The Impact of Host Rock Geochemistry on Bacterial Community Structure in Oligotrophic Cave Environments.


Abstract:

Despite extremely starved conditions, caves contain surprisingly diverse microbial communities. Our research is geared toward understanding what ecosystems drivers are responsible for this high diversity. To assess the effect of rock fabric and mineralogy, we carried out a comparative geomicrobiology study within Carlsbad Cavern, New Mexico, USA. Samples were collected from two different geologic locations within the cave: WF1 in the Massive Member of the Capitan Formation and sF88 in the calcareous siltstones of the Yates Formation. We examined the organic content at each location using liquid chromatography mass spectroscopy and analyzed microbial community structure using molecular phylogenetic analyses. In order to assess whether microbial activity was leading to changes in the bedrock at each location, the samples were also examined by petrology, X-ray diffraction (XRD) and scanning electron microscopy with energy dispersive X-ray spectroscopy (SEM-EDX). Our results suggest that on the chemically complex Yates Formation (sF88), the microbial community was significantly more diverse than on the limestone surfaces of the Capitan (WF1), despite a higher total number of cells on the latter. Further, the broader diversity of bacterial species at sF88 reflected a larger range of potential metabolic capabilities, presumably due to opportunities to use ions within the rock as nutrients and for chemolithotrophic energy production. The use of these ions at sF88 is supported by the formation of a corrosion residue, presumably through microbial scavenging activities. Our results suggest that rock fabric and mineralogy may be an important driver of ecosystem function and should be carefully reviewed when carrying out microbial community analysis in cave environments.

Keywords: caves, geomicrobiology, geochemistry, phylogenetics, oligotrophy

INTRODUCTION
Caves, with limited exception, form through the erosional processes of water. By the time caves are enlarged sufficiently to allow human access, the water has (generally) departed, leaving the cave exposed to an oxygenated atmosphere (Klimchouk et al., 2000). Without sunlight energy, the entry of nutrients into the system becomes a function of the geology and depth of the cave; significant organic input is limited to the entrance zone and areas fed by surface water entering the system through faults and fractures (Klimchouk et al., 2000). Due to extremely low biomass in these environments and the difficulty in extracting DNA from chemically complex geologic samples, most studies of microbial activity in cave environments have tended to examine areas of measurable energy input (Angert et al., 1998; Barton & Luiszer, 2005; Bottrell et al., 1991; Culver, 1982; Groth et al., 2001; Sarbu et al., 1996). In a recent study we used molecular phylogenetics to determine what, if any, microbial activity was occurring within an oligotrophic cave without measurable energy input (Barton et al., 2004). Our results suggested that a diverse microbial flora subsisted in this oligotrophic environment, in contrast to previous cultivation studies from similar environments (Groth & Saiz-Jimenez, 1999; Groth et al., 1999). Further, the microbial community within this environment appeared to subsist by using barely perceptible carbon and energy sources; these included organics entering the system through percolation, or the presence of volatile organic molecules within the atmosphere (Barton et al., 2004). The presence of bacterial phylotypes with identity to organisms capable of carrying out iron oxidation suggested the use of reduced iron as an energy source, while
a high proportion of nitrogen assimilating organisms suggested a source for nitrogen (Laiz et al., 1999). A broad phylogenetic distribution of bacterial species has been identified by other investigators in similarly oligotrophic environments (Chelius & Moore, 2004; Osman et al., 2005).

Together, our data led us to hypothesize that the large diversity of microorganisms found in oligotrophic cave environments may reflect mutualistic interactions to support community growth under such starved conditions (Barton & Jurado, 2007); due to the complex nature of the organic carbon and inorganic energy sources, not one organism is capable of carrying out all the energetically favorable reactions necessary to support growth (Juttner, 1984; Laiz et al., 1999). Rather, energetic restrictions allow certain reactions to proceed only through a close interaction with species that remove intermediates, allowing energy conservation in what would otherwise be an endothermic reaction (Schink, 2002). Mutualism has been described for microbial communities carrying out anaerobic ethanol fermentation, methane oxidation and the breakdown of complex aromatic compounds (Schink, 2002). Such interactions may also be a central issue in the unculturability of most microorganisms in the environment; mutualistic interactions make many organisms recalcitrant to cultivation, where appropriate growth conditions may be dependent on specific interactions with other species (Grotenhuysen et al., 1991; Mohn & Tiedje, 1992).

