The Nature of Bacterial Communities in Four Windows Cave, El Malpais National Monument, New Mexico, USA

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Abstract

One of the striking features of some lava tube caves is the extensive bacterial mats (a.k.a. lava wall slime) that cover the walls. Despite their prominence little is known about the nature of these bacterial communities. We have investigated the bacterial mats on the walls of Four Windows Cave, a lava tube in El Malpais National Monument, New Mexico, USA. These bacterial mats in the twilight zone adjacent to algal mats, and in the dark zone of the lava tube, cover from 25–75% of the wall. Their macroscopic and microscopic visual appearance suggests that these bacterial mats are composed of actinomycetes, bacteria that commonly inhabit caves. Vacuuming of bacterial mats and the adjacent algae revealed collembola and mites on the algae but no invertebrates were recovered from the bacterial mats. DNA was extracted from wall rock communities, purified, the 16S rRNA gene was amplified using PCR, cloned, and approximately 1000 bases were sequenced from thirty clones. Comparison of Four Windows bacterial sequences with the Ribosomal Database II revealed that some were most closely related to actinomycetes. Others grouped with members of the *Chloroflexi*, the *Verrucomicrobia*, and the *Betaproteobacteria*. Closest relatives of two of the clones were from Mammoth Cave samples. The latter appear to be novel bacterial species. The ability of bacteria cultured from these mats to withstand the effects of ultraviolet (UV) radiation revealed the microbes isolated from the lava tube were much more UV sensitive than the microbes isolated from the surface. However, all of the microbes tested displayed at least slight sensitivity to UV radiation. Based on the results, the bacterial colonies currently inhabiting the Four-Windows lava tube appear to be at least somewhat cave-adapted. Our studies of the actinomycete communities in Four Windows Cave reveal a diverse community of bacteria that appear to be unpalatable to invertebrates.

Introduction

A revolution in microbiology occurred with the introduction of 16S ribosomal methodology to discover the great diversity and distribution of life through genetic sequences. Standard culturing techniques used to cultivate microorganisms from caves, have met with limited success (Amann et al. 1995; Hugenholtz et al. 1998). Culture-independent molecular phylogenetic techniques allow us to reveal the diversity present in many varied environments (Pace 1997). Many novel prokaryotic species have been detected as a result of this new technology. Bacteria have been found in some of the most extreme areas including deep-sea thermal vents, within rock cores, and in caves. These microorganisms are important participants in the precipitation and dissolution of minerals, in caves (Northup and Lavoie 2001) and on the surface (Ehrlich 1999). However, we have barely begun to characterize the microbial diversity of caves and the roles of microorganisms in the subsurface.

Humid lava tube caves contain highly visible mats of bacteria and other microorganisms , nicknamed "lava wall slime," (Figure 1), but they have received even less attention than limestone caves (Northup and Welbourn 1997). These microbial mats do contain fungi and aerobic bacteria and serve as a habitat for arthropods that feed on nutrients captured in the slimes, e.g. springtails (Insecta: Collembola), mites (Arachnida: Acari), fly larvae (Insecta: Diptera), earthworms (Oligochaeta), a water treader (Insecta: Hemiptera), and carabid beetles (Insecta: Coleoptera) (Howarth, 1973, 1981). Stone and Howarth (Howarth, 1981) also have suggested that the slimes are important sites of nutrient recycling (e.g. nitrogen).

Ashmole et al. (1992) have found slimes present in humid caves in the Canary and Azore Islands, but never in dry caves. In the Northwestern USA (Washington) lava tube slimes consist of different species of bacteria, including actinomycetes in the genus *Streptomyces* (Staley and Crawford 1975). Staley and Crawford (1975) observed two main types: a white slime that is occurs alone, is hydrophobic, and occurs in warmer areas (>6 degrees C), and an orange slime that underlies the white slime and is seen in colder areas. Associated with the slime, Staley and Crawford (1975) found fly larvae (Diptera: Mycetophilidae), overwintering harvestmen (Arachnida: Opiliones), a troglobitic harvestman, *Speleonychia* sp. (Opilionides: Travuniidae), and a millipede (Diplopoda: Polyzoniidae).

We remain almost completely ignorant of the nature of these bacterial mats-due to the lack of culture-independent studies. Thus, this study was undertaken using culture-independent methods to characterize the nature of the lava wall slime in Four Windows Cave. We also investigated the sensitivity of cultured isolates to ultraviolet (UV) radiation to determine whether the bacteria of lava tubes have lost resistance to UV radiation in comparison to their surface bacteria. Previous studies of the sensitivity of deep subsurface bacteria found no differences in sensitivity between deep subsurface and surface bacteria (Arrage et al. 1993a,1993b). Most cave animals lose non-essential traits as they adapt to the subsurface environment, but this has never been investigated in bacteria inhabiting caves.

