence of mineral crystallization. The banding morphology was the most numerous macroscopic growth and Mn concentrating indicator. Most of the bands grew between 1 mm and 3 cm below the agar surface, indicating varying degrees of microaerophilic preference. The halo and fan morphologies are centered on the agar surface, implying a preference for higher oxygen partial pressures.

was done through the Washington University Sequencing Facility in St. Louis, Missouri, while the bulk of the sequencing was done at the University of New Mexico Molecular Biology Facility.

3.3.5. Phylogenetic Analysis

[16] Each sequence was submitted to the CHIMERA_CHECK program of the Ribosomal Database Project (RDP) [*Maidak et al.*, 2001] (http://rdp.cme.msu.edu/) or to the Mallard program (http://www.bioinformatics-toolkit.org/Mallard/index.html [*Ashelford et al.*, 2006]) to detect the presence of possible chimeric artifacts. All sequences were analyzed using BLAST (NCBI [*Altschul et al.*, 1997]) and SIMILARITY_RANK (RDP [*Maidak et al.*, 2001]) to determine the taxonomic groupings of clone sequences. Sequences were submitted to GenBank and assigned the accession numbers FJ595524–FJ595655. Each major taxonomic sequence group (e.g., *Proteobacteria*, *Acidobacteria*, etc.) was submitted to the Greengenes alignment tool to create an initial alignment, including the nearest-neighbor database sequences for each clone (http://greengenes.lbl.gov/cgi-bin/nph-index.cgi [*DeSantis et al.*, 2006a, 2006b]). Out-group sequences (*Aquifex aeolicus* AE000657, *Thermotoga maritime* AE000512, and *Thermus aquaticus* L09663) were added to each set of sequences, and the data sets were realigned using MAFFT version 6.611 [*Katoh and Toh*, 2008; *Katoh et al.*, 2002, 2005]. Ambiguous regions of each alignment were removed using GBlocks version 0.91 [*Castresana*, 2000; *Talavera and Castresana*, 2007]. A maximum likelihood phylogeny with branch support from 100 bootstrap replicates was constructed for each data set

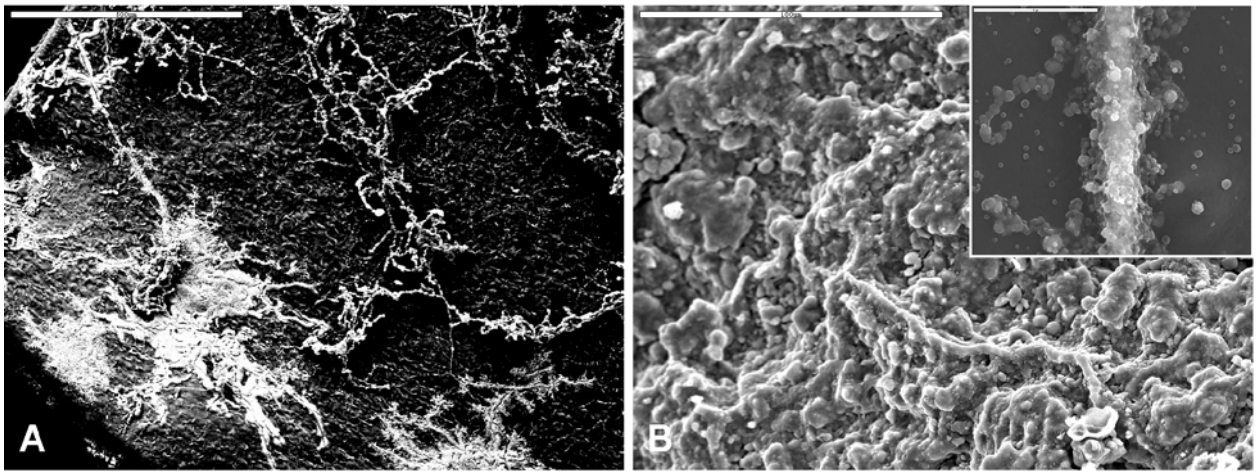

Figure 2. Electron micrographs of cultures. (a) Backscattered electron (BSE) image of surface of bacterial culture plate showing deposition of Mn oxides by bacteria (lighter areas). Scale bar is 500 μm. (b) Mn-coated tube-like structure on the surface of smooth varnish from site 2. Scale bar is 100 μm. Inset compares a similar structure from a bacterial culture taken from the same sample site. Scale bar is 1 μm.

(PhyML version 3.0 webserver [*Guindon and Gascuel*, 2003; *Guindon et al.*, 2005]) using the best fit model as determined by Modeltest version 3.7 [*Posada and Crandall*, 1998; *Posada and Buckley*, 2004]. In order to perform rarefaction analysis, a data set was constructed by taking the representative sequence from each RFLP pattern and duplicating that sequence in the data set to represent the number of clones found in that clone group. Using this data set, a distance matrix was constructed with the PHYLIP version 3.6 DNADIST module under the F84 model of evolution [*Felsenstein*, 1989], which was used to generate rarefaction curves at the 95% similarity level using DOTUR [*Schloss and Handelsman*, 2005]. The similarity between each clone library was calculated using S-Libshuff (http://www.plantpath.wisc.edu/joh/s-libshuff.html [*Schloss et al.*, 2004; *Singleton et al.*, 2001]) and SONS [*Schloss and Handelsman*, 2006]. Richness and diversity indices were computed using EstimateS (version 8.0, Colwell, http://viceroy.eeb.uconn.edu/estimates).

3.4. Microscopy

[17] In addition to bulk analytical techniques, samples were examined on a JEOL 5800 SEM equipped with an energy dispersive X-ray analyzer (EDX). Rock chips from the varnish and bare rock sites were mounted directly on SEM sample stubs and coated with Au-Pd metal for imaging. Small sections of cultures were examined with SEM/EDX to determine the presence of precipitated manganese. Several additional chips with surface varnish were embedded in epoxy, sectioned, and polished for cross-sectional imaging on a JEOL 8200 electron microprobe equipped with a backscattered electron (BSE) detector.

3.5. Chemistry and X-ray Diffraction

[18] Varnish was removed from the surface of the larger pieces using a reciprocating tungsten carbide-coated wire saw to remove as much varnish as possible in order to analyze the varnish separately from the underlying host rock. Major and trace elements were analyzed by means of atomic absorption spectroscopy on samples digested in HF and HNO_3. Mineralogical composition was determined on powdered samples prior to digestion by X-ray diffraction (XRD) using a Scintag Pad V diffractometer.

[19] A varnish sample from site 1 was analyzed using synchrotron-based X-ray diffraction (SR-XRD) at beam line 10.3.2 at the Advanced Light Source synchrotron at the Lawrence Berkeley National Laboratory (Berkeley, CA). X-ray energy was set to 17 KeV using a crystal monochromator. The crushed sample, mounted on Kapton tape, was exposed to a 5 by 5 μm spot for 300 s acquisition time.

[20] Black Canyon rainwater and pothole water samples were analyzed by atomic absorption (AA) spectroscopy and by inductively coupled plasma atomic emission spectrometry (ICP/AES). Sevilleta (see location information above) rainwater and dry deposition samples were analyzed for metals and trace elements by ion chromatography using a Dionex Ion Chromatograph DX-100, and by ICP/AES.

4. Results

4.1. Culturing

[21] Many of the minimum microbial consortia (MMC) that we isolated from environmental samples produced amorphous Mn oxides and eventually Mn crystalline minerals (Figure 2) when that element was provided in media (Figure 1). Of the 165 total MMC obtained on replicate media series, about 20% (33) produced black or dark brown bands, halos or fans in solid media within the first several months (Figure 1). An additional 45% (76) of the MMC produced similarly black or dark brown deposits within the first 18 months of incubation, albeit some at very low productivities. A remaining 56 cultures produced at least limited Mn morphologies over the course of an additional year (2.5 years total incubation time). Uninoculated media, serving as negative controls, produced no mineral precipitates, although 2 of a total of 20 negative controls (2 per 10 medium variants) did darken over time. Both of these were organic carbon containing media variants, $MnCO_3$ and $MnCO_3$/

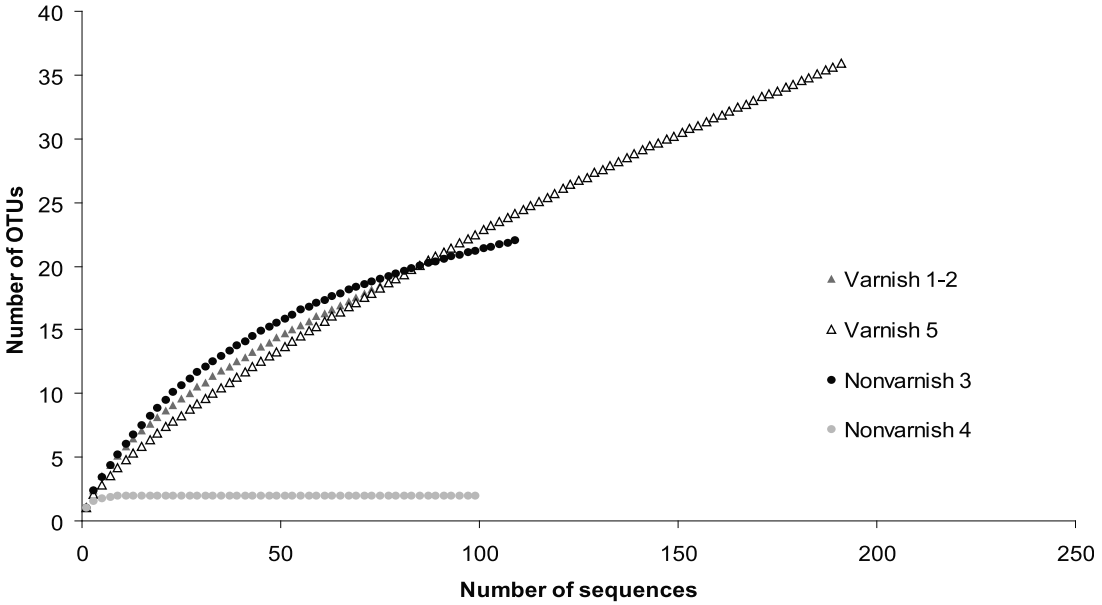

Figure 3. Varnish and nonvarnish sample rarefaction curves.

