

FEASIBILITY OF THE METAGENOMIC CHARACTERIZATION OF ROCK VARNISH

Final Report

JPL Task 1063

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A. OBJECTIVES

In recent years, the study of rock varnish has become important to JPL for two reasons: 1) rock varnish has been hypothesized to exist on Mars, based on images from the Viking landers and Mars Pathfinder [1,2], and 2) rock varnish has been shown to grow in both hot and cold deserts and semi-arid regions, and thus provides a potential Martian analog environment for microbial life. Little is known about the mechanisms by which varnish is formed, despite almost three decades of research employing both microanalytical and biological techniques [3,4].

The objective of this project was to investigate the feasibility of performing sequencing of 16S rDNA and metagenomic classification of the biodiversity of the microbes found in and on rock varnish. Our hypothesis is that varnishes may be formed from microbially derived manganese and iron oxides, or manganese and iron salts that undergo natural sol-gel processing and essentially “shrink-wrap” clay particles and other debris delivered by wind and rain to form the very thin layers of ceramic that are observed [5]. If this hypothesis is valid, then characterization of the DNA from rock varnish microbial communities should reveal the presence of iron- and/or manganese-oxidizing bacteria. Thus, the objectives of this work are to (a) isolate and characterize some of the extremeophylic bacteria living in desert rock varnish, (b) construct rDNA clone libraries for bacteria, archaea, and eukarya using the polymerase chain reaction (PCR) and appropriate primer sets, (c) sequence representative clones from each library and characterize these sequences according to the closest phylogenetic matches available in the rDNA databases (e.g., Genbank at the National Center for Biotechnology Information; NCBI), (d) perform a count of the number of bacteria cells per gram in a representative rock varnish sample provided by JPL, and (e) estimate the feasibility of performing comparative genomic hybridizations of DNA from rock varnish communities as described in the recent paper from the University of Idaho group [6].

B. PROGRESS AND RESULTS

1. *Science Data*

Site and Varnish collection. The JPL team performed aseptic collection of several varnished clasts in February 2003 near Parker, Arizona and south of Death Valley, California. A sterile brush was used to remove loosely attached soil, and varnish was removed from the rock

surface using a flame-sterilized Dremel tool in a laminar flow hood in the JPL Planetary Protection Laboratory. Soil from the area around the clasts was also collected.

Microbial enumerations. An essential part of determining the feasibility of metagenomic analysis of rock varnish is the determination of the number of viable cells that can be recovered. A sample of powdered rock varnish (0.1 g) was added to 1 mL of sterile double-distilled H₂O in a 1.5-mL sterile Eppendorf microcentrifuge tube. Serial 1:10 dilutions were made giving a range of dilutions from 10⁻¹ to 10⁻³. The dilution samples were fixed with 2% ice-cold HPLC-grade methanol, vortexed well, and incubated at room temperature for 30 minutes. The samples were stained with 60 µL/mL of a stock DAPI (or Acridine Orange at 50 µg/mL) solution. DAPI-stained samples were incubated at room temperature for 30 minutes in the dark before filtration. The most appropriate volume for analysis was determined to be 500 µL of the 10⁻³ dilution. Samples were filtered onto 25-mm Millipore Isopore 22-µm-pore-size black polycarbonate filters with Whatman 25-mm GF/F filters used for support. Fluorescing cells were counted on a Zeiss Research epifluorescence microscope equipped with an Osram Xenon short-arc photo-optic lamp XBO 75W, and Chroma #31000 filter set for DAPI/Hoechst/AMCA.

The mean field (n) counted per sample was 57.16. The field standard deviation per sample was 7.48. The rock varnish had an average DAPI direct count of 9.0x10⁷ cells gram⁻¹ (standard deviation = 1.2E+07). There was no determinable difference between DAPI and Acridine Orange direct counts.

Molecular biology techniques. The DNA was extracted from 500 mg of varnish or surrounding soil at JPL using a Fast Soil DNA extraction kit (Bio101) and Beadbeater (Savant). Approximately 2 µg and 6 µg of DNA were obtained from 500 mg of varnish and soil, respectively. This DNA was provided to the UI team for some of its work. rDNA libraries also were prepared from the isolated DNA by JPL, using standard procedures and primer sets specific for Eubacteria and Archaea; 100-200 clones were prepared for each library. The Polymerase Chain Reaction (PCR) was performed to amplify 16S or 18S ribosomal RNA (rRNA) genes using DNA from soil or varnish as template with primers specific for Bacteria (27f and 1492r), Archaea (20f and 1492r), and Eukarya (338f and 907r). PCR products were obtained for each reaction except for the eukaryotic primers with DNA from soil as template. Each clone library was further classified by placing clones into RFLP-restriction-group patterns, by treating individual clones with two restriction enzymes and examining electrophoretic banding patterns. A representative clone from each distinct RFLP group was sequenced. Clone libraries of bacterial- and archaeal-specific PCR product from desert varnish were constructed by cloning product using a TOPO TA cloning kit (Invitrogen) and transforming into *E. coli*. Resulting clones were screened by amplified ribosomal DNA restriction analysis (ARDRA) using restriction enzymes RsaI and MspI. Clones with similar restriction patterns in both digests were designated to a common operational taxonomic unit (OTU). Full-length (single-strand) sequence was obtained by sequencing the same primers used for PCR amplification. Only a small subset of the screened clones has been sequenced thus far.