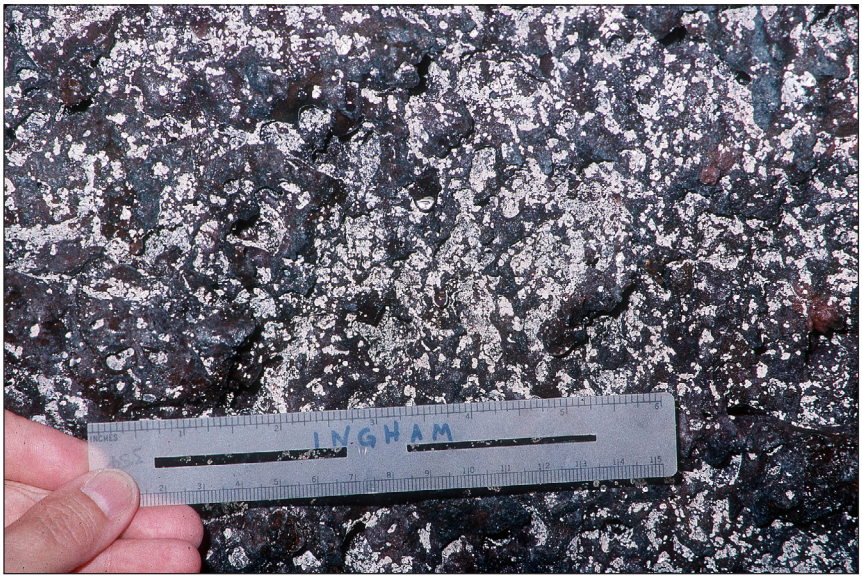

Figure 1. Close-up view of the bacterial colonies on the walls of Four Windows Cave. Photo by Kenneth Ingham.

During a previous investigation of the arthropod community inhabiting Four Windows Cave (Northup and Welbourn 1997), we noted the presence of mites on the algal mats on the walls of the twilight zone, but not on the bacterial mats. A more systematic vacuuming experiment was undertaken to document this anecdotal observation that might suggest that the bacterial mats are distasteful or toxic to invertebrates.

These preliminary studies of the microbial mats present in one lava tube, Four Windows Cave, will allow us to more fully understand the lava tube ecosystem and will lay the groundwork for future studies in other lava tubes.

Experimental Methods

Cave Description

Four Windows Cave, located in El Malpais National Monument, New Mexico, USA is a moderately long lava tube with four skylights that give the cave its name. An extensive invertebrate community exists in the moss garden growing under the skylights. These skylights provide light for the moss garden directly below them and the algal communities on the walls of the twilight zone, both of which support moderately diverse invertebrate communities. Four Windows is cold, ranging from -2 to +2° C with ice stalagmites form in the winter. During the rainy season (July and August), moisture seeps into the cave through cracks and supplies moisture and organic matter for the microbial and invertebrate communities.

The walls and ceiling of Four Windows Cave have extensive deposits of bacterial mats. The distribution of bacterial colonies is patchy (Figure 1), but appears to be most dense in areas of lower light and possibly where moisture enters the cave through cracks. Mat coverage ranges from isolated, individual colonies to dense mats several mm thick (Lavoie and Northup 1994). The visible color of both individual and massed colonies was predominately whitish-tan, but a few gold colored colonies and veins of colonies occur. Observation shows that colonies are hydrophobic, with water or secreted fluids beading up on the surface. This water often reflects light, causing the colonies to appear reflective. Senger and Crawford (1984) associate the hydrophobicity to the presence of spores produced by the bacteria.

Sample Collection for DNA Extraction and Invertebrate Study

Small samples of wall rock covered with bacterial slime were collected from Four Windows Cave in July, 1996 under a National Park Service collecting permit. These samples were chipped from the parent wall rock with an ethanol-dipped, flame-sterilized rock hammer. The samples were then caught in a sterile container, sealed, and placed on dry ice for transport. Upon arriving at the lab, the samples were stored in a -80° C freezer.

Both algae and bacteria were vacuumed with an Insect Vac (BioQuip) to examine the invertebrate communities that inhabit each environment. First, the collection tube of the vacuum was cleaned with ethanol and a sterile filter placed inside. Bacteria patches were vacuumed for one minute, the collection chamber was washed thoroughly with ethanol, and its contents repeatedly transferred to an appropriately labeled, sterile tube. The vacuum filter also was caught in this container and sealed. This procedure was repeated on an algae patch adjacent to the bacterial mats. Bacterial and algal washes were analyzed separately microscopically.