$FeCO_3$. Electron microscopy of cultures revealed that mineralogies ranged from amorphous oxides through various crystalline phases (described below). Concentrations of Mn were confirmed with EDS of harvested material from one replicate from each original culture type. Some of the crystalline morphologies resemble crumpled tissue paper-like and star-like shapes that we have seen in other environments in prior work [*Spilde et al.*, 2005, 2009; *Boston et al.*, 2001].

4.2. Molecular Phylogeny

[22] Rarefaction curves show that the nonvarnish clone libraries approached saturation, particularly for site 4, while the varnish sites are still accumulating diversity (Figure 3). Although the clone libraries at each site represent significantly different communities ($p < 0.0001$), nine of the OTUs at the 95% similarity level were found at more than one of the different sampling sites. Seven OTUs were found in both varnish and nonvarnish sites, while two other OTUs were found at both of the nonvarnish sites. Interestingly, no similar OTUs were found in both of the varnish sites.

[23] Overall, our clone library from Black Canyon varnish site 1-2 was substantially different from varnish site 5's clone library. *Chloroflexi* and *Ktedobacteria* (unclassified) were numerous in the site 1-2 clone library, while the clone library from site 5 had many *Cyanobacteria* clones. All three sites contained *Actinobacteria* and *Alphaproteobacteria*, while the site 5 clone library lacked *Betaproteobacteria*, but contained a small number of *Gemmatimonadetes* and *Bacteroidetes* clones. Nonvarnish site clone libraries contained numerous *Actinobacteria* clones, and to a lesser extent, *Cyanobacteria*. Nonvarnish sites also were the only samples to contain *Deinococcus-Thermus* sequences (Table 1).

[24] Clones whose closest relatives were from desert or other soil environments were numerous in site 1-2 (varnish), while the site 5 (varnish) clone library contained many clones whose closest relatives were from other rock varnish environments and clean room studies (Figure 4). Interestingly, the clone library for site 1-2 (varnish) also contained a greater number of closest relatives from basalt environments than did site 5's (varnish) clone library. Some closest relatives were from studies of aerosols, especially in the site 5 (varnish) clone library. Sites 3 and 4 (nonvarnish) clones exhibited a much greater portion of desert soil closest relatives than did the varnish sites and showed a greater diversity in the habitats of closest relatives.

[25] The phylogenetic analyses show an intermixing of nonvarnish and varnish clones in most phyla (Figures 5, 6, and 7 and Table 1). The exceptions to this pattern include

Table 1. Distribution of Clones Across Sample Sites by Phylum

Phylum	Nonvarnish Site 3	Nonvarnish Site 4	Varnish Site 1-2	Varnish Site 5	Cultured Phylotypes
Actinobacteria	11	22	3	3	3
Gemmatimonadetes	2				2
Deinococcus-Thermus		4			
Cyanobacteria	8	2	4	27	
CFB	2	1		4	4
Ktedobacter (unclassified)	1		14		
Chloroflexi	2		2	2	
Firmicutes	1				1
Alphaproteobacteria	6	7	3	7	6
Betaproteobacteria, *Gammaproteobacteria*	2				11
Deltaproteobacteria	2				

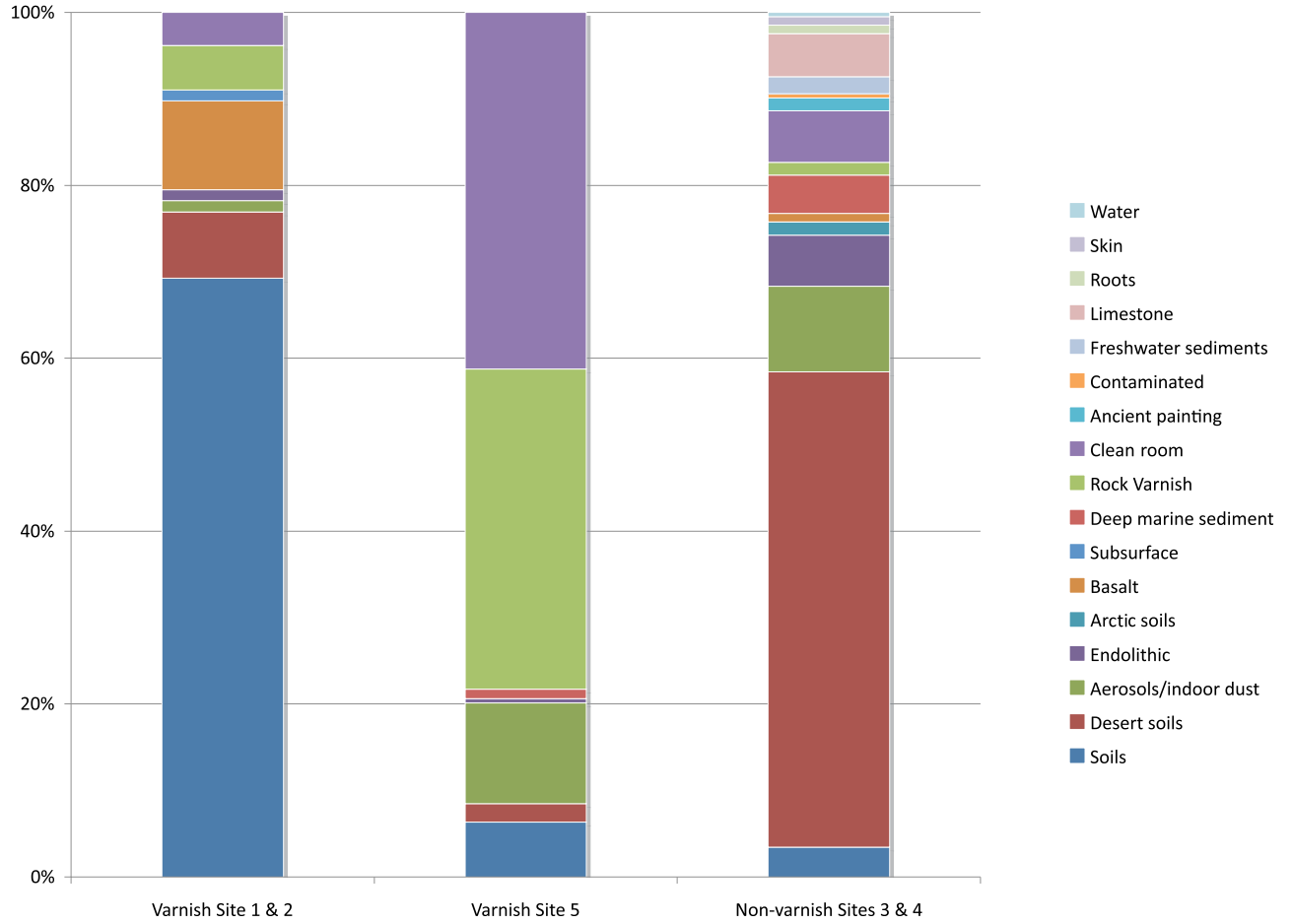

Figure 4. Black Canyon Desert varnish and nonvarnish clones by habitat of closest relative. A qualitative analysis of Black Canyon clones by habitat.

the *Deinococcus-Thermus*, which only included nonvarnish clones and the *Acidobacteria*, which only included varnish clones. Our analyses show that a new family level group that clusters with the *Ktedobacter* has been detected in the rock varnish environmental clones (Figure 5).

[26] Cyanobacterial clones group with some cultured relatives in the genera *Anabaena*, *Nostoc*, and *Chroococcidiopsis*, while many of the clone sequences appear to be novel, both within the *Cyanobacteria* and the *Chloroflexi*. Some of the varnish and nonvarnish clones in the *Chloroflexi* and *Cyanobacteria* group with sequences from a study of the Atacama Desert varnish [*Kuhlman et al.*, 2008].

[27] Within the *Alphaproteobacteria*, clone sequences group with the genera *Methylobacterium*, *Sphingomonas*, *Afipia*, and *Rhizobium*, while those in the *Betaproteobacteria* group with *Janthinobacterium* and *Achromobacter* (Figure 6).

[28] Cultured representatives are found in the *Cytophaga-Flexibacter-Bacteroides* (CFB), *Proteobacteria*, *Actinobacteria*, and *Gemmatimonadetes* (Figures 6 and 7).

