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IDENTIFICATION OF THE MICROBIAL COMMUNITIES ASSOCIATED WITH ROOTS IN LAVA TUBES IN NEW MEXICO AND HAWAI`I

JESSICA R. SNIDER[1], MONICA MOYA[1], MATTHEW G. GARCIA[1], MICHAEL N. SPILDE[2], DIANA E. NORTHUP[1]

[1]Department of Biology, MSC03 2020, University of New Mexico, Albuquerque, NM 87131, USA
[2]Institute of Meteoritics, MSC03 2050, University of New Mexico, Albuquerque, NM 87131, USA

Although roots have been found to be an essential energy source in lava tubes around the world, the role of the roots and their microbial communities in the cave environment is largely unknown. We investigated bacterial communities found on roots and walls in two lava tubes in the El Malpais National Monument, New Mexico, USA, and two lava tubes in Hawai`i, USA, using culture-independent methods. Root, wall, soil and water samples were collected to determine carbon levels and for DNA extraction. Root and wall samples were collected for scanning electron microscopy (SEM) to look for presence of microorganisms. All samples were collected with permits or permission of landowners. Samples of these communities were taken aseptically and stored on site in sucrose lysis buffer to lyse the cells and for preserve the DNA. DNA was extracted and purified using the MoBio Power Soil DNA Extraction Kit, amplified using polymerase chain reaction (PCR), cloned using Topo TA Cloning and sequenced using Big Dye Terminator v1.1 sequencing. Closest relatives were identified through searches of the NCBI BLAST database. Alignment was done using Greengenes and neighbor joining phylogenetic trees with 100 bootstrap replicates were constructed using Paup version 4.0b10. Bacterial communities of the roots and walls were compared using presence/absence charts. Preliminary results show that the water drips collected from the roots had three times the amount of dissolved organic carbon as drips collected from the walls, suggesting that the roots are an area of increased nutrients in the lava tube. SEM analysis found evidence of bacteria and fungus associated with the roots while only bacteria were noticed on the samples of wall using SEM. Both root and wall samples from the New Mexico lava tubes had closest relatives within the *Acidobacteria*, *Alphaproteobacteria* and *Actinobacteria*. However, only the wall bacterial communities had closest relatives in the *Gammaproteobacteria* and *Firmicutes,* while only the roots had closest relatives in *Deltaproteobacteria*, *Bacteroidetes* and *Betaproteobacteria*. This study suggests that the roots support a diverse microbial community in the lava tubes and is one of the first projects to look at root-associated microorganisms in cave environments.

1. Introduction

Few studies have examined the role of roots growing into caves, aside from their use as the sole food source of the troglobitic planthopper *Oliarus polyphemus* in Hawaiian lava tubes (Howarth, 1972), and no studies have investigated the microbial communities associated with the roots growing into caves. Traditionally cited sources of energy in caves include organic debris entering the cave via sinking streams, gravity or floods (Poulson, 2005), drip water percolating down into the cave, or remains of any type left by trogloxenes visiting the cave (Gillieson, 1996). In addition, chemoautotrophs, use elements from the cave walls or soils as an energy source (Barton and Northup, 2007). However, it is unlikely that roots growing into caves have no effect or bearing on the cave environment and food web. Indeed, we believe roots are another significant source of food for the cave environment, especially in oligotrophic, or nutrient poor, lava tubes.

While plant roots withdraw and store essential nutrients from the surrounding soil matrix, they can also affect the local area around them. Surrounding all plants' roots is an area called the rhizosphere, a layer of soil up to 20 mm thick that is affected biologically, chemically and physically by the presence of the roots and is rich in microorganisms directly or indirectly associated with the plant root. Roots excrete numerous exudates and produce dead material in the form of fine root turnover, and this nutrient input results in higher levels of microbial diversity and activity in the rhizosphere compared to that of root/rhizosphere free soils (Madigan et al., 2008). Roots growing into a lava tube cave bring in their at least part of their rhizosphere, including its load of carbon, nutrients and numerous new microbes, into the cave environment. While the presence of roots affects the cave environment, growing into the cave environment also affects the rhizosphere of the roots. As the root grows out of the soil of the epikarst and into open

air of the cave atmosphere the rhizosphere loses much of it accompanying soils. It can be assumed that the microbial community associated with the root is altered by this change in the rhizosphere; however, no research has looked at how the rhizosphere changes as it grows into the cave. The roots' (and rhizosphere's) incursion into the lava tube cave may "seed" the cave with novel microorganisms. In order to shed more light on the role of roots growing into the lava tube cave environment, we plan to address the following questions:

1. What is the composition of the microbial communities associated with roots growing into lava tubes in New Mexico and Hawaii?
2. Are the roots an area of increased nutrient (e.g. carbon, nitrogen and phosphorus) levels in the lava tube environment?
3. How related are the wall and root microbial communities? Which groups are similar or different in the two cave habitats?