Scanning Electron Microscopy

Samples of the lava tube wall rock covered with microbial colonies were

Figure 2. Cal Welbourn sampling invertebrates from algal colonies on the wall of Four Windows Cave. Photo by Kenneth Ingham.

examined on a JEOL 5800 scanning electron microscope (SEM) equipped with an Oxford (Link) Isis energy dispersive x-ray analyzer (EDX). Rock samples with adherent bacterial colonies were mounted directly on an SEM sample stub while in the cave and then coated by evaporation with Au-Pd in the lab prior to imaging.

Molecular Characterization of the Bacterial Community

Extraction of DNA. Nucleic acids were extracted and purified from two 0.5 gm aliquots of sample by using the bead-mill homogenization procedure described by Kuske et al. (1997). Following bead-mill disruption and centrifugation, the supernatant was transferred and the bead pellet was washed once with 1 ml of TE buffer (10 mM Tris [pH 8.0], 1 mM EDTA), re-homogenized for 5 sec, and centrifuged again. This supernatant was pooled with the original supernatant. Nucleic acids were precipitated from the solution by using 0.1 volume of 3 M sodium acetate (pH 5.2) and 2.5 volumes of ethanol, incubated on ice, and centrifuged for 30 min at 12,000 x g. Precipitated nucleic acids were suspended in TE. DNA was purified using Sephadex G-200 spin columns equilibrated in TE, as described previously (Kuske et al. 1997). The clear column eluate containing DNA was precipitated and suspended in TE buffer. Negative control samples were prepared with TENS buffer alone containing no sample addition and were subjected to the same procedures as used with the samples.

PCR amplification of small subunit rRNA genes from environmental DNAs. The forward primer used was 533F and the reverse primer used was the 1492R primer (Lane 1991). Amplification reaction mixtures contained 30 mM Tris-HCL (pH8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 5 μg bovine serum albumin (Boehringer-Mannheim), 200 μM (each) deoxynucleoside triphosphates, 100 pmol of each primer, and 5 U of Taq polymerase (AmpliTaq LD; Perkin-Elmer, Foster City, Calif.) in a final reaction volume of 100 μl. PCR was conducted with a Perkin-Elmer 9600 thermal cycler as follows: 2 min at 94 °C (denaturation), followed by 35 cycles of 60 sec annealing at 48 °C (annealing), 60 sec at 72 °C (extension), and 5 sec at 94 °C (denaturation), with a final 60 sec at 48 °C (annealing) and 5-min at 72 °C (extension) step after cycling was complete. Five microliters of each reaction mixture was analyzed on 1% SeaKem agarose gels and the desired PCR amplification products were ferified by ethidium bromide staining and UV illumination of the gels.

Small-subunit rDNA libraries. A clone library of small subunit rRNA gene copies was generated from the Four Windows sample. PCR products from 533F-1492R amplification reactions were ligated into pGEM-T plasmid vectors (Promega, Madison, Wis.) using T4 DNA ligase and overnight incubation at 4 ºC, according to the manufacturer's protocols. Recombinant plasmids were transformed into *Escherichia coli* JM109 competent cells (Promega), and colonies containing plasmids with inserts were identified by blue/white color selection on LB/ampicillin/IPTG/XGal agar plates.

RFLP. To assist in determining the genetic diversity of the bacterial colony, the 16S ribosomal DNA of seventeen clones were cut with enzymes to produce RFLPs (restriction fragment length polymorphisms): one μl of plasmid DNA, two μl of React Buffer 3, sixteen μl of double distilled water, and one μl of enzyme were used to digest the DNA. Enzymes used were *EcoR1*, *Bstu 1*and *RSA1*, with one enzyme per reaction. Sheared DNA patterns were visualized using a 4% Metaphor (FMC Rockland, Maine) electrophoresis gel in TAE, stained with 1 μl of ethidium bromide and exposed to UV light.

DNA Sequencing. PCR products from 32 clones with inserts of the correct size (approximately 1.0 kb) 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 thermo sequenase dye terminator cycle sequencing pre-mix kit (Amersham Life Science, Inc., Cleveland, Ohio) and ABI PRISM dye terminator cycle sequencing kit (Perkin-Elmer, Foster City, Calif.) on an ABI 377. Primers used for sequencing were T7 and SP6. Full-length insert sequences were obtained for a subset of clones by using primers for internal sequencing (906F, 907R, and 765F) of the rRNA gene.