[29] Clones from varnish, nonvarnish, and cultures were generally not closely related to known manganese oxidizers [*Tebo et al.*, 2004], such as *Pseudomonas* spp., *Leptothrix discophora*, *Caldimonas manganoxidans*, *Pedomicrobium manganicum*, *Aurantimonas manganoxydans*, *Erythrobacter* sp., *Bacillus* spp., and *Arthrobacter* spp. (Table 2). Identity values of our clones to known manganese oxidizers generally fell between 82% and 93%, with some values as low as

Figure 5. Maximum likelihood tree with branch support from 100 bootstrap replicates of varnish and nonvarnish sequences with their closest relatives from the *Cyanobacteria*, *Chloroflexi*, *Cytophaga-Flexibacter-Bacteroides* (CFB), *Acidobacteria*, *Firmicutes*, and unclassified divisions. Each RFLP clone group is colored by the site where it was found: red, varnish site 1-2; orange, varnish site 5; light blue, nonvarnish site 3; dark blue, nonvarnish site 4. Sequences in purple, containing the MN designation, were isolated from manganese cultures. Clades containing mainly sequences from this study have been collapsed, and the representative triangle is colored to represent the proportion of groups from each site. Colored numbers next to each collapsed clade represent the number of RFLP clone groups from each site. The total number of clones represented by the RFLP groups from each site is given in parentheses.

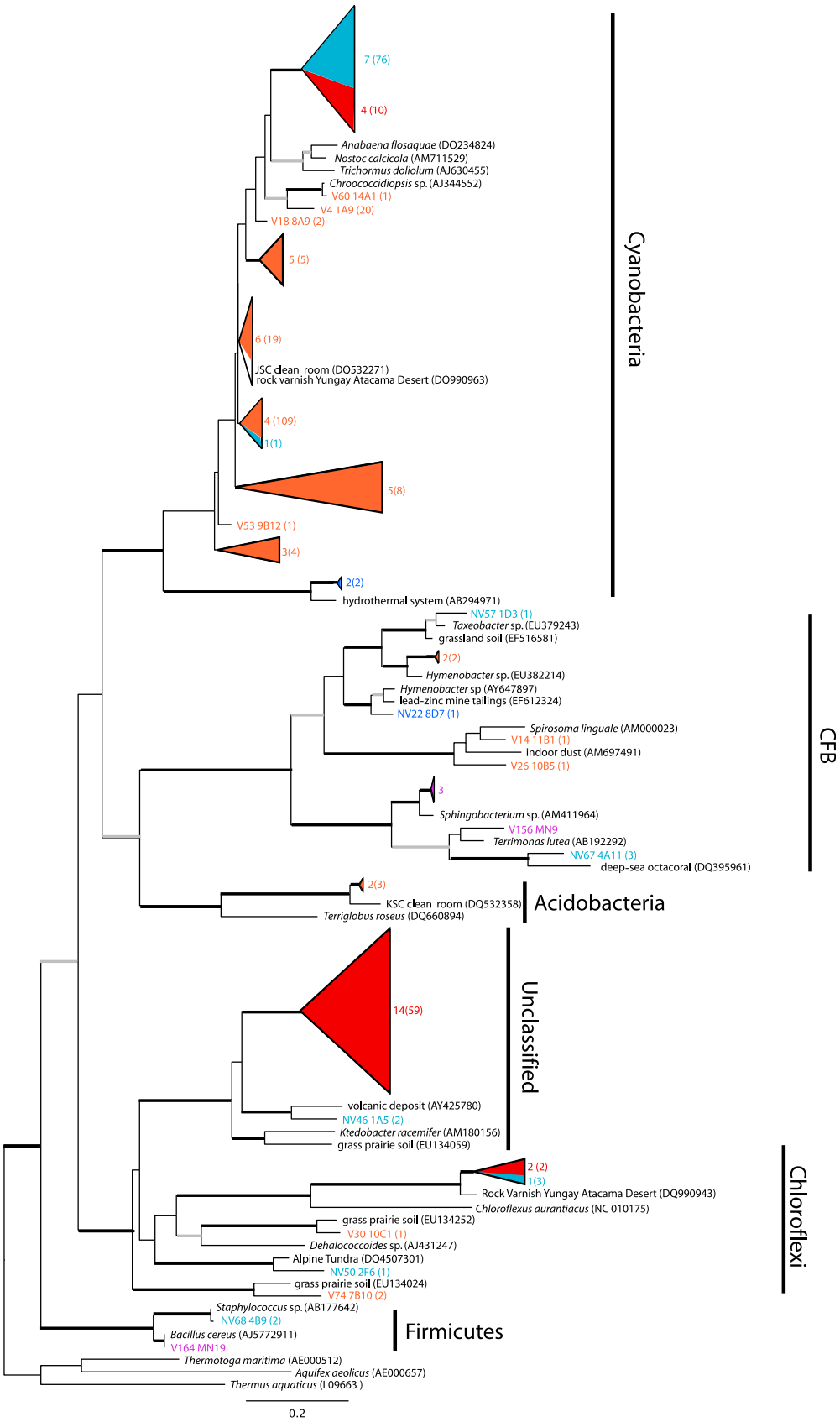
7 (76)
4 (10)
Anabaena flosaquae (DQ234824)
Nostoc calcicola (AM711529)
Trichormus doliolum (AJ630455)
Chroococcidiopsis sp. (AJ344552)
V60 14A1 (1)
V4 1A9 (20)
V18 8A9 (2)
5 (5)
6 (19)
JSC clean room (DQ532271)
rock varnish Yungay Atacama Desert (DQ990963)
4 (109)
1(1)
5(8)
V53 9B12 (1)
3(4)
2(2)
hydrothermal system (AB294971)
Cyanobacteria
NV57 1D3 (1)
Taxeobacter sp. (EU379243)
grassland soil (EF516581)
2(2)
Hymenobacter sp. (EU382214)
Hymenobacter sp (AY647897)
lead-zinc mine tailings (EF612324)
NV22 8D7 (1)
Spirosoma linguale (AM000023)
V14 11B1 (1)
indoor dust (AM697491)
V26 10B5 (1)
3
Sphingobacterium sp. (AM411964)
V156 MN9
Terrimonas lutea (AB192292)
NV67 4A11 (3)
deep-sea octacoral (DQ395961)
CFB
2(3)
KSC clean room (DQ532358)
Terriglobus roseus (DQ660894)
Acidobacteria
14(59)
volcanic deposit (AY425780)
NV46 1A5 (2)
Ktedobacter racemifer (AM180156)
grass prairie soil (EU134059)
Unclassified
2 (2)
1(3)
Rock Varnish Yungay Atacama Desert (DQ990943)
Chloroflexus aurantiacus (NC 010175)
grass prairie soil (EU134252)
V30 10C1 (1)
Dehalococcoides sp. (AJ431247)
Alpine Tundra (DQ4507301)
NV50 2F6 (1)
grass prairie soil (EU134024)
V74 7B10 (2)
Chloroflexi
Staphylococcus sp. (AB177642)
NV68 4B9 (2)
Bacillus cereus (AJ5772911)
V164 MN19
Firmicutes
Thermotoga maritima (AE000512)
Aquifex aeolicus (AE000657)
Thermus aquaticus (L09663)
0.2