To further examine drivers of microbial diversity in starved environments, this study attempts to determine what role rock fabric and mineralogy plays in community diversity under oligotrophic conditions. This was done by carrying out a comparative analysis of two microbial communities within Carlsbad Cavern, New Mexico, USA, where bacterial species exist on disparate geologic surfaces. We used a molecular and geochemical approach to examine community structure at each location and compared microbial interactions with the rock matrix of the cave.

**MATERIALS AND METHODS**

**Sample sites and geology**

Carlsbad Cavern was formed in the Capitan Reef complex by hypogenic sulfuric acid speleogenesis, with a postulated biogenic origin (Engel et al., 2004; Hill, 1990; Palmer, 2000). The Carlsbad cave system is mostly within the Capitan limestone, where the relatively impermeable iron-rich, silty Yates Formation traps oxygenated groundwater and releases it into the Capitan Formation (Palmer, 2000). Upper portions of the cave are also located in the Yates Formation, which contains numerous calcareous siltstones, sandstones and secondary minerals. The cave is located in a desert area, does not contain any surface streams and is not prone to flooding.

Samples for collection were identified within Carlsbad Caverns based on a number of parameters, including geologic location, altered bedrock or secondary mineralization (Fig. 1). The first site, WF1, is along the Main Corridor and located in the limestone of the Massive Member of the Capitan Formation (CaCO$_3$), with an average annual temperature of 12.5˚C (Forbes 2000) and a relative humidity (RH) of 95%, measured using an RH300 Digital Psychrometer (Extech Instruments, Waltham, MA). The second site, sF88, is located within the Yates Formation, with the sample collection site directly above a calcareous siltstone bed (Fig. 1), with an average annual temperature of 16.3˚C and measured RH of 99% (Forbes 2000). The Yates is comprised a fine-grained, laminated psilomic dolomite in thin beds, inter-layered with thin layers of calcareous red quartz siltstones and fine-grained sandstones (Borer & Harris, 1991; Brown & Loucks, 1993; Duchene, 2000; Mutti & Simo, 1993). The Yates is rich in magnesium and iron, with its red color due to the presence of hematite (Fe$_2$O$_3$), which is not generally detectable by scanning electron microscopy energy dispersive X-ray spectroscopy (SEM-EDX) (Borer & Harris, 1991). Three 5 g rock samples were collected from each location using a sterilized Dremel drill tool and each sample was preserved in an appropriate manner for the subsequent tests: DNA extraction in 70% alcohol / -20°C; chemical samples were collected in gamma-irradiated clean tubes and stored at 4°C; rock samples were collected in 50 ml plastic tubes.

**Fig. 1.** Profile (line plot facing north) of the Carlsbad Cavern cave system (approximately 48 km of passage is represented) with the corresponding geologic units of the Capitan Reef complex overlain. The two sample locations (WF1 and sF88) are indicated by the filled circles. The entrance and ‘Big Room’ are designated. Courtesy of the Cave Resource Office, Carlsbad Caverns National Park.
Chemical Analysis

Total organic carbon (TOC) was measured by extracting crushed rock with dH₂O and then determining the TOC g⁻¹ of rock material using a Shimadzu TOC-VCSN analyzer at Waters Laboratory, Western Kentucky University, KY. Analyses of sample extracts for organic carbon were carried out using high performance liquid chromatography-mass spectrometry (HPLC/MS). The system consisted of an Alliance 2695 HPLC system, 2996 photodiode array detector and a ZQ single quadrupole mass spectrometer (all equipment was from Waters Corp., Milford, MA). Approximately 100mg of sample from each of the sites was extracted in 1 ml of a 50/50 water and acetonitrile solution by sonication (all solvents were of HPLC grade or better). Samples were then allowed to stand and settle prior to the top (clear) layer extracted and analyzed by HPLC/MS. A standard gradient was run using an Xterra C18 column (2.1X100mm 3.5μm particle) using a formic acid and acetonitrile gradient over 30 minutes.