Phylogenetic analysis. Each sequence was submitted to the CHIMERA_CHECK program of the Ribosomal Database Project (RDP; Maidak et al. 2001; (http://rdp8.cme.msu.edu/html/)) to detect the presence of possible chimeric artifacts. All sequences were initially analyzed using BLAST (NCBI; Altschul et al. 1997) and SIMILARITY_MATCH (RDPII; Maidak et al. 2001) to identify related sequences available in public databases and to determine phylogenetic groupings of clone sequences. Clone insert representatives of each phylogenetic group identified were sequenced in their entirety. Alignment of the final dataset was accomplished using the RDP II alignment software and manually using the BioEdit editor (http://www.mbio.ncsu.edu/BioEdit/bioedit.html), guided by 16S primary and secondary structure considerations. Identity values were generated by the similarity identity matrix program in BioEdit (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). Distance analyses were performed using PAUP (version 4.0b10, distributed by Sinauer; http://paup.csit.fsu.edu/) with the Jukes-Cantor model. The tree of highest likelihood was found by repeated tree building using random sequence input orders. Bootstrap analyses were conducted on 1000 resampled datasets using PAUP.

UV Sensitivity Experiments

Bacterial inoculation, isolation and growth. To obtain bacterial isolates from the rock walls and surface rocks, we swiped polyester fiber-tipped swabs across the rock and inoculated thirty R2A medium (low-nutrient) plates using the standard streak isolation method. We obtained water samples with sterile 5 ml syringes from a pool of water that had accumulated inside the cave and dispensed 0.2ml of the water onto ten R2A plates, which were spread with a flame-sterilized glass spreader onsite in the cave. Inoculated plates were incubated in the cave for 16 hours before transport to a 3°C incubator in the laboratory where they remained in the dark for two weeks. Surface inoculates were stored for just under three weeks at 37°C. Morphologically unique colonies from both sets of plates were sub-cultured to provide pure cultures for UV experiments. In addition, we sub-cultured the surface colonies onto nutrient-rich LB plates.

UV Radiation Treatment. Once the subcultures were grown, we chose twelve of the most interesting cave colonies and six of the surface colonies to expose to UV light. Interesting was defined as the most morphologically different and slowly growing (likely to be more cave-adapted) colonies. Three replicate plates of colonies per R2A plate were inoculated for each of two treatments plus the control. Immediately after inoculating the plates, we placed the plates, with lids off, under the sterile hood and exposed the plates to a UV light from a germicidal lamp for 100 seconds (1 Dose) or 50 seconds (½-dose plates). After the treatment, we covered the plates, wrapped them in foil to prevent photoreactivation and placed them in the appropriate incubators. The control replicates that were not exposed to UV light were also wrapped in foil and incubated. We monitored the growth of the cultures with visual checks of colony growth for six days, and documented them with a digital camera.

Results

Invertebrate Vacuuming

Visual observation of the bacterial colonies in Four Windows Cave revealed no macroscopically visual invertebrates. Therefore, bacterial and algal mats were vacuumed as described above to more thoroughly investigate the presence of invertebrates. No invertebrates were found within the bacterial mat collection tube. The algal mat collection tube contained fourteen collembola. Eleven of these belonged to the family Hypogasturidae and three belonged to the family Entomobryidae. Previous vacuuming had also yielded Acari (mites) in the Nanorchestidae, and undetermined Oribatida and insects in family Chironomidae (Diptera) were also found.

Scanning Electron Microscopy

Examination by Scanning Electron Microscopy (SEM) of samples of white bacterial mat samples from Four Windows Cave revealed a dense mat of bacteria (Figure 3), some of which were tentatively identified as actinomycetes from their visual appearance. Additional morphologies observed with SEM (not shown) resembled planctomycete-like or *Verrucomicrobium*-like bacteria.

RFLP Analysis and Nucleotide sequences

All eleven RFLP clones examined exhibited unique banding. Several clone sequences appeared to be chimeras and were removed from the analysis. Comparison of our sequences with those in the Ribosomal Database II and Blast revealed that some Four Windows bacterial sequences are most closely related to *Actinobacteria*, as suspected. Other clones grouped with members of the *Chloroflexi*, the *Verrucomicrobia*, and the *Betaproteobacteria*. Two of the closest relatives to our clones were sequenced from Mammoth Cave samples. The latter appear to be novel bacterial species. Figure 4 shows a phylogenetic tree of representative clone sequences and their closest relatives.

UV Sensitivity

Six days after the UV treatments, we scored the UV sensitivity of the different strains based on comparisons with the control strains. Each replicate was rated from one to three, with three being the most sensitive. Overall, every strain showed at least some sensitivity to the 1 dose (100 sec) of UV radiation and all but four strains (all surface) showed some sensitivity to ½ dose (50 sec) of UV. All of the cave strains showed significantly more sensitivity than the surface strains and seven of the cave replicates showed no growth at all with both 1 and ½ doses. All cave bacteria replicates were scored a three. Figures 5 and 6 show the dramatic differences in growth after UV exposure in surface and cave isolates respectively.