Figure 5

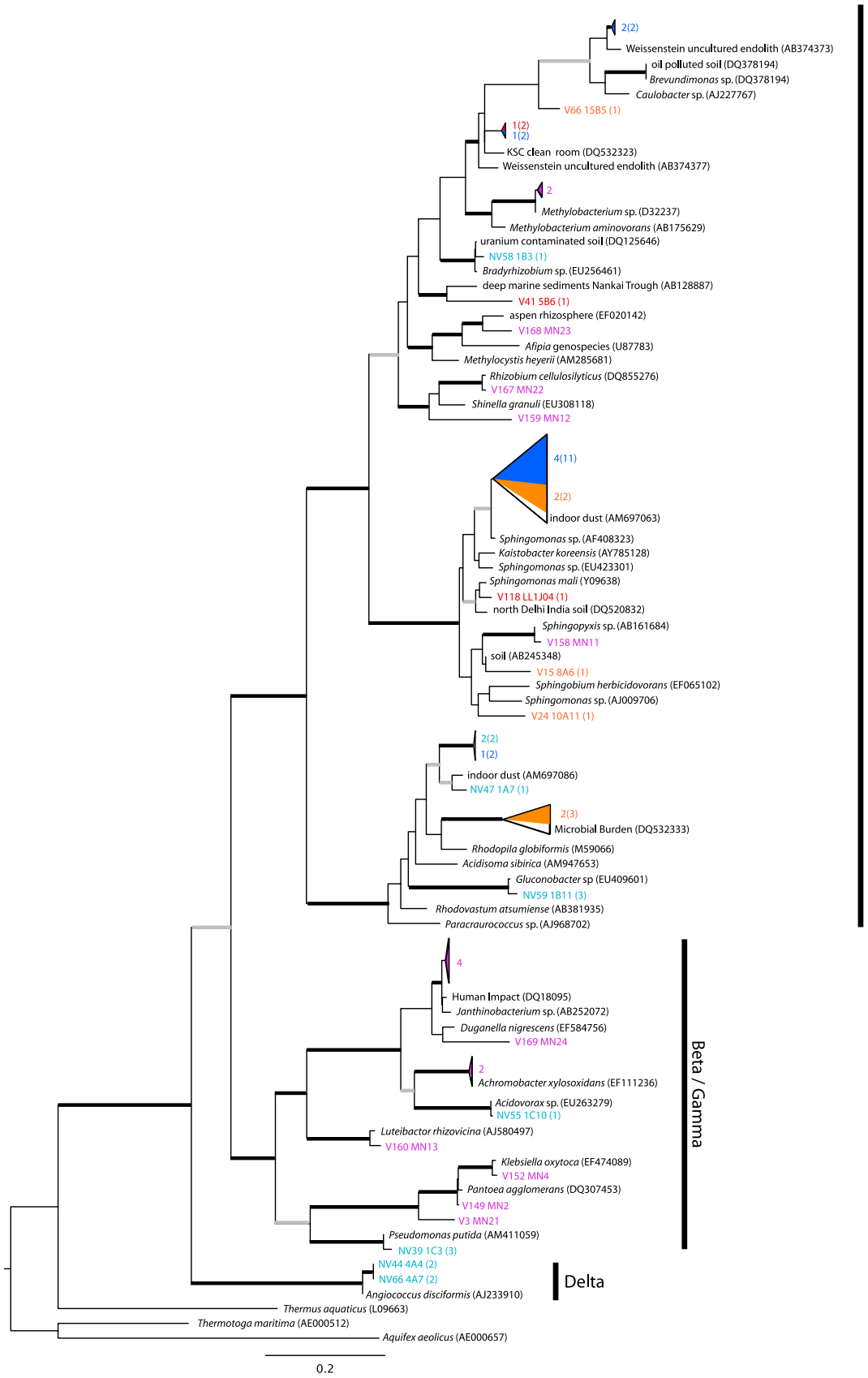
2(2)
Weissenstein uncultured endolith (AB374373)
oil polluted soil (DQ378194)
Brevundimonas sp. (DQ378194)
Caulobacter sp. (AJ227767)
V66 15B5 (1)
1(2)
1(2)
KSC clean room (DQ532323)
Weissenstein uncultured endolith (AB374377)
2
Methylobacterium sp. (D32237)
Methylobacterium aminovorans (AB175629)
uranium contaminated soil (DQ125646)
NV58 1B3 (1)
Bradyrhizobium sp. (EU256461)
deep marine sediments Nankai Trough (AB128887)
V41 5B6 (1)
aspen rhizosphere (EF020142)
V168 MN23
Afipia genospecies (U87783)
Methylocystis heyerii (AM285681)
Rhizobium cellulosilyticus (DQ855276)
V167 MN22
Shinella granuli (EU308118)
V159 MN12
4(11)
2(2)
indoor dust (AM697063)
Sphingomonas sp. (AF408323)
Kaistobacter koreensis (AY785128)
Sphingomonas sp. (EU423301)
Sphingomonas mali (Y09638)
V118 LL1J04 (1)
north Delhi India soil (DQ520832)
Sphingopyxis sp. (AB161684)
V158 MN11
soil (AB245348)
V15 8A6 (1)
Sphingobium herbicidovorans (EF065102)
Sphingomonas sp. (AJ009706)
V24 10A11 (1)
2(2)
1(2)
indoor dust (AM697086)
NV47 1A7 (1)
2(3)
Microbial Burden (DQ532333)
Rhodopila globiformis (M59066)
Acidisoma sibirica (AM947653)
Gluconobacter sp (EU409601)
NV59 1B11 (3)
Rhodovastum atsumiense (AB381935)
Paracraurococcus sp. (AJ968702)
4
Human Impact (DQ18095)
Janthinobacterium sp. (AB252072)
Duganella nigrescens (EF584756)
V169 MN24
2
Achromobacter xylosoxidans (EF111236)
Acidovorax sp. (EU263279)
NV55 1C10 (1)
Luteibactor rhizovicina (AJ580497)
V160 MN13
Klebsiella oxytoca (EF474089)
V152 MN4
Pantoea agglomerans (DQ307453)
V149 MN2
V3 MN21
Pseudomonas putida (AM411059)
NV39 1C3 (3)
NV44 4A4 (2)
NV66 4A7 (2)
Angiococcus disciformis (AJ233910)
Thermus aquaticus (L09663)
Thermotoga maritima (AE000512)
Aquifex aeolicus (AE000657)
0.2
Alpha
Beta / Gamma
Delta

Figure 6

76%. One nonvarnish clone was more closely related at 99% identity to *Pseudomonas putida*.

4.3. SEM

[30] Rock varnish from Black Canyon is discontinuous in hand samples and is often isolated into surface depressions. The varnish is dark brown to black, although with a more matte texture than that found in other deserts, such as the Mojave, where varnish often displays a vivid sheen. In SEM images, the varnish tends to display two distinct forms: relatively smooth surfaces and patches of botryoidal protrusions or pinnacles. The smooth varnish surfaces exhibit a somewhat lumpy texture at high magnification. X-ray spectra from the surface usually contain peaks for Si, Al, Mn, Fe, O, Ca, K and C, in order of peak intensity. These surfaces are commonly crosscut by tube-like features (Figure 2b), which are significantly enriched in Mn compared to the surrounding varnish.

[31] The second common form of varnish observed on the Black Canyon samples is a mass of botryoidal shapes. These are patches or clumps of protrusions or short pinnacles a few micrometers tall (Figure 8). The patches are distributed mainly into low spots on the surface and are on the order of hundreds of micrometers across but discontinuously scattered across the rock surface. This type of structure is somewhat less common than the smooth surface varnish. The patches have higher concentrations of Mn than the smooth surface varnish, and in X-ray maps, Mn is usually limited to the coverage of the botryoidal patch (Figure 8c) with little Mn outside of that area. Close examination of these botryoidal clusters revealed a large amount of organic and mineral debris that had been collected on the pinnacles (Figure 8b). In addition to random organic debris such as pollen grains, an abundance of fine filaments, possibly dehydrated microbial exopolysaccharides, were seen looping around between the pinnacles. This network of filaments appears to be responsible for trapping the debris associated with the pinnacle structures.

[32] Another common surface form observed in SEM were small, grape-like clusters, usually in pits or holes in the surface (Figure 8a), identified as putative microorganisms. The grape-like clusters are sometimes spatially associated with the varnish but contain no Mn in X-ray spectra.

[33] In cross section, varnish from the Black Canyon sites displays micrometer-scale layering that can be broken into three different types: (1) uniformly layered varnish, representing the smooth surface varnish observed in Figures 9a and 9b; (2) botryoidal varnish, representing the patches of pinnacle varnish seen in Figure 9c; and (3) chaotic layering, which had no counterpart observed on the surface. The smooth varnish, shown in Figure 9a, exhibits laterally continuous, regular layers. The layers are generally uniform in thickness and in backscattered electron (BSE) intensity, indicating constant composition. A few layers appear brighter in the BSE image, indicating a higher atomic number due to increased Mn content. The botryoidal form, shown in cross section in Figure 9c contains micrometer-scale lamellae that are organized into a pinnacle but laterally discontinuous. In many cases, the pinnacles exhibit arcs of lamellae that climb concentrically upward throughout the botryoidal pinnacle. Despite the lateral discontinuities, some bright layers (in BSE images) may be present from pinnacle to pinnacle, and some layers, such as the bright, high manganese layer immediately below the pinnacles in Figure 9b, can be traced across the entire sample. The third type, chaotic layering, was associated with both the layered and botryoidal forms particularly in the lower portions. The chaotic layering is distinguished by disorganized laminae that may form concentric circles of various sizes or nonoriented layering (a wavy texture). In the uniformly layered varnish, there may be a transition to the chaotic form, as in the lower portion of Figure 9a or remain separate and blanket the chaotic region below.

[34] As a form of control sample, we compared rocks with no macroscopically visible varnish (sites 3 and 4) to those with varnish (sites 1–2 and 5). Despite the fact that both samples appeared devoid of varnish to the unaided eye, each had microbial colonies visible in the SEM (Figure 10). The 60 year old blasted surface (site 4) displayed colonies of microorganisms that strongly resemble known microcolonial fungi examples hidden in micropits in the surface. Most of the colonies were less than 20 μm across and contained less than 15 spheres attached to one another to form the colony (Figure 10a). The naturally weathered sample surface (site 3) contained colonies that were larger than those on the younger blasted surface (site 4), but again hidden in pits in the surface. These colonies measured as much as 70 by 40 μm and contained many more attached spheres, indicating that the colonies were probably older and more developed (Figures 10b and 10c). Though not quantitatively measured, the colonies on the natural rock sample (site 3) were more common than those on the blasted rock surface (site 4).

[35] Surrounding some of the colonies on the natural rock sample (site 3), small Mn- and Fe-rich areas were observed in the SEM (Figures 10d, 10e, and 10f). These features, which were not noticeable when the sample was collected, were only a few hundred micrometers in diameter and consist of smooth patches containing as much as 34 wt % MnO_2 and 15 wt % Fe_2O_3. Most of these patches partially or completely surround colonies in pits; the Mn-rich area themselves are not necessarily within pits or depressions, although they may extend into the pits. The exception is a small 100–150 μm patch at the bottom left side of Figure 10d that sits by itself and is not associated with a colony.

Figure 6. Maximum likelihood tree with branch support from 100 bootstrap replicates of varnish and nonvarnish sequences with their closest relatives from the *Proteobacteria*. Each RFLP clone group is colored by the site where it was found: red, varnish site 1-2; orange, varnish site 5; light blue, nonvarnish site 3; dark blue, nonvarnish site 4. Sequences in purple, containing the MN designation, were isolated from manganese cultures. Clades containing mainly sequences from this study have been collapsed, and the representative triangle is colored to represent the proportion of groups from each site. Colored numbers next to each collapsed clade represent the number of RFLP clone groups from each site. The total number of clones represented by the RFLP groups from each site is given in parentheses.