2. Materials and Methods

2.1 Cave description and sample collection

Two caves in New Mexico and two caves in Hawai`i, all with active root growth, were selected as collection sites. Roots Galore Cave and Pahoehoe Cave are located in the El Malpais National Monument in northwestern New Mexico. Thurston Lava Tube and Kula Kai Caverns are located on the Hawaiian Islands. Root samples from each cave were collected aseptically from active root growth near the root apex. Small samples of rocks from the walls and floors at least 2 meters from any noticeable root growth were collected from each cave. All samples were collected aseptically, under an official collecting permit or landowner permission and were stored on site in sucrose lysis buffer (SLB) to ensure preservation of the DNA. Samples were then transported back to the lab and stored at -80° C until DNA extraction. Additional samples were collected aseptically and stored on site in dry tubes for scanning electron microscopy and nutrient analysis.

2.2 SEM imaging and nutrient analyses

Samples for SEM imaging were coated with Au/Pd and viewed using a JEOL 5800 LV SEM at the University of New Mexico. Dry samples collected for nutrient analysis were desiccated, ground, and inorganic carbon was removed by HCl fumigation (Harris et al, 2001). Percent nitrogen and percent carbon was determined by high temperature combustion; the resulting gases were eluted on a gas chromatography column, detected by thermal conductivity and integrated to yield carbon and nitrogen content. Analysis was performed on a ThermoQuest CE Instruments NC2100 Elemental Analyzer (ThermoQuest Italia Sp.A., Rodano, Italy (Pella, 1990)). Soil extractable nitrogen was determined by extraction with 2N KCl followed by analysis for NH_4-N using method 98–70W(1a), 4500-NH_3-G(2) and NO_3-N using method 100–70W (1b), 4500-NO_3-F (2), on a Techincon AutoAnalyzer II (Mulvanery, 1996). Total phosphorus was determined by combustion at 500° C for one hour, followed by addition of 1N HCl and incubation at 80° C for 30 minutes. After dilution the aliquots were analyzed for PO_4 using method 94-70W (1c), 4500-P-F (2), on a Technicon AutoAnalyzer II. Total organic carbon (TOC) samples were analyzed using the persulfate digestion method (APHA, 1998) method on a Shimadzu TOC-5050A instrument. All analyses were completed at the UNM Biology Annex Labs.

2.3 Phylogenetic studies of microbial communities associated with walls and roots

DNA was extracted and purified using the MoBio Power Soil DNA Extraction Kit (MoBio Laboratories, Inc., California). The 16S rRNA gene was amplified using universal bacterial primers 46 forward (5'-GCYTAAYACATGCAAGTCG-3') and 1409 reverse (5'-GTGACGGGCRGTGTGTRCAA-3') with an amplification reaction mixture that contained 30mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl2, 5 mg bovine serum albumin (Boehringer- Mannheim), 200 mM (each) deoxynucleoside triphosphates, 100 pmol of each primer and 0.5 U of *Taq* polymerase (AmpliTaq LD; Perkin-Elmer) in a final reaction volume of 25µl. Amplicons were cleaned and purified using the Qiagen PCR cleanup kit (Qiagen, Germantown, MD) and were cloned using the TOPO TA Cloning kit (Invitrogen, Carlsbad, Calif). RG71 samples were sequenced using ABI PRISM Big Dye Terminator v1.1 sequencing kit (Perkin-Elmer, Foster City, Calif), while RG88 and PH1 and TH10 samples were sent to Washington University Genome Sequencing Facility for sequencing with primers M13F and M13R. Orientation of all sequences was checked using Orientation Checker (http://www.bioinformatics-toolkit.org) and sequences were screened for possible chimeric artifacts using Mallard (http://www.bioinformatics-toolkit.org). Closest relatives of the genetic sequences were selected using NCBI Blast. Alignment using 650bp was developed using GreenGenes (http://greengenes.lbl.gov/cgi-bin/nph-index.cgi) and manually refined using BioEdit multiple sequence editor (http://www.mbio.ncsu.edu/BioEdit/ BioEdit.html). Neighbor joining and unweighted maximum parsimony phylogenetic analysis was performed using PAUP version 4.0b10. Bootstrap analyses were conducted on 100 resample datasets.