DNA extraction

DNA extractions were carried out in a laminar-flow hood, using aseptic techniques and aerosol resistant tips to reduce the chance of contamination from outside sources (Barton et al., 2006). Unless stated otherwise, all chemicals were obtained from Sigma-Aldrich (St. Louis, MO) and reagents used were prepared from Fluka ultrapure DNase/RNase Free water, followed by filtration through a 0.2 μm cellulose filter to prevent contamination. In cases where contaminating DNA may be introduced from reagents, these were subjected to 3000 μJ cm⁻² of UV radiation using a Stratalinker 2400 (Stratagene, La Jolla, CA). To extract the small amount of DNA present in the rock, a modified bead beating method was used.

To extract the DNA, approximately 0.5g of sample was crushed using a flame-sterilized plattner’s mortar and pestle (Humboldt Manufacturing, Norridge, IL). To this 500μl 2X buffer EA [200 mM Tris (pH 8.0), 300 mM EGTA, 200 mM NaCl], 3 mg/ml lysozyme and 10 μg/ml poly-dIdC were added, and incubated at 37°C for 30 min. Proteinase K (to 1.2 mg/ml) and 100 μg/ml sodium dodecyl sulfate (SDS to 0.3% wt/vol) were added, mixed gently and incubated at 50°C for 30 min. Subsequently, 200μl of 20% SDS and 500μl phenol-chloroform-isoamyl alcohol (24:24:1) was added before disruption using a Mini-bead beater (Biospec, Bartlesville, OK) on low setting for 2 min and high for 30 s. Samples were centrifuged at 13,000 x g in a micro-centrifuge for 3 min at 4°C to deposit the sample debris; the supernatant (approximately 700-800μl) was then removed and the DNA by the addition of 2 μg poly-dIdC, 0.3 M sodium acetate and 2 volumes of cold ethanol. Isolated DNA was further purified by dialysis against 100 ml of 20 mM EGTA at 4°C for 4 hours in a Slide-A-Lyzer mini dialysis unit (3500 MWCO; Pierce, Rockford, IL) to remove any remaining calcium carbonate. The concentration of the final DNA preparation was determined using a Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE).

Polymerase Chain Reaction (PCR) and cloning

To isolate individual 16S rRNA gene clones, PCR amplification was used. The bacterial 16S rRNA gene specific 27F (5’ – AGA GTT TGA TCC TGG CTC AG – 3’) and universal 805R (5’ – GAC TAC CAG GGT ATC TAA T – 3’) primers were used in reaction mixtures containing 1 X PCR buffer (Perkin Elmer), 2.5 mM MgCl₂, 200 μM of each deoxynucleoside triphosphate, 300 nM of each forward and reverse primer, and 0.025 U of AmpliTaq Gold (Perkin Elmer) per μl. Reaction mixtures were incubated on a Mastercyber Gradient thermal cycler (Eppendorf Scientific) at 94°C for 12 min for initial denaturation and activation of the AmpliTaq Gold. PCR was then carried out with 34 cycles of 94°C 30 s, 58°C 45 s, 70°C 1 min, and a final extension period of 72°C 2 min. PCR products were then digested using HindPI1 and MspI restriction enzymes in NEB buffer 2 (New England Biolabs, Beverly, MA). The restriction digest was incubated at 37°C for 2 hours before being run on a 2% wt/vol agarose gel containing ethidium bromide, purified using a Qiagen PCR clean up kit (Qiagen, Valencia, CA) and cloned into an pCR2.1-TOPO cloning vector according to the manufacturer’s recommendations (Invitrogen, Carlsbad, CA).

Screening of rDNA clones by restriction length polymorphism (RFLP) and DNA sequencing