Discussion

The lack of invertebrates on the bacterial mats while invertebrates were found on adjacent algal mats suggests that the bacterial mats may contain toxic or distasteful compounds. Scanning electron microscopy and molecular phylogenetic analysis suggest the presence of actinomycete (*Actinobacteria*) bacteria in the bacterial mats. Actinomycetes are a highly varied group of Gram-positive bacteria that have the unusual characteristics of filamentous growth and exospore production. They may make up 10–33% of total soil microbes,

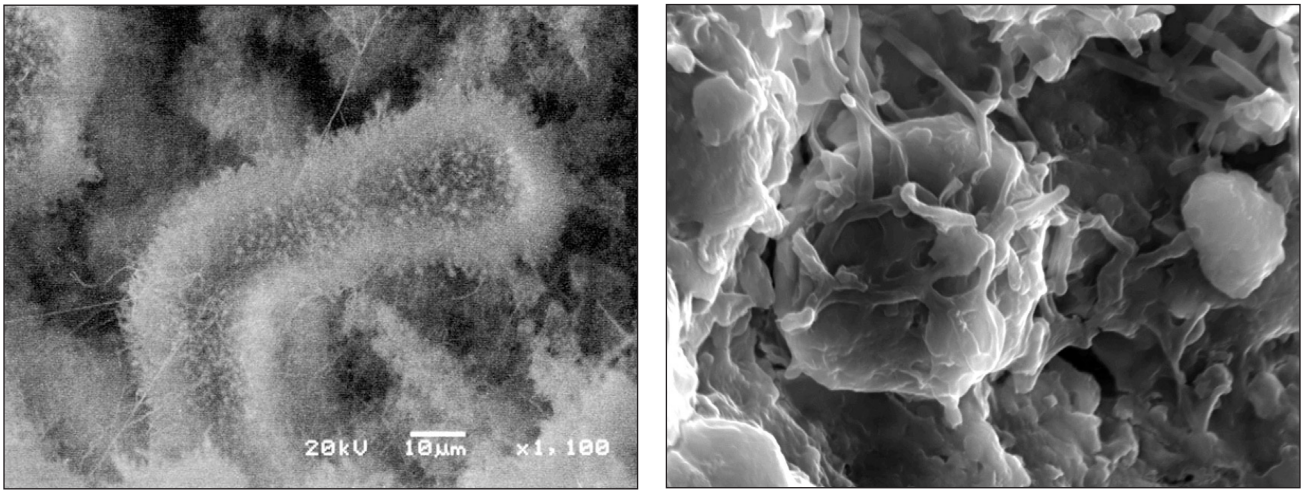

Figure 3. Scanning electron micrographs of sampled white bacterial colonies showing the presence of unusual morphologies (left) and filamentous (right). Photomicrographs by M. Spilde.