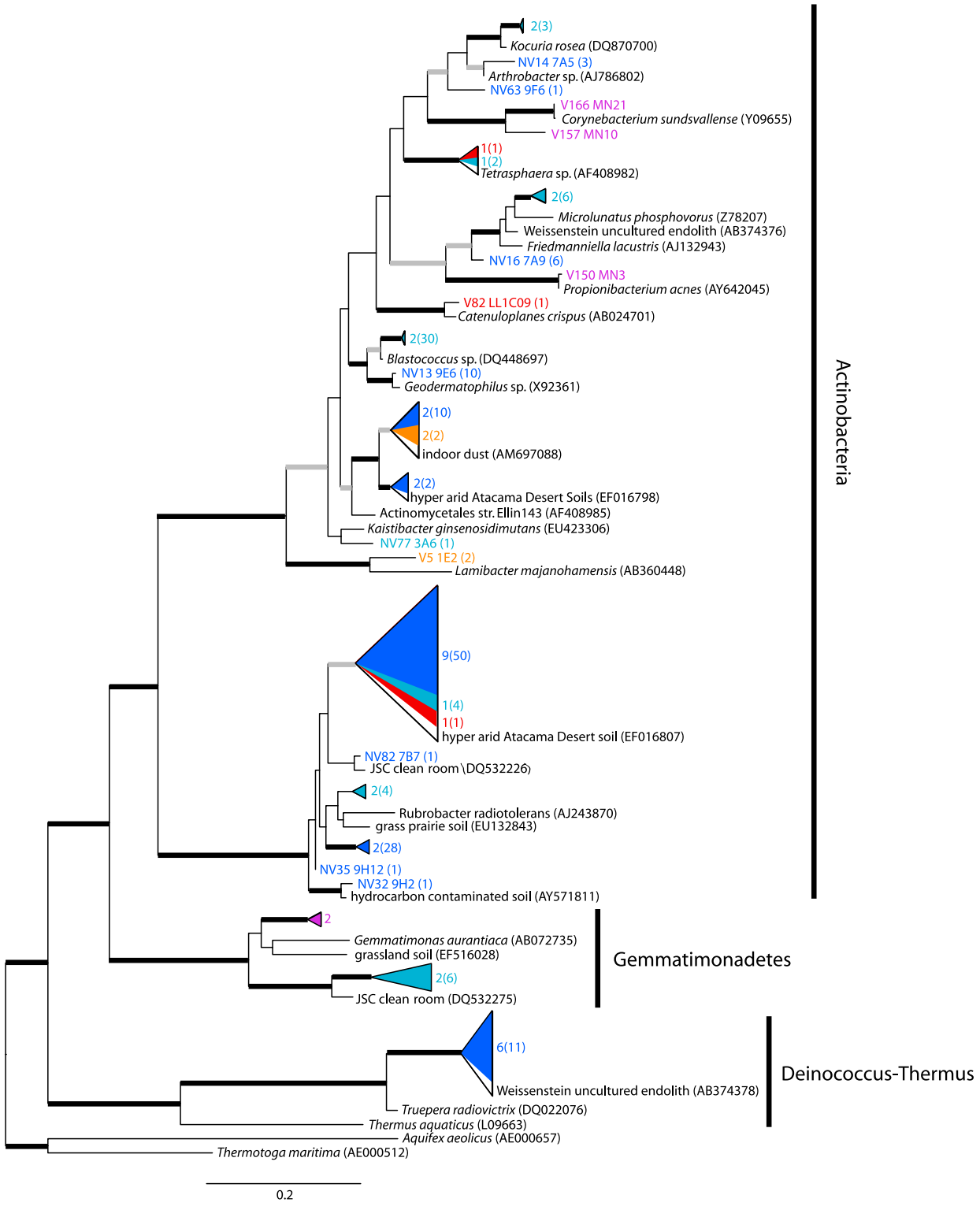

Figure 7. Maximum likelihood tree with branch support from 100 bootstrap replicates of varnish and nonvarnish sequences with their closest relatives from the *Actinobacteria*, *Gemmatimonadetes*, and *Deinococcus-Thermus*. Each RFLP clone group is colored by the site where it was found: red, varnish site 1-2; orange, varnish site 5; light blue, nonvarnish site 3; dark blue, nonvarnish site 4. Sequences in purple, containing the MN designation, were isolated from manganese cultures. Clades containing mainly sequences from this study have been collapsed, and the representative triangle is colored to represent the proportion of groups from each site. Colored numbers next to each collapsed clade represent the number of RFLP clone groups from each site. The total number of clones represented by the RFLP groups from each site is given in parentheses.

Table 2. Pairwise Sequence Similarity Values for Clones Within a Bacterial Group Relative to Known Mn Oxidizing Bacteria[a]

Known Mn Oxidizer	Identity to Varnish Clone (%)	Identity to Nonvarnish Clone (%)	Identity to Cultured Clone (%)
Firmicutes			
Bacillus sp. MB-11 (AF326360)	94	91	
Bacillus sp. PL12 (AF326366)	94	90	
Bacillus sp. PL26 (AF326369)	95	91	
Bacillus sp. SG-1 (AF326373)	95	91	
Alphaproteobacteria			
Aurantimonas manganoxydans (AJ786360)	81–89	82–89	77–91
Erythrobacter sp. SD-21 (AF325445)	79–91	79–92	77–91
Pedomicrobium manganicum (X97691)	80–87	81–87	79–87
Betaproteobacteria, *Gammaproteobacteria*			
Caldimonas manganoxidans (AB008801)		82–93	76–92
Leptothrix discophora SP-6 (L33974)		81–94	76–91
Leptothrix discophora SS-1 (NR_025916)		81–94	76–91
Pseudomonas sp. GP11 (AF326375)		82–96	79–86
Pseudomonas putida (AM411059)		82–99	78–86
Pseudomonas chlororaphis (D84011)		81–96	78–85
Actinobacteria			
Arthrobacter crystallopoietes (X80738)	84–93	87–96	86–88
Arthrobacter globiformis (AB089841)	83–92	87–95	86–89

[a]Mn oxidizing bacteria from *Tebo et al.* [2004]. GenBank accession numbers are given in parentheses following the name.

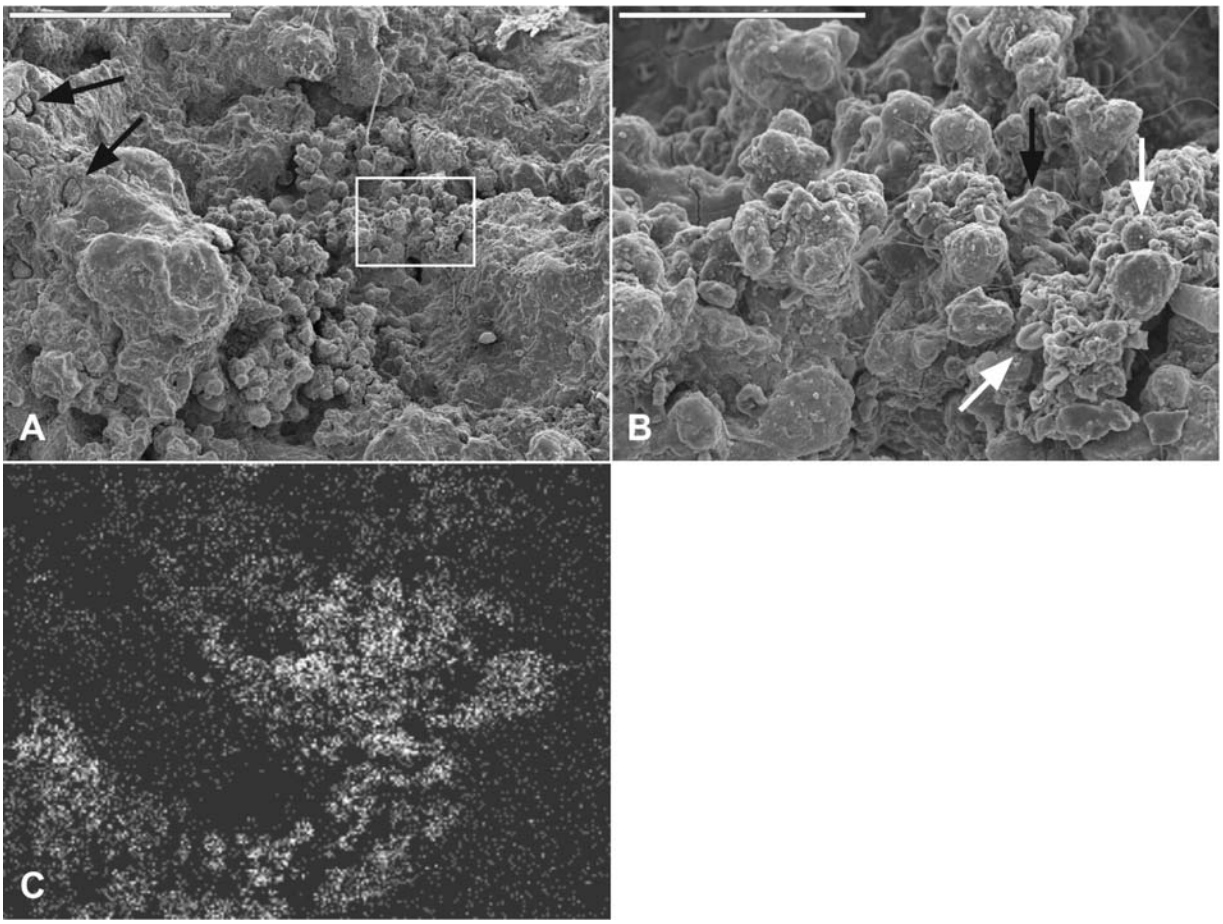

Figure 8. Secondary electron image (SEI) of varnish from Black Canyon site 1. (a) Botryoidal colonies are present in the center portion of the image, and small clusters of putative microorganisms can be seen along the left side of the image (arrows). Scale bar is 500 μm. (b) Close-up of a portion of the botryoidal varnish in Figure 8a (outline area). Note the presence of mineral debris (such as the particle at the black arrow), pollen grains (white arrows), and abundant microbial filaments attached to the surface of the varnish colony. Scale bar is 100 μm. (c) Mn X-ray map of the area in Figure 8a. Note the association of manganese with the botryoidal features but not the small putative microbial clusters.