The 16S rRNA gene inserts were PCR re-amplified using 100 ng T3 forward and T7 reverse primers under standard conditions and amplified using 94°C for 4 min for initial denaturation, then 38 cycles of 94°C 1 min, 52°C 45 s, 72°C 1 min, with an extension period of 72°C for 8 min. PCR products were then digested using HindPI1 and MspI restriction enzymes in NEB buffer 2 (New England Biolabs, Beverly, MA). The restriction digest was incubated at 37°C for 2 hours before being run on a 2% wt/vol SeaKem LE agarose gel (FMC BioProducts) and visualized with ethidium bromide staining with UV illuminescence. The unique RFLP patterns were grouped visually and a representative was selected for sequencing. Sequencing was carried out using the Thermo Sequenase Cycle Sequencing kit (USB, Cleveland, Ohio) according to the manufacturer’s guidelines. For areas that were problematic due to regions of high GC content, a SequiTherm Excell II DNA sequencing kit (Epicenter Technologies, Madison, WI) was used. Sequencing was carried out using fluorescently labeled sequencing primers M13 and T7 on a Long Gradient thermal cycler (Eppendorf Scientific) at 94˚C for 4 min for initial denaturation and activation of the AmpliTaq Gold. PCR was then carried out with 34 cycles of 94°C 30 s, 58°C 45 s, 70°C 1 min, and a final extension period of 72°C 2 min. PCR products were then digested using HindPI1 and MspI restriction enzymes in NEB buffer 2 (New England Biolabs, Beverly, MA). The restriction digest was incubated at 37°C for 2 hours before being run on a 2% wt/vol SeaKem LE agarose gel (FMC BioProducts) and visualized with ethidium bromide staining with UV illuminescence. The unique RFLP patterns were grouped visually and a representative was selected for sequencing. Sequencing was carried out using a formic acid and acetonitrile gradient over 30 minutes.

Phylogenetic Analysis

Sequences were compared to available databases by use of the BLAST (Basic Local Alignment Search Tool) network service [http://www.ncbi.nlm.nih.gov/ BLAST; Altschul et al., 1997] Partial sequences of the 16S rRNA gene were compiled using the AlignIR 2.0 Fragment Assembly and Contig Editor software (Li-Cor, Inc). Compiled sequences were examined for chimeric
sequences by use of the CHIMERA_CHECK program [http://rdp.cme.msu.edu/html/analyses.html] and by phylogenetic branching order discrepancies. Before further phylogenetic analysis, those sequences displaying similar BLAST hits were directly compared using the pairwise BLAST alignment tool [http://www.ncbi.nlm.nih.gov/blast/bl2seq/bl2.html]. Any sequences that demonstrated ≥ 98% identity toward each other were considered representatives of the same phylogroup and grouped accordingly; the remaining sequences screened for contaminants against the LBC database (Barton et al., 2006). Sequence alignments were carried out using the ARB Software Package [http://mpi-bremen.de/molecol/arb], with additional sequences from the Ribosomal Database Project (Maidak et al., 2000). Sequence alignments used for phylogenetic inference were minimized by use of the Lane Mask, which removes hypervariable regions of the 16S rRNA gene sequence from the analysis for Bacterial data sets. Due to the size of the 16S rRNA clones isolated in this study (~800 bp), only representative sequences from position 40 to 790 (E. coli numbering) were used in subsequent phylogenetic analyses. All presented dendrograms were constructed by use of ARB with evolutionary distance (neighbor-joining) and parsimronous (heuristic) algorithms. The robustness of inferred topologies was tested by bootstrap resampling (1000 replicates) of phylogenetic trees, calculated for both algorithms using PAUP* software (Sinauer Associates Inc., Sunderland, MA).}
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**Fig. 2.** Consensus dendogram of the 16S rRNA gene sequence phylotypes identified from the Carlsbad clone libraries WF1 (white boxes) and sF88 (black boxes). Phylogenetic analyses were carried out using both distance (Neighbor-Joining) and parsimonious (Heuristic) searches, with the robustness of inferred topologies determined by bootstrap analysis (1000 replicates); the consensus dendogram of both methods is shown. Branch points supported (bootstrap values >70%) in both phylogenetic analyses are indicated by closed circles, while marginal branch support (bootstrap values >50% but >70%) in both analyses are show by an open circle. The bar indicates 10% sequence divergence.
the sites using liquid chromatography coupled mass spectrometry (LC/MS). A preliminary analysis of the LC/MS peaks indicates that the organic matter present is low (measured as $3 \mu g g^{-1}$ at sF88), although slightly higher at WF1 (results not shown). The data suggest that the organic material is of a phenolic and aromatic nature, which is in accord with the structure of the organic material commonly found in soils and the postulated origin of each of the organic carbon observed in caves (Saiz-Jimenez & Hermosin, 1999; Sylvia et al., 1999).