Pure cultures were examined for their closest-known phylogenetic affiliations by using PCR to amplify their 16S rDNA genes, sequencing the PCR products, and comparing the sequences to known sequences in databases at the National Center for Biological Information

(NCBI) using BLASTN 2.2.6. Prior to PCR, cells were lysed directly using the following procedure: A cell suspension (50 µl) was added to 100 µl TE buffer (pH 8) containing 1% Triton-X 100 (final concentration: v/v). The suspension was boiled for 10 minutes in a water bath, cooled for 1 minute, and vortexed. Cell debris was removed by centrifugation at 3,000 x G for 15 seconds, and 2 µl of the supernatant was used directly for PCR. Each 50-µl PCR reaction contained the following components: HPLC-grade water up to volume, 1/10 volume of 10x PCR buffer (Promega), MgCl₂ (2 µM) (Promega), deoxyribonucleotide triphosphates (dNTPs, 0.2 mM) (Invitrogen), 1x bovine serum albumin (1 µl) (Boehring Mannheim), forward and reverse primers (05 µM each) (Integrated DNA Technologies), Taq DNA polymerase (1.25 U) (Promega), and 2 µl of prepared cell extract. Universal eubacterial primers 338f (5'-ACT CCT ACG GGA GGC AGC -3') and 907r (5'- CCG TCA ATT CMT TTR AGT TT -3') were used, with M being a 1:1 Mixture of A and C. The PCR protocol we used consisted of a 5-minute denaturation step at 95°C, followed by 32 cycles of denaturation (45 s, 95°C), primer annealing (45 s, 55°C) and primer extension (45 s, 72°C), finishing with a final extension step (5 min, 72°C). The presence of appropriately sized PCR products was visualized on 1% agarose gels. PCR products were purified from PCR reaction mixtures using the Qiaquick PCR Purification Kit (Qiagen) and sequenced at the Washington State University Laboratory for Biotechnology and Bioanalysis.

The uncultivated microbial community of Death Valley rock varnish. The team at JPL generated three rDNA libraries from the Death Valley rock varnish community DNA and control libraries from soil adjacent to the varnished rock. Varnish 16S rDNA libraries were prepared for Eubacteria and Archaea. We have been unsuccessful thus far in producing an 18S rDNA library for Eukarya. The control soil libraries were prepared for Eubacteria and Archaea. Between 100 and 200 clones were prepared for each library. The clones within each library were arranged into related subgroups through examination of their RFLP patterns, and 16S rDNA PCR products of representative members of each subgroup were sequenced. Results of sequencing of representatives of these rDNA libraries are shown in Tables 1-4.

Table 1. Preliminary sequence analyses of representative clones of a Eubacterial rDNA library of rock varnish from Parker, Arizona.

DRV Eubacteria

<i>Rubrobacter</i> sp. (92%)	Uncultured alpha protoebacterium (98%)
<i>Chelatococcus asaccarovorans</i> (98%)	<i>Craurococcus roseus</i> (93%)
<i>Rubrobacter</i> sp. (93%)	<i>Rubrobacter</i> sp. (93%)
<i>Rubrobacter</i> sp. (94%)	<i>Polyangium cellulorum</i> / <i>Chondromyces</i> (96%)
<i>Sphingomonas</i> sp. (97%)	

Table 2. Preliminary sequence analyses of representative clones of an Archaea rDNA library of rock varnish from Parker, Arizona.

DRV Archaea

Uncultured archaeon clone (97%)

Uncultured crenarchaeote (97%)

Table 3. Preliminary sequence analyses of representative clones of the surrounding soil Eubacterial 16S rDNA library.

Surrounding Soil Eubacteria

<i>Rubrobacter</i> sp. (94%)	Thermal soil bacterium (91%)
Uncultured bacterium (98%)	<i>Burkholderia</i> sp. (89%)
Uncultured bacterium (92%)	<i>Methylosinus</i> sp. (93%)
Uncultured soil bacterium (89%)	Uncultured soil bacterium (97%)
Uncultured delta protoebacterium (94%)	<i>Methylobacterium</i> sp. (93%)

Table 4. Preliminary sequence analyses of representative clones of the surrounding soil Archaea 16S rDNA library.

Surrounding Soil Archaea

Unidentified archaeon (96%)
Uncultured archaeon (98%)

C. SIGNIFICANCE OF RESULTS

Rock varnish is a thin coating (< 500 μm) composed of Mn, Fe and clay minerals that are ubiquitous in deserts [7] and believed by some to exist on Mars [1,2]. Bacteria have been implicated in the formation of rock varnishes due to the dominance of Mn, but the mechanism of varnish growth remains poorly understood and highly controversial [8]. A recent study of varnish using environmental scanning electron microscopy (ESEM) reports rod-shaped objects within desert varnish with various degrees of degradation [9]. However, it is not clear whether the purported bacterium has a role in the formation of the varnish, or is merely encased in the varnish upon death. Preservation of atmospheric signatures in rock varnish has recently been studied by Bao, et al. (2001) [10]. They concluded that rock varnishes or other surface deposits might provide a record of paleoclimatic information and sulfur biogeochemical cycles. This could be useful for those within the scientific community who have an interest in understanding long-term climate variations.