3. Results and Discussion

3.1 SEM imaging and nutrient analyses

There were significant differences in root length and lushness of associated fungal and microbial mats between New Mexican and Hawaiian lava tube caves in which we sampled. In Roots Galore Cave, roots grew approximately 20 cm into the cave and were covered with white fungal and bacterial mats (Fig. 1). Roots growing into Pahoehoe Cave were shorter, with only 5 to 8 cm of growth and showed minimal white microbial mats. The roots in the Hawaiian lava tubes typically were between one and four meters, some even growing through the cave and back into the floor of the cave. However, they did not show the thick white fungal mats found on the roots growing into the New Mexico lava tubes, as seen in Figure 2. Samples from Roots Galore Cave viewed by SEM showed fungal growth associated with the root, included one fungal mass appearing to grow into the root (Fig. 3). The root also appeared to have a thick mat of microorganisms growing over most to of the root outer surface (Fig. 4).

Figure 1: Macroscopic photographs of the roots in Roots Galore Cave, New Mexico. The roots have about 20 cm of growth and the thick white microbial maps are visible in the photograph. Photos copyright © 2007 and 2008, Kenneth Ingham.

Figure 2: Macroscopic photographs of the roots in Kula Kai Caverns, Hawai`i. Unlike the roots in Figure 1, the Hawaii roots are 2 meters long and do not appear to have microbial mats associated with them. Photos copyright © 2007 and 2008, Kenneth Ingham.

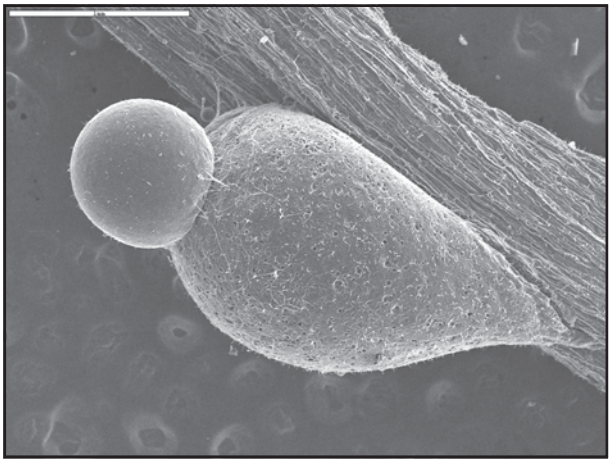

Figure 3: SEM images of root samples from Roots Galore. The scale bar in the image is 1mm long. Notice how the fungus appears to be growing into the root at the bottom of the image while other strands anchor the mass to the roots.

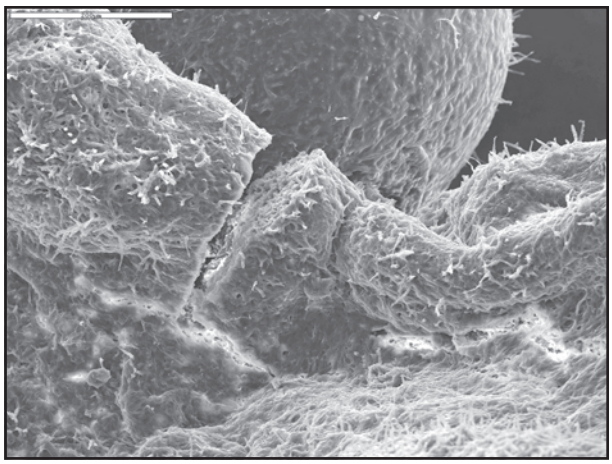

Figure 4: SEM images of root samples from Roots Galore. The scale bar is 200μm long. Microbial mats, including both fungus and bacteria, cover the surface the root and help increase the surface area exposed to soil of the root.