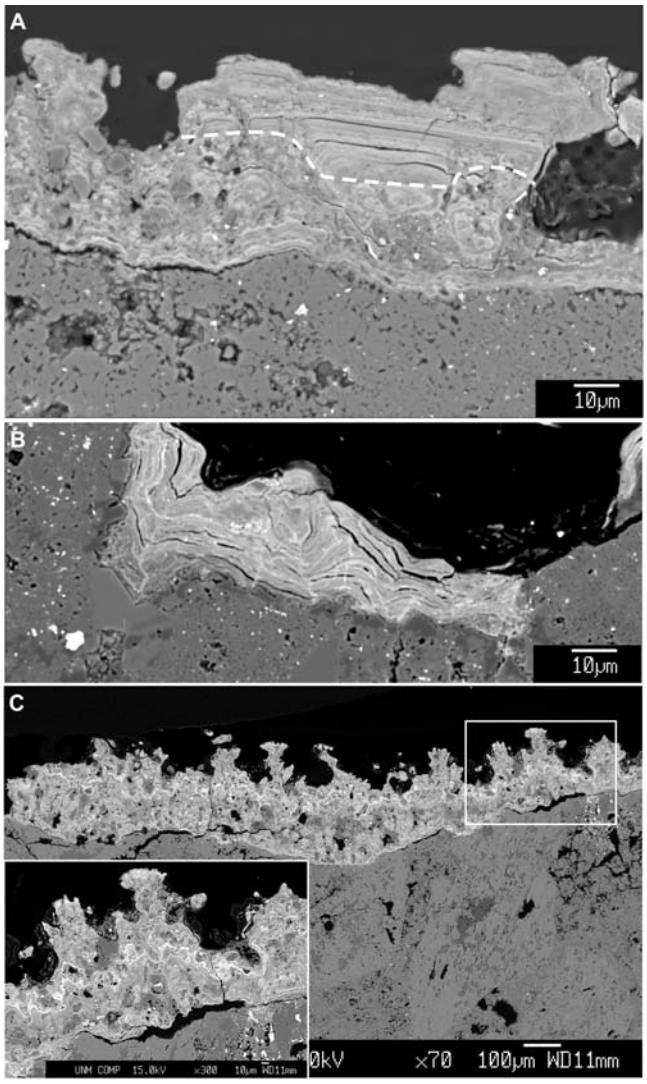

Figure 9. BSE images from polished sections of rock varnish from the Black Canyon sites. (a) Layered varnish from site 2. Chaotic layering is present in the lower portion of the varnish, below the uniformly layered region (dashed line). (b) Layered varnish from site 5. A discontinuous layer of silica is present on the surface of the varnish. (c) Botryoidal varnish from site 2. Inset shows a close-up of several pinnacles in the white outlined box. In all images, the varnish is lighter gray and the substrate rock is darker gray.

4.4. Chemistry and XRD

4.4.1. Rainwater/Standing Water Chemistry

[36] The results indicate that the standing and flowing water contains dissolved Si, K, and other cations and moderate sulfate, nitrate, and chloride (4, 7, and 2 ppm, respectively). The rainwater had low dissolved cations, mainly Ca and detectable levels of Ba (0.3 ppm), but not enough sample from Black Canyon was available for further analysis. Only the drip water contained detectable dissolved metals, specifically Fe (13.5 ppm) and Mn (0.8 ppm). The rainwater and dry deposition samples from the Sevilleta site showed no detectable Fe or Mn.

4.4.2. XRD

[37] XRD results yielded amorphous Mn oxides with a weak peak identified as 7-Angstrom phyllomanganate (possibly birnessite). We were unable to identify any further specific manganese phases.

4.4.3. Synchrotron Analysis

[38] SR-XRD analysis of varnish from site 1 shows the manganese oxide to have a phyllomanganate (layered manganese oxide) basal plane spacing of 7.17 Å and an XRD pattern consistent with hexagonal birnessite. Birnessite consists of MnO_6 sheets or layers with interlayer cations filling the space between the sheets; interlayer cations include Na, K, Ca, Mn(II), and H_2O [*Post*, 1999]. Hexagonal birnessite contains vacancies in the MnO_6 sheet that are charge balanced by substitution of higher-valence cations into the interlayer sites. Monoclinic birnessite, on the other hand, contains MnO_6 layers that are relatively vacancy free.

5. Discussion

5.1. Biogenicity of Varnish

[39] The strongest arguments for varnish biogenicity are the presence of organisms able to precipitate Mn [*Dorn and Oberlander*, 1981; *Krumbein and Jens*, 1981; *Taylor-George et al.*, 1983; *Palmer et al.*, 1986; *Jones*, 1991; *Hungate et al.*, 1987; *Raymond et al.*, 1992]. Our cultivation results show that there are numerous organisms present in the varnish layers of the Black Canyon sites that have the ability to produce manganese oxides in culture. This provides a strong piece of circumstantial evidence that supports a biogenic component to desert varnish production at this location. An active role of the microorganisms in mineralization processes is suggested by the time series killed controls, which show that the minerals being produced can be halted if the organisms are killed (details not reported here). A simple, passive precipitation on cell surfaces is much less likely in light of these observations.

[40] Another interesting potential indicator of a biological origin that has been cited is the stromatolitic texture of varnishes [*Perry and Adams*, 1978]. The Black Canyon varnish sites that we examined are characterized in many cases by stromatolitic textures. Such layering may also indicate fluctuations in environmental conditions, which can have both chemical and biological consequences.

[41] Mn solubility and precipitation is largely controlled by Eh and pH conditions [*Taylor et al.*, 1964; *Nealson*, 1983; *Jones*, 1991]. Namely, low Eh and pH usually leads to dissolution of Mn whereas high Eh and pH will promote precipitation. Two models have been proposed to explain how Mn is concentrated so effectively in varnish. The biotic model assumes that bacteria capable of bioconcentrating Mn favor a relatively wet environment with low pH and alkalinity, whereas the abiotic model proposes that in a relatively dry environment that has high pH and alkalinity, there is no effective way to release Mn from airborne dust [cf. *Smith and Whalley*, 1989]. Both models, though different in mechanism, suggest a climatic influence over the enhancement and depletion of Mn in varnish, supported by the work of *Liu et al.* [2000], who concluded that varnish microlaminations are correlated with environmental fluctuations. They posit that Fe-rich layers accumulate during dry con-