If manganese- and iron-oxidizing bacteria are involved in varnish formation, we should observe community DNA sequences of microorganisms involved in manganese and/or iron oxidation. Manganese is the second most abundant transition metal in the Earth's crust. Mn(II) is the soluble form that is available to organisms and is stable in the pH 6-to-9 range. Mn(III) and Mn(IV) primarily form insoluble oxides and oxyhydroxides. Microbial Mn(II) oxidation thus could result in the formation of manganese oxides that are important mineral phases not only in varnishes but also in soils, sediments and waters [11,12]. Iron-oxidizing bacteria have been known for over 100 years [13]. Like manganese oxidation, iron oxidation occurs at the exterior of the cell surface. Iron hydroxides are often deposited on the remains of biogenic structures, such as sheaths and stalks [14](Emerson, 2000). The deposition of ferric hydroxides

on the sheath is a way for iron-oxidizing organisms to prevent becoming encrusted in iron-oxide precipitates [15]. Such precipitates might be incorporated in a varnish matrix through the activities of iron-oxidizing bacteria.

An alternative explanation for the high numbers of bacteria seen in varnish samples (our observation showed 9.0×10^7 cells per gram of varnish) is that this material represents a habitat that is simply occupied by extremophilic bacteria. If this were the case, we would expect to have seen molecular evidence of extremophiles in the varnish, but not necessarily organisms involved in metal oxidation. Our results support this alternative hypothesis, though it is very possible that products produced by varnish bacteria (e.g., pigments) may play important roles in varnish morphogenesis.

Within the 16S rDNA libraries of the uncultivated microbial community of Death Valley rock varnish, we observed a number of sequences related to extremophiles. One of the most common sequences observed was closely related to the genus *Rubrobacter*. *Rubrobacter* species are of the Actinobacteria lineage and are known as inhabitants of masonry and lime wall paintings where they cause a rosy discolorization [16]. Diverse, yet-to-be-cultured members of the *Rubrobacter* subdivision are widespread in Australian arid soils [17]. Numerous sequences of uncultivated bacteria we observed (Table 2) are related to *Rubrobacter radiotolerans* and *Rubrobacter xylanophilus*, bacteria known for their exceptional resistance to gamma radiation [18].

The clone libraries from the control soils (non-varnish) showed sequences which were mostly unrelated to those observed in the varnish, with the exception of an occurrence of a *Rubrobacter* sequence (Table 4). This confirms that the microbial populations of varnish are distinct from those of soil.

One likely rock-varnish-inhabiting genus observed (Table 2) was *Chondromyces* or *Polyangium* (both 96% similarities). This group is in the lineage of Myxococcales [19] and contains many members known to inhabit extreme environments [20] and to make bioactive substances [21].

A likely representative of the genus *Sphingomonas* (Table 2) was observed in the uncultivated rock varnish community. This genus is well known for inhabiting extreme environments such as the deep subsurface [22] and has recently been observed within an endolithic community in Antarctica where it was found within translucent gypsum crusts on the surface of ice-free sandstone boulders [23]. This, like rock varnish, represents an environment exposed to high levels of UV irradiation. The genus *Sphingomonas* contains many representatives that are able to degrade exotic molecules, including numerous xenobiotic compounds [24].

A moderately good match of 93% similarity was observed (Table 2) for a potential member of the genus *Craurococcus*, a novel aerobic bacteriochlorophyll a-containing bacterium found in a soil environment. A similar level of similarity was seen for clone #27 for the related obligately aerobic, bacteriochlorophyll a-containing bacterium *Rhodopila globiformis*. The environment of rock varnish would be conducive to the development of photosynthetic life

forms, so the presence of a bacterium such as *Craurococcus roseus* or *Rhodopila globiformis* is not surprising.

As is normal for this type of investigation, several sequences (Table 2) showed similarities to sequences in the databases seen previously only in uncultured bacteria. This is simply confirmation that many or perhaps most of the bacteria in rock varnish, like other environments, have never been cultured. The large number of cells and diversity of the microbial community indicate that it will be feasible to perform metagenomic analyses on rock varnishes, and future work will focus on verifying the community structure, culturing and characterizing previously uncultivable species.

D. FINANCIAL STATUS

The total funding for this task was \$30,000, all of which has been expended.

E. PERSONNEL

Other JPL personnel who participated in this research included Dr. Robert Anderson (JPL 322), Myron La Duc (354) and Gregory Kuhlman (354). Other University of Idaho personnel who assisted in this research included Dr. William Fusco (Environmental Biotechnology Institute, Manager of the Molecular Ecology and Genomics Laboratory), Carina Jung (graduate student), Tara Stuecker (technician), David Newcombe (graduate student), Lisa Allenbach (technician) and Christopher Ball (graduate student).

F. PUBLICATIONS

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