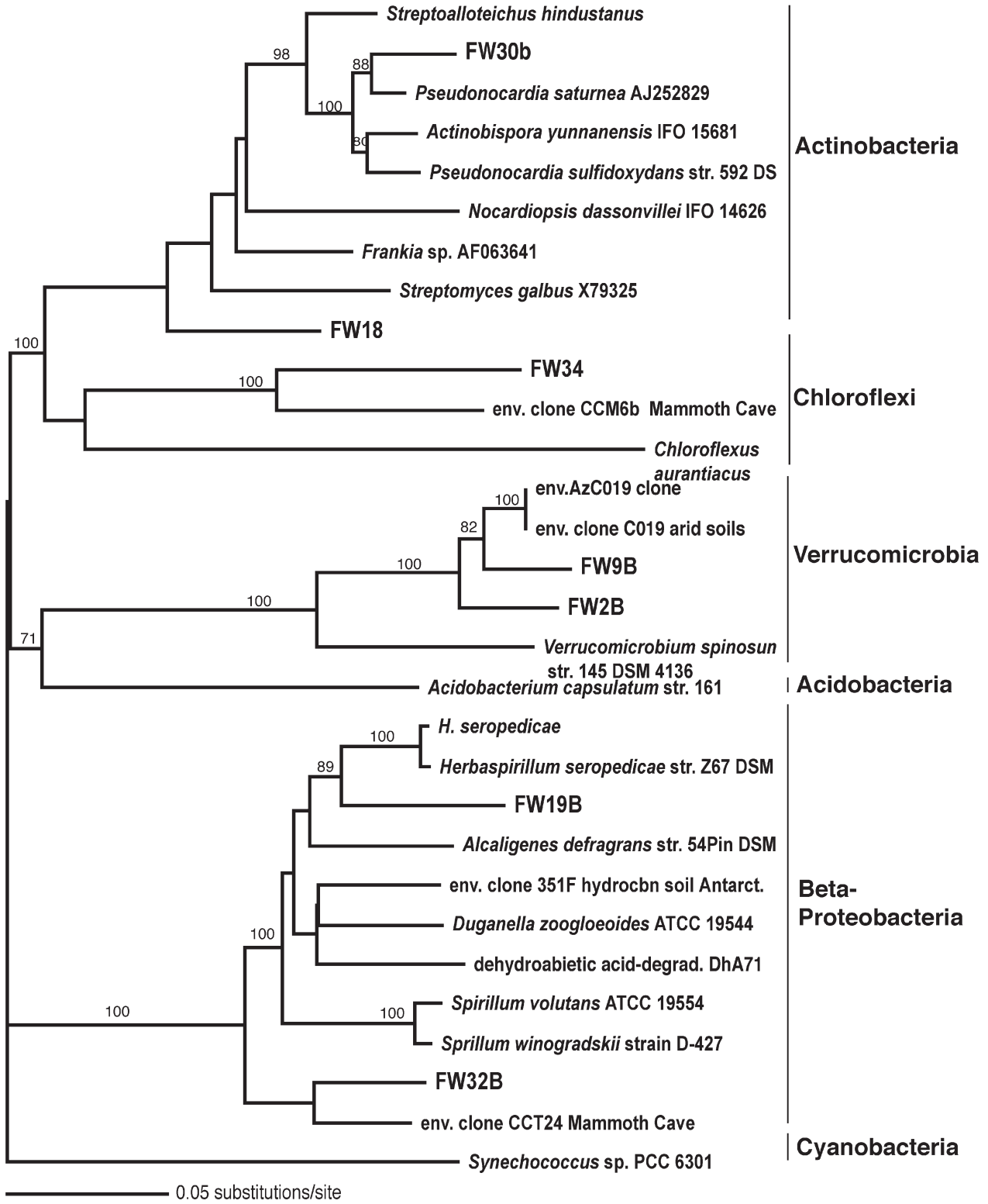

Figure 4. Phylogenetic tree of bacterial rDNA clone sequences from Four Windows Cave lava wall microbial mats. Partial rRNA gene sequences (ca. 1000 nucleotides) from clones (designated "FW" in bold type) were analyzed with most closely related sequences obtained from the databases, as well as other representatives of major bacterial groups. *Synechococcus* sp. PCC 6301 was used as the outgroup. The tree was inferred by maximum likelihood analysis of homologous nucleotide positions of sequence from each organism or clone. Numbers indicate percentages of bootstrap resamplings that support branches in maximum likelihood (above branch) and maximum parsimony (below branch) analyses. Bootstrap results are reported only for those branches that attained >70% support with at least one of the methods used.

with *Streptomyces* and *Norcardia* being the most abundant genera. They are relatively resistant to desiccation, and prefer alkaline or neutral pH environments. Metabolically, their main role in nature is in decomposition of organic matter and they thrive in environments where nutrients are sparse and conditions extreme. Many actinomycetes are known to fix atmospheric nitrogen either in association with some plant roots or as free-living cells. The role of Actinomycetes in nitrogen fixation in caves has not been explored (Lavoie per. comm. 1993). With a temperature of -2 to +2°C and seeping organic matter for nourishment, Four Windows Cave provides an excellent habitat for these bacteria. Some types of actinomycetes are medicinally and agriculturally significant because they excrete antibiotic products to repel invaders. The antibiotic properties of many bacteria species make them interesting to the medicinal industry. The lack of invertebrate life on the bacterial slime communities could be an indicator that the environment may be excreting antibiotic compounds toxic or distasteful to these small animals.

The molecular phylogenetic analysis of bacteria adhered to the rock walls of Four Windows Cave revealed that the community is not merely actinomycetes, but contains organisms from three other major bacterial groups: *Chloroflexi*, *Verrucomicrobia*, and the *Betaproteobacteria*. None of these relationships are especially close as evidenced by the long branch length for many of the Four Windows Cave clones. The closest relatives are those from Mammoth Cave environmental isolates and other soil environmental isolates, indicating the novel nature of these isolates. Clone FW34 grouped with the *Chloroflexi*, a group of generally phototrophic, filamentous organisms. However, other studies have shown cave bacteria grouping with the *Chloroflexi* (Engel, personal communication 2005), and in this case, the association is not a close one. The closer relative of clone FW34 is an isolate from the soils of Mammoth Cave in Kentucky. The lack of a close relationship to a cultivated bacterial species and the fact that close relatives can have different physiologies does not allow us to draw any conclusions concerning this clone. Several clones, as represented by FW2b and FW9b, group with the *Verrucomicrobia*, a recently proposed division that has been elevated to phylum status within the Bacterial Domain (Schlesner et al. 2001). The genera *Verrucomicrobium* and *Prosthecobacter* within the *Verrucomicrobia* are prosthecate with fimbriae (finger or hair-like appendages) extensions from their tips. Their morphology is similar to some of the morphologies seen in the SEM photomicrographs of Four Windows samples. The *Verrucomicrobia* have been found in a variety of aquatic and terrestrial habitats worldwide. While most cultivated members are heterotrophic, we are just beginning to learn about their physiology. Thus, little can be said about the physiology of the Four Windows clones based on their association with the *Verrucomicrobia*.