Preliminary TOC levels were determined for drip water in Roots Galore Cave. Our results showed that the TOC levels in the water dripping from the roots to be three times greater than that dripping down from the ceiling where no roots were growing. These preliminary results suggest that the roots are providing additional carbon to the cave.

3.2 Phylogenetic studies of microbial communities associated with walls and roots

The microbial communities on roots in the New Mexico lava tubes were associated with *Pinus ponderosa* roots growing into the lava tubes. Closest relatives to the microbial communities associated with the roots grouped within five phyla: *Bacteroidetes, Actinobacteria, Acidobacteria, Alphaproteobacteria,* and *Betaproteobacteria*. Figure 5 shows a phylogenetic tree of the Roots Galore root microbial mats. 44% of the 18 unique clone sequences grouped within the *Acidobacteria*, while 33% grouped within the *Alphaproteobacteria*. A number of the clones had closest relatives that were known rhizosphere bacterial species. For example, RG88B09, RG88B02 and RG71A10 all had closest relatives that have been associated with trembling Aspen roots. Other sequences were more closely related to soil microbes, such as RG71B12, which had a closest relative from Holocene lake sediment. Surprisingly, some microbes found on the roots had closest relatives that were common in other caves with no root samples. RG88B11 has a closest relative found in a Hawaiian lava tube bacterial mat and RG71C09 grouped with bacteria found in Roman catacombs. Closest relatives of clones from the Pahoehoe Cave wall sample grouped in six phyla: *Actinobacteria, Alphaproteobacteria, Gammaproteobacteria, Acidobacteria, Firmicutes* and *Chloroflexi* (Fig. 6). The closest relatives of the clones were fairly evenly distributed among the six phyla. Most of the clones had closest relatives that resided in other lava tube caves or soils, such as one clone with a closest relative found associated with trembling Aspen.

Bacteria found on roots in Hawai`i lava tubes had closest relatives that grouped in *Alphaproteobacteria*, *Betaproteobacteria* and *Cyanobacteria*. A majority of the clone sequences had closest relatives that live associated