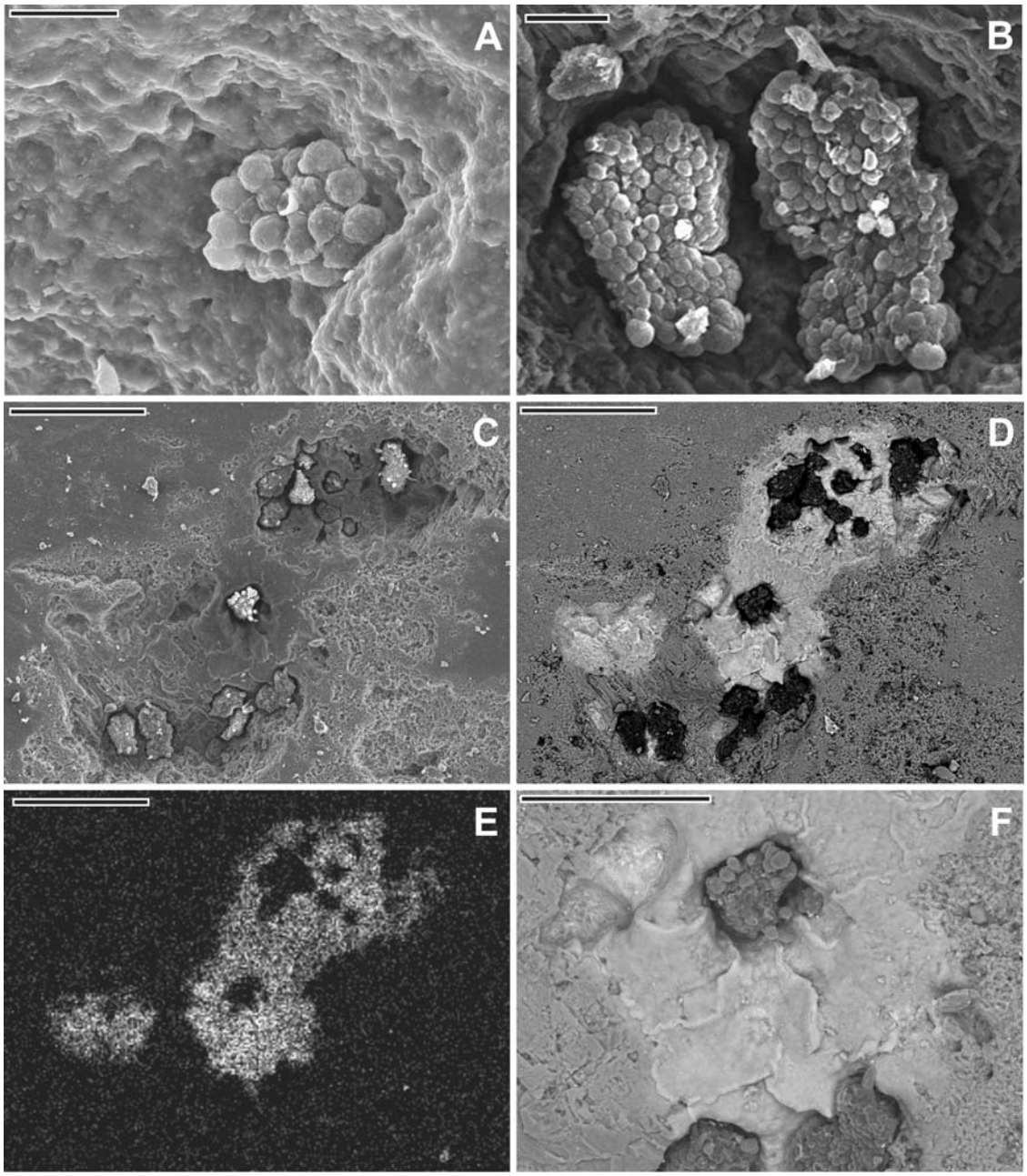

Figure 10. Electron micrographs of blasted and naturally bare rock samples. (a) Microbial colony (putative microcolonial fungi) in pit on surface of the blasted rock sample from site 4. (b) Microbial colonies within pits on the naturally bare rock sample from site 3. (c) Cluster of microbial colonies on the bare rock sample. (d) BSE image of the same area in Figure 10c showing the distribution of Mn-rich varnish (light areas). (e) X-ray map of Mn on the same area as Figures 10c and 10d. (f) Higher-magnification image of the center of Figure 10d showing the smooth, uniform surface texture of the Mn-rich varnish compared to the rock surface. Figures 10a–10c are SEI, and Figures 10e and 10f are BSE images. Scale bars are 20 μm (Figures 10a and 10b), 200 μm (Figures 10c–10e), and 100 μm (Figure 10f).

ditions whereas darker Mn-rich layers accumulate during wet periods. However, the abiotic model does not take into account that it may be the bacteria that are affecting Eh and pH conditions [*Jones*, 1991]. Such a response to moisture could also be a biological response, with the moisture enabling manganese oxidizers to enter a phase of high biological activity. In our field observations at the site reported here and elsewhere, there is frequently a clear correlation of visibly heavier Mn varnishing on preferential water pathways over rock surfaces, down cliff faces, and in transient rock pothole pools (a.k.a. *tinajas*, in the Southwest U.S.).

[42] Our molecular analysis of the cultures has demonstrated that most of the species present in manganese oxide precipitating MMC are not previously known manganese oxidizers (Table 2) and include *Rhizobium*, *Methylobacterium*,

Afipia, *Gemmatimonas*, *Sphingobacterium*, *Shinella*, *Carynebacterium*, *Sundsvallense*, and *Gemmatimonas* (Figures 5, 6, and 7, clones whose names include MN). Several of these genera, *Methylobacterium*, *Afipia*, *Gemmatimonas*, overlap with those found in the Atacama Desert varnish study by *Kuhlman et al.* [2008]. One genus, which was found in the manganese precipitating varnish cultures, *Bacillus*, is a documented manganese oxidizer in various habitats, including desert varnish [*Perry and Adams*, 1978; *Hungate et al.*, 1987; *Tebo et al.*, 2004]. This evidence is suggestive, rather than conclusive, and it is possible that some of these organisms provide nutrients to other organisms that do the actual precipitation of manganese in these mixed cultures. Further detailed analyses of individual MMC strains will be essential to unraveling this complex ecological and geomicrobial relationship. However, we can conclude that the community-wide capability to conduct Mn oxidation and crystallization processes is abundant and widespread, which is consistent with the ubiquity of this material in arid environments worldwide.

5.2. Comparison With Other Varnish and Ferromanganese Communities

[43] Our phylogenetic results for the varnish sites are strongly congruent with those of *Kuhlman et al.* [2008] in their study of the rock varnish community from the Yungay region of the Atacama Desert, despite substantial differences in the degree of aridity of the two sites. Both culture-independent studies document the presence of *Alphaproteobacteria*, *Betaproteobacteria*, and *Gammaproteobacteria*, *Cyanobacteria*, *Chloroflexi*, *Actinobacteria*, *Gemmatimonadetes*, and CFB. Our results also document the presence of *Firmicutes*, which includes the manganese-oxidizing genus, *Bacillus*. Both studies included one unclassified group, which differed between the two sites. Even at the level of genus, a great deal of overlap between the two sites was observed. Both the Black Canyon and the Yungay varnish contained *Methylobacterium*, *Afipia*, *Bradyrhizobiacea*, *Janthinobacterium*, *Chrococcidiopsis*, *Anabaena*, *Nostoc*, *Hymenobacter*, *Actinomycetales*, *Geodermatophilus*, *Chloroflexus*, and *Gemmatimonadetes* as closest relatives of varnish clones.

[44] The degree to which rock varnish communities overlap with other ferromanganese deposits and with soil communities is also of interest for interpreting our results. Manganese and iron oxides occur widely in sediments and soils worldwide. One type of such deposits in soils, ferromanganese nodules, overlaps to some degree with rock varnish communities in bacterial community composition. In a study of both nodules and the soil surrounding nodules, *He et al.* [2008] found that both the soil and the nodules have communities dominated by *Acidobacteria* and *Proteobacteria*. Surrounding soil had more *Acidobacteria* and *Verrucomicrobia* than found in nodules, while soils do not contain the *Firmicutes* observed in nodules. A major difference between the soil ferromanganese nodules and our varnish communities is the lack of *Acidobacteria* in the Black Canyon varnish communities. Groups found in common between the soil nodules and varnish communities include the *Proteobacteria* (*Beta*, *Alpha*, *Gamma*, and *Delta* subdivisions), the *Gemmatimonadetes*, *Actinobacteria*, CFB, and *Firmicutes*. The only known manganese-oxidizing genus in common between the studies was *Bacillus*. Not unexpectedly, neither *Cyanobacteria* nor *Chloroflexi* found in the Black Canyon varnish, were found in the soil ferromanganese nodules. The close congruence at the genus level observed between the Atacama Desert varnish [*Kuhlman et al.*, 2008] and the Black Canyon varnish, was not seen with the soil ferromanganese nodules, despite some overlap at the phylum level.

[45] Soils, in general, are dominated by *Proteobacteria*, *Acidobacteria*, *Actinobacteria*, *Verrucomicrobia*, *Bacteroidetes*, *Chloroflexi*, *Planctomycetes*, *Gemmatimonadetes*, and *Firmicutes*, with *Proteobacteria* making up the largest percentage (39% on average) of soil bacterial communities [*Janssen*, 2006]. Varnish communities, both in the Atacama in Chile and Black Canyon in New Mexico, USA, overlap to some degree with soil communities at the phylum level, but also exhibit major differences. Black Canyon varnish communities contain no *Acidobacteria*, *Verrucomicrobia*, or *Planctomycetes*. Thus, marked differences occur between Black Canyon varnish communities and both soil and ferromanganese deposits that occur in soils [*He et al.*, 2008]. These observations, plus the strong congruence with the Atacama varnish community and the presence of organisms capable of precipitating manganese in culture, suggest that these varnish communities are not simply derived from soil bacterial communities, but are a complex community that contains novel manganese oxidizers.

5.3. Overlap Among Varnish, Nonvarnish, and Cultured Sequences

[46] Nonvarnish and varnish sequences intermixed within several of the phyla found in our sites, with some notable exceptions (Table 1). *Deinococcus-Thermus* phylotypes were only found in nonvarnish site 4, the recently blasted rock. Because species in this group are known for their resistance to ultraviolet radiation, we believe that these may be early colonizers on the "cleaned" rock, which lacks any visible varnish and may not tolerate being covered by varnish layers. The *Ktedobacter* (unclassified group), on the other hand, were predominantly found in varnish site 1-2. The actinobacterial phylotypes are predominantly found in the nonvarnish sites, but a few actinobacterial phylotypes were also found from varnish sites. The greatest number of actinobacterial phylotypes was found in the recently blasted rock (site 4) at the Black Canyon site. Many of the *Actinobacteria* are heterotrophic and may provide needed nutrients to other community members and may be pioneering species.

[47] Major differences were found between the two nonvarnish sites. Overall, many more phylotypes (eight of the nine phyla documented) were found in nonvarnish site 3, the natural rock with no macroscopically visible varnish, than in nonvarnish 4, which contained only five of the nine phyla documented across all sites. Because site 4 represents a "cleaned" surface, the phylogenetic differences between these two sites provide insights into colonization. More clones from the *Cyanobacteria* and *Chloroflexi* phyla were found in the older, noncleaned nonvarnish site 3, possibly indicating these organisms flourish in a more developed community.