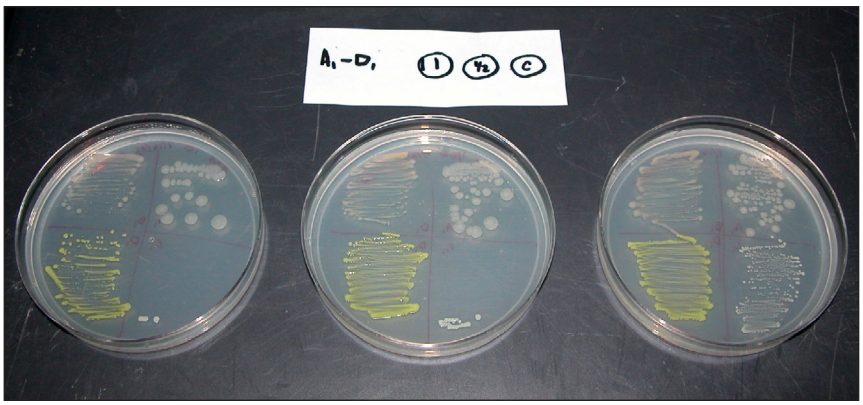

Figure 5. Comparison of replicate 1 surface strains A – D. 1= full UV dose, ½= half UV dose, C= no UV. Replicates 2 and 3 showed similar UV sensitivity (data not shown).

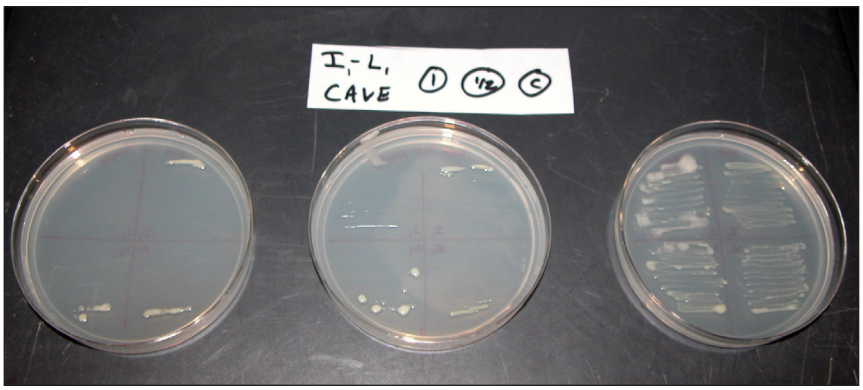

Figure 6. Comparison of replicate 1 cave strains I – L. 1= full UV dose, ½= half UV dose, C= no UV. Replicates 2 and 3 showed similar UV sensitivity (data not shown).

The grouping of isolate FW19B with *Herbaspirillum seropedicae* in the *Betaproteobacteria*, probably reveals an isolate from the surface rhizosphere. Bacteria in the *Herbaspirillum* are usually associated with plant roots, often as nitrogen-fixers. Isolate FW32B groups with another Mammoth Cave environmental isolate within the *Betaproteobacteria*.

Overall, the molecular phylogenetic analysis of a small clone library from Four Windows Cave points to the novel nature of the isolates and the need to learn more about their physiology through enrichment culture studies. Of note is the observation that the closest relatives come from another cave, Mammoth Cave in Kentucky. It is tempting to speculate that this is a small bit of evidence for the existence of an indigenous cave microbial community, but much remains to be learned about the microbial diversity of caves.

Our UV sensitivity experiments with cultured isolates from Four Windows Cave showed a marked sensitivity to UV radiation in comparison to surface cultured isolates, showing a different trend than that seen by Arrage et al. (1993b).

To further test these findings in a more rigorous manner, we are repeating the experiments with isolates from other caves with quantification of the starting inoculum and final growth amounts. If the finding is confirmed, the loss of UV sensitivity may represent an adaptation to the cave environment by bacteria that have no need of UV radiation resistance in the dark environment of the cave. Many of the same genes (*recA*) that control for UV resistance/repair also control repair for other environmental stresses such as desiccation.

This study represents some small steps in adding to our understanding of the bacterial mats that coat the walls of many lava tubes worldwide. We have established that there is a morphologically and genetically diverse community in these mats, that the culturable bacteria are UV sensitive, and that these mats are distasteful to invertebrates who preferentially feed on adjacent algal mats. These studies will hopefully spark interest in these interesting and novel communities, allowing us to further investigate their nature.