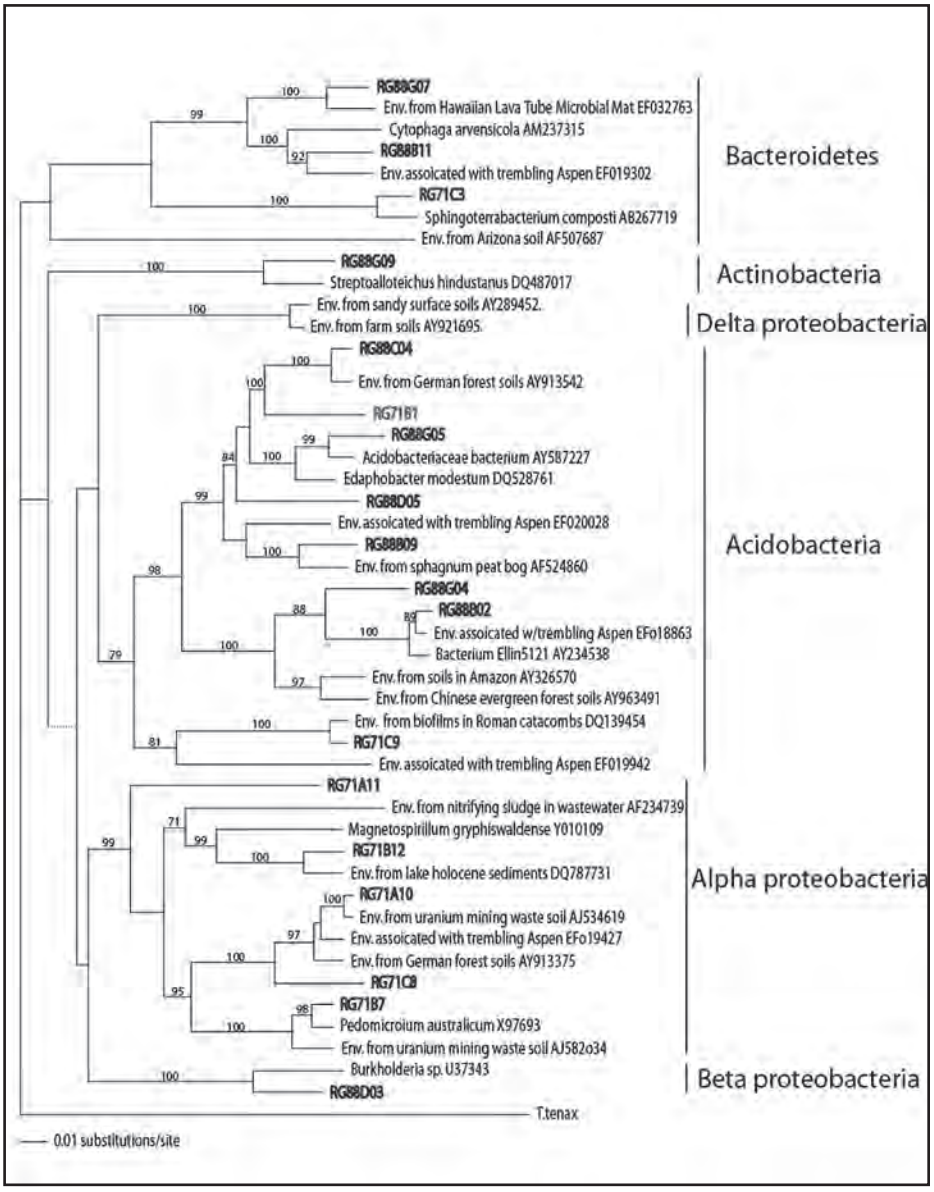

Figure 5: Neighbor joining tree of bacteria associated with roots in Roots Galore Cave. Note that while most of the clones have closest relatives found in soils, two of the clones have closest relatives that reside in other cave environments.

Phylum	NM cave roots	NM cave walls	Hawai`i cave roots	Hawai`i cave walls
Actinobacteria	X	X		X
Planctomyocetes	X			X
Firmicutes	X	X		X
Bacteroidetes	X			
Alphaproteobacteria	X	X	X	X
Betaproteobacteria	X		X	X
Deltaproteobacteria	X			X
Gammaproteobacteria	X	X		X
Acidobacteria	X	X		X
TM7	X			
Verrucomicrobia	X			X
Gemmatimonadetes	X			
Chloroflexi		X		
Cyanobacteria			X	
Nitrospira				X

Figure 6: Microbial populations by phyla from New Mexico and Hawaii cave roots and walls. The New Mexico cave roots and Hawai`i cave walls appear to have the most diverse communities of microorganisms.

with dolomite rocks, soils and Hawaiian volcanic deposits. Closest relatives of clones from the roots in Kula Kai Caverns in Hawai`i grouped in *Actinobacteria, Acidobacteria, Bacteriodetes* and *Gammaproteobacteria,* with 75% of the clones having closest relatives in the *Actinobacteria*. Most of the clones from Kula Kai Caverns have closest relatives that were associated with dolomite deposits in the Alps or with other soils. Figure 6 shows a chart of all phyla found in the roots and walls of New Mexico and Hawai`i lava tubes.

4. Conclusions

Comparisons of the phylogenetic trees from roots and walls in lava tubes show that while most of the bacterial sequences on the roots are typical root-associated bacteria, some bacteria more commonly found in bacterial mats on the walls of caves were found on the roots. In addition, evidence that some of the soil bacteria more commonly found associated with soil and plant roots have been also been found on the walls of the lava tubes. For example, RG88B11 and RG71C09 clones both group with bacteria mats found in Hawaiian lava tubes and Roman catacombs, respectively. Clones with these similar closest relatives have also been found on the walls of lava tubes in New Mexico. Such results may lend support to the suggestion that there are organisms that are indigenous to subsurface environments, such as caves and catacombs. This suggested that the roots could be picking up the bacteria as they grew through the ceiling of the cave, that the roots are introducing, or seeding, bacteria into the cave environment or that both the roots and the cave walls are acquiring bacteria from the soil overlaying the cave. These preliminary results suggest that the microbial communities on the roots and the walls are related and may be acquiring microbes from each other or another source. In addition, preliminary results of the nutrient analysis suggest that the roots represent an area of carbon enrichment in the cave and water dripping off of the roots is also carbon enriched. Our analyses suggest a diverse community of microorganisms inhabits both the root masses entering the lava tubes and the walls of the lava tubes.

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