Molecular Phylogenetic Analysis of WF1 versus sF88 Communities

In order to determine whether the differences in geologic chemistry affected the structure of the microbial communities found at WF1 and sF88 we carried out a molecular phylogenetic study. Due to the difficulty of obtaining DNA from calcium rich samples, along with the small biomass associated with these extremely starved environments, we have developed a new extraction protocol for calcium rich samples from caves (Barton et al., 2006). While this protocol allows us to reproducibly obtain DNA from extremely low-biomass environments, we still routinely obtain less than 500 ng of community DNA/g of material. We therefore use the 8F and 805R 16S rRNA gene primer set for amplification, which provides the most reliable PCR amplification at these low DNA concentrations. While the subsequent rDNA product is short (~800 bp in length), it still provides sufficient information for statistically significant phylogenetic placement (Nei et al., 1998).

Clone libraries were created for both sF88 and WF1 locations by ligating the PCR product into the plasmid vector and transforming into chemically competent E. coli cells; for sF88 144 clones were isolated, while at WF1, 96 representative clones were used. The 16S rRNA gene sequence of each clone library was screened by RFLP analysis to identify unique phylotypes. The final clone libraries contained 49 unique phylotypes for sF88 and 38 unique phylotypes for WF1 and were grouped into operational taxonomic units (OTUs), demonstrating >98% identity for tree building. The sequences of these phylotypes were compared with the NCBI database and the closest cultivated relative was identified (Table 2).

Surprisingly, many of the phylotypes we identified shared a greater degree of identity with previously cultivated species than in a past cave study (Barton et al., 2004); however, this may simply reflect the increase in size of the 16S rRNA gene sequence database. In order to confirm the identity of these identified phylotypes, they were phylogenetically aligned using the ARB sequence analysis program, followed by statistical analysis of the resultant dendogram using the PAUP* software program. The consensus tree for each location confirmed the phylogenetic placement of the identified species (Fig. 2). Interestingly, the distribution of phylotypes identified correlates well with previous cave environments and similarly starved locations (Barton et al., 2004; Chelius & Moore, 2004; Osman et al., 2005). It is also interesting to note that many of the sF88 phylotypes share the closest identity to 16S rRNA gene sequences identified in the WF1 library, suggesting the shared ancestry of the two communities within this cave environment.

While the identity of unique phylotypes at sF88 and WF1 demonstrates a similar distribution among the bacterial divisions, the dendogram does not reflect the relative abundance of the phylotypes identified in each location, which are represented in Fig. 3. It is interesting that this comparative pie-chart demonstrates a much greater species distribution within the chemically complex sF88 environment, even while there is a higher absolute number of bacterial cells at the WF1 site. In order to determine whether there were statistically significant differences in community structure between each site, we created rarefaction curves using the Chao 2 non-parametric estimator to determine true species richness (Fig. 4). These rarefaction curves did indeed suggest that there were differences in the absolute diversity between the two microbial communities; however, the 95% confidence levels suggest that the sample sizes need to be increased to determine the significance of these differences.

Geologic Samples: Thin-sections and X-ray powder diffractometry (XRD)

One of the most striking differences between WF1 and sF88 sites was the presence of a corrosion residue
at the more starved sF88 site. The two localities were compared for mineralogic alteration using thin-section petrography, XRD and SEM-EDX. Petrologically, both localities were quite different, reflecting the difference in composition between the Capitan and Yates formations (Fig. 5A and 5B). SEM images confirmed a calcite bedrock with an apparent microbial biofilm at WF1, but no significant corrosion surface (Fig. 5A and 5C). At sF88, however, the surface of the rock underwent a number of mineralogical and crystallographic changes, resulting in a poorly consolidated corrosion residue (Fig. 5B and 5D). This corrosion residue is comprised of dolomite recrystallized into coarser crystals, with fine-grained clay minerals and other opaque minerals present between them.

Microbial species often change the chemical nature of the environment on which they live through catabolic processes (Banfield & Nealson, 1997). In order to determine if such transformations were occurring at WF1 or sF88 we carried out an SEM-EDX analysis of insoluble particulate matter in the rock. In order to identify such minerals, we extracted the rock in each location with 1M hydrochloric acid to remove the overwhelming carbonate minerals and examined individual particulate grains. At WF1 this material is comprised of clay particles (representative EDX spectra in Fig. 5E and 5F) normally associated with the Massive Member of the Capitan Formation while at sF88 this material comprised of iron oxides and elemental iron (representative EDX spectra in Fig. 5G and 5H). These iron forms are too small and/or amorphous to be detected in our XRD analyses (Fig. 6), suggesting a biogenic origin. These results support the theory that iron oxidation may be one mechanism of energy production at the sF88 location, indicating that the microorganisms living in these environments may be acquiring energy from the host rock itself.