References

Altschul SF, Madden TL, Schäffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Research 25:3389-3402.

Amann, R. I., Ludwig ,W., and K. Schleifer. 1995. Phylogenetic Identification and In Situ Detection of Individual Microbial Cells without Cultivation. Microbiological Reviews **59**: 143-166.

Arrage, A.A., Phelps, T.J., Benoit, R.E., Palumbo, A.V. and White, D.C. 1993a. Bacterial sensitivity to UV light as a model for ionizing radiation resistance. Journal of Microbiological Methods **18(2)**: 127-136.

Arrage, A.A., Phelps, T.J., Benoit, R.E., Palumbo, A.V. and White, D.C. 1993b. Survival of subsurface microorganisms exposed to UV radiation and hydrogen peroxide. Applied and Environmental Microbiology **59(11)**: 3545-3550.

Ashmole, N. P., Orom, P., Ashmole, M. J., and Martin, J. L. 1992. Primary faunal succession in volcanic terrain: Lava and cave studies on the Canary Islands: Biological Journal of the Linnean Society **46**: 207-234.

Ehrlich, H.L. 1999. Microbes as geologic agents: Their role in mineral formation. Geomicrobiology Journal **16**(2): 135-153.

Howarth, F. G. 1973. The cavernicolous fauna of Hawaiian lava tubes, 1. Introduction. Pacific Insects 15: 139-151.

Howarth, F. G. 1981. Community structure and niche differentiation in Hawaiian lava tubes; pp. 318-336. *In*: Mueller-Dombois, D., Bridges, K. W., and Carson, H. L. (eds.). *Island Ecosystems: Biological Organization in Selected Hawaiian Communities*. US/IBP Synthesis Series 15: Stroudsburg (PA): Hutchinson Ross Publishing Company.

Hugenholtz, P., Goebel, B.M., and Pace, N.R. (1998) Impact of culture-independent studies on the emerging phylogenetic view of bacterial diversity. Journal of Bacteriology **180**:4765-4774.

Kuske, C., Barns, S., and J. Busch. 1997. Diverse uncultivated bacterial groups from soils of the arid southwestern United States that are present in many geographic regions. Applied and Environmental Microbiology **63**: 3614-3621.

Lane DJ. 1991. 16S/23S rRNA sequencing. p. 115-175. *In*: Stackebrandt E., Goodfellow M., (eds.) *Nucleic Acid Techniques in Bacterial Systematics*. New York: John Wiley and Sons.

Lavoie, K.H. and Northup, D.E. 1994. Distributional survey of actinomycetes in a limestone cave and a lava tube cave. pp.44-45. In: Sasowsky I.D., Palmer M.V. (eds.) *Breakthroughs in karst geomicrobiology and redox geochemistry: abstracts and field-trip guide for the symposium held February 16 through 19, 1994 Colorado Springs, Colorado*. Special Pubication **1**. Charles Town, WV: Karst Waters Institute, Inc.

Maidak, B.L., Cole, J.R., Lilburn, T.G., Parker Jr., C.T., Saxman, P.R., Farris, R.J., Garrity, G.M., Olsen, G.J., Schmidt, T.M., Tiedje, J.M. 2001. The RDP-II (Ribosomal Database Project). Nucleic Acids Research **29**:173-174.

Northup, D.E. and Lavoie, K.H. 2001. Geomicrobiology of caves. Geomicrobiology Journal **18**(3): 199-222.

Northup, D.E. and Welbourn, W.C. 1997, Life in the twilight zone: Lava tube ecology. New Mexico Bureau of Mines & Mineral Resources Bulletin **156**: 69-82.

Pace, N.R. 1997. A molecular view of microbial diversity and the biosphere. Science **276**(5313): 734-740.

Schlesner, H., Jenkins, C. and Staley, J.T. 2001. The phylum Verrucomicrobia: A phylogenetically heterogeneous bacterial group. *In*: M. Dworkin et al., eds., *The Prokaryotes: An Evolving Electronic Resource for the Microbiological Community*, 3rd edition, release 3.19, Springer-Verlag, New York, http://link.springer-ny.com/link/service/books/10125/.

Senger, C.M. and Crawford, R.L. 1984. Biological inventory: Mount St. Helens Cave basalt flow area: Final Report. Unpublished report prepared for the Gifford Pinchot National Forest, Mount St. Helens National Volcanic Monument, St. Helens Ranger District. 526 pp.

Staley, J. T. and Crawford, R. 1975. The biologist's chamber: lava tube slime: Cascade Caver **14** (2-3): 20-21.

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