[48] Our culturing efforts were particularly effective with the *Alphaproteobacteria*, *Betaproteobacteria*, and *Gammaproteobacteria*, and to a lesser extent with the *Actinobacteria*,

Gemmatimonadetes, CFB, and *Firmicutes*. We have now documented several new phylotypes that are capable of manganese oxide production, which were not previously reported to possess this capability and future studies will investigate the interactions of these different species and their mineral precipitates.

[49] We were not successful in growing *Cyanobacteria*, *Deinococcus-Thermus*, *Chloroflexi*, or *Deltaproteobacteria* that were capable of producing manganese oxides. We hypothesize that both the *Cyanobacteria* and the *Chloroflexi* may be organisms that provide nutrients and carbon compounds to other community members. The large number of cyanobacterial phylotypes in varnish site 5 may be due to the increased level of moisture in this site, which is an ephemeral watercourse.

[50] Our ability to culture a putative manganese-oxidizing *Bacillus*, while not isolating *Bacillus* phylotypes from the varnish clone libraries may reflect the difficulty of lysing *Firmicutes* cells. Several additional cultured phylotypes represent unique diversity not observed in the clone libraries, which potentially would not have been detected without culturing. These results parallel those seen by *Donachie et al.* [2007], who suggest that a polyphasic approach to characterizing diversity is necessary in order to avoid the biases inherent in different approaches.

[51] The presence of the microscopic incipient varnish on our "nonvarnish" samples would explain the extensive overlap between phyla found in the varnish and nonvarnish sites. Although not visible to the unaided eye, there is sufficient biomass to be picked up during DNA extraction and amplification.

5.4. Microstructure of Varnish

[52] Our microscopy studies of both nonvarnish sites (Figure 10) revealed the presence of incipient varnish that we believe represents early stages of varnish formation. Larger colonies were observed in nonvarnish site 3 than in the blasted nonvarnish site 4. Figure 10 also shows the tongues of manganese that are coming out from beneath the colonies from site 3, while no apparent manganese was observed in site 4. Most of the incipient varnish was found surrounding colony-occupied pits in the site 3 sample, suggesting that rock varnish starts near the colonies or is associated with them. Because of the resemblance of the colonies to microcolonial fungi (MCF) [*Gorbushina*, 2003, 2007], additional studies are being undertaken to study the fungi associated with the varnish deposits. It is also possible that these microcolonial growth shapes may represent colonies of *Cyanobacteria* or *Actinobacteria*, which are known to sometimes mimic microcolonial fungi in external appearance [*Gorbushina*, 2003]. Both *Cyanobacteria* and *Actinobacteria* are found in the varnish clone libraries. The colonies themselves are slightly enriched in iron and not in manganese, but may provide necessary conditions for Mn-oxidizing microorganisms to take hold. These samples also imply that microorganisms can quickly colonize rock surfaces, since the blasted rock has been exposed for only about 60 years. This observation certainly agrees with observations of *Krumbein and Jens* [1981], who scraped varnish from rocks in the Negev Desert, Israel and noted regrowth in 14 years. The varnish on these samples probably represents an early stage of development, where the varnish has not yet covered the entire rock surface or become macroscopically visible to the unaided eye. This observation that varnish starts growing at nucleation centers and spreads laterally agrees with the observations of *Perry and Adams* [1978], although they make no mention of any microorganisms at the varnish nucleation centers.

[53] The microstructure of the rock varnish yields some significant clues to its origin. The botryoidal varnish and chaotic layering represent deposition by upright or pinnacled colonies such as those in Figure 8. These botryoidal structures have been described in varnish from several localities [*Krinsley et al.*, 1995; *Nagy et al.*, 1991; *Perry et al.*, 2003], and *Perry and Adams* [1978] interpreted these structures as proof that varnish grows vertically and laterally. In many ways, the botryoidal structures in cross section in Figure 9 resemble miniature stromatolites, a texture often argued as indirect evidence of deposition by living organisms [*Allwood et al.*, 2006]. Certainly the botryoidal varnish pinnacles in Figure 8 represent microbial colonies that are distinctly different, larger and upright, from the nearby MCF colonies that are sunken into the rock. While it has been speculated that MCF may aid in varnish formation by trapping dust [*Dorn and Oberlander*, 1981; *Taylor-George et al.*, 1983], it is clear from Figure 8 that the pinnacles of the botryoidal varnish are trapping significant amounts of mineral and organic debris. Numerous mineral particles can be seen between the botryoidal pinnacles in addition to pollen and organic debris. Mineral grains are also observed that are trapped within the chaotic and botryoidal layers in cross section in Figure 9. The trapped dust is the likely source of the manganese, iron, and other materials that make up the varnish [*Allen*, 1978; *Dorn and Oberlander*, 1981; *Perry and Adams*, 1978; *Thiagarajan and Lee*, 2004]. We suggest that metals such as Fe, Mn, Co, Ni, Pb and other trace elements are dissolved from the dust minerals by rainwater (pH around 5.7 [*Thiagarajan and Lee*, 2004]) and manganese-oxidizing microbes oxidize the dissolved manganese, leaving behind altered silicates (mainly clay and silica) and the oxidized manganese. Furthermore, the trapped organic material may provide nutrients and carbon for a microbial community building rock varnish.

[54] The transition from chaotic layering to uniform laminations (Figure 9a) suggests that conditions of deposition changed or diagenesis of the varnish has taken place after deposition. *Krinsley et al.* [1995] studied the micrometer- to nanometer-scale layering in desert varnish in detail and determined that the oxides are remobilized and reprecipitated as nanometer-scale layers, eliminating cell wall and other evidence of microbes. Furthermore, the harsh environmental conditions to which varnish is exposed are not conducive to preservation of cellular material and only the manganese oxide respiratory waste would remain. *Garvie et al.* [2008] concluded that the chaotic nature of varnish layering and the different manganese phases present in the varnish was an indication that varnish does not reach mineralogical equilibrium and is subject to continuous change. The smooth varnish may then represent reworking or weathering of the botryoidal varnish after deposition. This can be seen in the chaotic layers in Figure 9a that likely represent remnant botryoidal layers that have been reformed into smooth layers by weathering, planing off of the botryoidal pinnacles and redeposition of the manganese oxide,

along with clays and silica, into thin laminar layers characteristic of the smooth varnish.

5.5. Significance of Varnish Studies

[55] An understanding of the origin of Mn-rich rock varnish is important to understanding the biogeochemical cycling of manganese in the terrestrial environment and perhaps planetary environments such as Mars. The recent robotic exploration of the surface of Mars has renewed interest in terrestrial rock varnish. Some of the images returned by the recent MER missions and the previous Pathfinder have led to speculation about coatings on rock surfaces on the red planet. S. Murchie et al. (unpublished data, 2004) pointed out that certain features at the Pathfinder site resembled rock varnish. Considerable work has been done to compare terrestrial desert varnish with potential varnish that may be present on Mars, in the belief that varnish may preserve biofabrics [*Allen et al.*, 2004; *Bao et al.*, 2001; *Gorbushina et al.*, 2002; *Goudie*, 1980] or may be a location that harbors extant life [*DiGregorio*, 2002]. The surface environment of Mars is believed to be highly inhospitable for life. The Viking landers found strongly oxidizing conditions [*Zent and McKay*, 1994] and the thin atmosphere provides little protection against solar ultraviolet radiation. However, several recent discoveries make rock varnish an important target in the search for life on the surface of Mars. First, there is evidence that manganese-depositing organisms may be less susceptible to radiation. Among the most radiation-resistant bacterial groups, *Deinococcus*, *Enterococcus*, *Lactobacillus*, and *Cyanobacteria* accumulate Mn(II), while bacteria that accumulate iron, such as *Shewanella oneidensis* and *Pseudomonas putida*, are much more sensitive to radiation [*Daly et al.*, 2004]. Thus rock varnish may provide a niche for radiation-resistant life forms [*Kuhlman et al.*, 2005], although we only found *Deinococcus* spp. in nonvarnish samples. Second, manganese oxides may provide protection from the oxidative surface conditions. Manganese has been shown to act catalytically as an antioxidant by associating with anions including phosphate, and metabolic intermediates such as lactate or malate [*Archibald and Duong*, 1984; *Archibald and Fridovich*, 1981, 1982; *Inaoka et al.*, 1999]. Furthermore, *Horsburgh et al.* [2002] describe evidence that suggests the accumulation of manganese may form the basis for an alternative, catalytic detoxification of harmful reactive oxygen species via several, recently identified transporters. Understanding the origin of rock varnish may therefore be an important step toward looking for life on Mars.

6. Conclusions

[56] Our cultivation experimental results suggest that organisms are present in the varnish layers of the Black Canyon sites that have the ability to produce manganese oxides in culture, including one known manganese oxidizer, *Bacillus*. Additionally, we have identified several new groups of bacteria that were present in the varnish that demonstrated the ability to precipitate manganese oxides. Our culture-independent characterization shows significant differences between varnish and nonvarnish communities, but also shows some overlap between the two communities, which may be explained by the presence of incipient varnish revealed by scanning electron microscopy. Our results also show extensive congruence at both the phylum and genus level with those of the Atacama Desert study conducted by *Kuhlman et al.* [2008], which is startling given the differences in aridity and physical distance between the two sites. Taken together, these results suggest that microorganisms play a role in the formation of rock varnish. The recent discovery of possible varnish coatings on rocks on the Martian surface and the potential for varnish to provide a refuge for life from ultraviolet radiation and other hostile surface conditions, provide a stimulus to understand the role of microorganisms in the formation of varnish.

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