In order to compare the changes in geologic structure at the two localities, samples were analyzed using comparative XRD (Fig. 6). At each location, the sample collected was broken down into two components; the interior bedrock and the surface layers. The interior rock was >1 cm away from any observable surface feature (as demonstrated by the thin-section analysis).
and was considered representing the bedrock mineralogy. Each layer was clearly marked in hand sample, allowing for segregation of the layers prior to powdering for XRD. The results (Fig. 6) demonstrated that, in agreement with our thin-section analysis, that there were no significant changes in the mineral structure of the surface rock at WF1, when compared with the host-rock matrix; however, there was an accumulation of clay sized particles. We have seen such clays accumulating at other sites within Carlsbad Cavern that demonstrate biogenic activity (Bertog et al., unpublished results). Heating of the sample to 350°C for 30 min led to the loss of the clay peak, suggesting kaolinite. At sF88 there appeared to be more significant mineral changes; dolomite in the bedrock had been removed with an increase in the relative abundance of the non-soluble bedrock material, such as quartz and other silicates (Fig. 6) as would be expected if the calcareous cements of the Yates Formation had been dissolved. SEM images of sF88 confirmed a crystallographic change in the corrosion residue, with a fine (<1μm) powdery residue (Fig. 5D).

DISCUSSION

The majority of caves contain little available carbon, making them an ideal environment in which to study oligotrophic microbial interactions and geochemical processes on exposed surfaces (Laiz et al., 1999). Such geomicrobial activity is thought to be indicated by the presence of corrosion residues: areas of fabric and mineralogical change in the bedrock, characterized by a color change and softening or powdering of the rock (Boston et al., 2001; Canaveras et al., 2001; Northup et al., 2003). While these residues do contain an observable microbial population, a mechanism of formation remains to be determined (Canaveras et al., 2001; Northup et al., 2003).

At the geochemically simple WF1 site (Capitan), the microbial community is dominated by members of the Actinobacteria, a broad class of high G+C, gram-positive bacteria found predominantly within soil. Of these, representative phylotypes of the Pseudomonocordaria appear to be the most abundant, representing half of all the identified Actinobacteria and over 80% of the total community of bacteria found at this location (Fig. 3). While the identity between the predominant phylotype and the next closest cultivated species is only 97%, we can postulate on a general function of this species in the environment (Achenbach & Coates, 2000; Pace, 1997). Members of the Pseudomonocordaria are aerobes that demonstrate a wide metabolic range for the degradation of complex plant matter, such as cellulose, suggesting that the community at WF1 is primarily using soil detritus for growth (Dworkin, 2002). Interestingly, other phylotypes identified at WF1 share similarity to Acinetobacter johnsonii, able to mobilize phosphate from inorganic sources, and Comamonas spp., which degrade a number of nitrogen-containing aromatic compounds, with the release of usable nitrate and ammonia (Dworkin, 2002; Itoh & Shiba, 2004). Both of these groups similarly display saprophytic lifestyles and are routinely found in the environment under nutrient limiting conditions (Dworkin, 2002).

In contrast to the relatively simple microbial diversity identified at WF1, the clone library generated at the more geochemically complex sF88 site (Yates) was more diverse, with representatives from the Alpha-, Beta- and Gammaproteobacteria (Fig. 3); very similar in structure to other oligotrophic cave environments (Barton et al., 2004; Chelius & Moore, 2004). Among the phylotypes identified, there was significant representation by members of the genera Brevundimonas, Massilia and Stenotrophomonas; 26%, 18% and 17% respectively. Representative Brevundimonas spp. are from the Caulobacter family, which are oligotrophic organisms able to adapt to extremely starved environments (Dworkin, 2002; Li et al., 2004). Members of the genus Massilia are able to utilize a large number of carbohydrates and other complex organic molecules as carbon and energy sources, with the subsequent production of acids (Dworkin, 2002). Such activity may explain some of the significant structural changes observed in the Yates host rock, where the calcareous cements of the siltstones are easily dissolved by acids, leading to the formation of the observed corrosion residues. The large number of phylotypes representative of Stenotrophomonas and Delftia spp. identified at sF88 (Table 2) is less easily explained, as members of these genera carry out denitrification reactions, with the conversion of ammonia to nitrous oxide (Dworkin, 2002). These organisms also play an important role in the denitrification of complex organic compounds, such as nitrobenzene. Such denitrification activity...
is difficult to explain in the context of nitrogen starvation, unless a key energy conservation activity is nitrate reduction and/or the reduction of nitrogen-containing aromatic compounds.

One interesting observation through the SEM-EDX analyses was the selective enrichment of iron oxides within the corrosion residues observed at sF88, while our clone library does not demonstrate the presence of any ‘classic’ iron-oxidizing species (Table 2). One explanation may be that the iron-oxidation that is observed on the surface of the rocks could be the direct result of autodissolution, wherein reduced iron within the host rock is exposed to the oxygenated atmosphere of the cave through microbial processes (Ehrlich, 2002). Nonetheless, it would be surprising if the oligotrophic community at sF88 did not harness Fe(II) as an electron donor before its loss. The absence of well-known iron-oxidizing species may reflect the need for a more exhaustive phylogenetic examination of this site (Ehrlich, 2002; Ley et al., 2006). As with the WF1 community, nitrogen and phosphorus must be growth limiting factors at sF88. It is then hardly surprising that phylotypes related to the nitrogen assimilating species Herbaspirillum frisingense and Janthinobacterium agaricidamnosum were found. Interestingly Acinetobacter spp., which can also mobilize inorganic phosphate, were identified at sF88 and may provide an important clue for nutrient acquisition in these starved ecosystems (Van Groenestijn et al., 1988).

In attempting to understand the mechanisms that support the often surprising levels of microbial diversity in very starved cave environments, our results suggest that community structure may be greatly affected by the chemical nature of the rock on which these organisms grow. In the case of the WF1 community, which grows on limestone, the rock has little potential for additional energy sources. As a result, the community appears to rely more heavily on heterotrophic growth from allochthonous energy sources. The clay particles seen with XRD at this site may be due to the production of organic acids by microbial species, utilizing these reduced compounds for growth, which leads to the accumulation of these insoluble particles. At sF88, the geochemical complexity of the rock may provide additional energy sources, allowing species to use chemolithotrophic mechanisms to conserve energy. The trace elements available at sF88 could also prove essential to the growth of microbial species, allowing the formation of co-enzymes critical in intermediate metabolism. Indeed, we have known for decades that many cell types cannot grow without the addition of specific mineral supplements (Conway de Macario et al., 1982; Morgan, 1958; Roth et al., 1996). It is therefore no surprise that the geochemistry of the bedrock can impact both the microbial species capable of growth as well as the types of energy conservation reactions observed. The necessity for trace elements and inorganic energy sources in growth is apparent at the sF88 site, where microbial metabolic transformation has led to extensive mineralogic alterations of the Yates rock fabric and the formation of a corrosion residue. Our results suggest that such variations in geochemistry may have a profound affect on microbial community structure in cave environments. As a result, care should be taken when choosing sample sites for microbial study within caves, as the geologic setting may add unforeseen complexity to analyses or complicate the interpretation of comparative studies. Not only does this study hint at the high microbial diversity in caves, in which niche biogeochemistry may be an important driver of species diversity (Begon et al., 1998), it also emphasizes the need for a thorough understanding of the geologic conditions when studying such environments.

ACKNOWLEDGEMENTS

The authors wish to thank Brad Lubbers for excellent technical assistance, Karl Hagglund and Brenda Racke for assistance with the SEM and EDX analyses, Matthew Zacate for assistance in establishing the ARB database and running the PAUP software, Michael Queen for excellent assistance in interpretation of the geology of Carlsbad Caverns, and Harvey DuChene and an anonymous reviewer for critical comments that significantly improved the manuscript. We would also like to thank the staff, in particular Paul Burger, at the Cave Resource Office at Carlsbad Caverns National Park for their invaluable assistance with sample collection.

This work was supported in part by the Kentucky EPSCoR Program, the Kentucky Academy of Science, the Center for Integrative Natural Science and Mathematics (CINSAM) at NKU, and the National Park Service. Infrastructure support was provided, in part, by the National Institutes of Heath KY INBRE program (5P20RR016481-05). NMT and MPK were additionally supported by NKU SURG awards.

REFERENCES


Table 1. EDX Bulk Elemental Analyses at WF1 and sF88

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<th>sF88 Average Wt%*</th>
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ND=none detected

*a=the average was taken from two separate analysis for 50 seconds at 2 kV (take of angle 38,3° count min^-1

Table 2. Summary of the unique phylotype groups identified in the sF88 and WF1 clone libraries.

<table>
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<th>Phylogenetic Group</th>
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<th>Clones Group</th>
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<td>Alphaproteobacteria</td>
<td>NMTsF8</td>
<td>26/32</td>
<td>Brevundimons nasdae</td>
<td>99%</td>
<td>DQ066606</td>
</tr>
<tr>
<td></td>
<td>NMTsF27</td>
<td>5/32</td>
<td>Brevundimons vesicularis</td>
<td>94%</td>
<td>DQ066612</td>
</tr>
<tr>
<td></td>
<td>NMTsF32</td>
<td>1/32</td>
<td>Caulobacter subvibrioides</td>
<td>99%</td>
<td>DQ066613</td>
</tr>
<tr>
<td>Betaproteobacteria</td>
<td>NMTsF1</td>
<td>18/29</td>
<td>Massilia sp.</td>
<td>98%</td>
<td>DQ066600</td>
</tr>
<tr>
<td></td>
<td>NMTsF3</td>
<td>6/29</td>
<td>Delftia tsuruhtasis</td>
<td>99%</td>
<td>DQ066602</td>
</tr>
<tr>
<td></td>
<td>NMTsF7</td>
<td>2/29</td>
<td>Acidovorax sp.</td>
<td>98%</td>
<td>DQ066605</td>
</tr>
<tr>
<td></td>
<td>NMTsF13</td>
<td>2/29</td>
<td>Herbaspirillum frisingense</td>
<td>100%</td>
<td>DQ066607</td>
</tr>
<tr>
<td></td>
<td>NMTsF44</td>
<td>1/29</td>
<td>Janthinobacterium agaricidamnosum</td>
<td>99%</td>
<td>DQ066617</td>
</tr>
<tr>
<td>Gammaproteobacteria</td>
<td>NMTsF2</td>
<td>17/31</td>
<td>Stenotrophomomas sp.</td>
<td>98%</td>
<td>DQ066601</td>
</tr>
<tr>
<td></td>
<td>NMTsF6</td>
<td>1/31</td>
<td>Uncultured Acinetobacter sp.</td>
<td>99%</td>
<td>DQ066604</td>
</tr>
<tr>
<td></td>
<td>NMTsF16</td>
<td>1/31</td>
<td>Uncultured bacteria clone FS117-02</td>
<td>89%</td>
<td>DQ066608</td>
</tr>
<tr>
<td></td>
<td>NMTsF19</td>
<td>7/31</td>
<td>Cellvibrio ostraviensis</td>
<td>98%</td>
<td>DQ066609</td>
</tr>
<tr>
<td></td>
<td>NMTsF26</td>
<td>4/31</td>
<td>Xanthomas retroflexus</td>
<td>99%</td>
<td>DQ066611</td>
</tr>
<tr>
<td></td>
<td>NMTsF36</td>
<td>1/13</td>
<td>Pseudomonas borealis</td>
<td>99%</td>
<td>DQ066614</td>
</tr>
<tr>
<td>Cytophagales</td>
<td>NMTsF40</td>
<td>1/1</td>
<td>Uncultured Bacteroidetes bacterium</td>
<td>98%</td>
<td>DQ066616</td>
</tr>
<tr>
<td>Actinobacteria</td>
<td>NMTsF4</td>
<td>2/7</td>
<td>Nocardioides sp.</td>
<td>98%</td>
<td>DQ066603</td>
</tr>
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<td>2/7</td>
<td>Rhodococcus sp.</td>
<td>100%</td>
<td>DQ066610</td>
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<td>Mycobacterium gordonae</td>
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<td>DQ066615</td>
</tr>
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<td>Curtobacterium sp.</td>
<td>99%</td>
<td>DQ066618</td>
</tr>
<tr>
<td>WF1 Library</td>
<td>NMT-WF15</td>
<td>1/1</td>
<td>Methylobacterium aquaticum</td>
<td>99%</td>
<td>DQ228717</td>
</tr>
<tr>
<td>Alphaproteobacteria</td>
<td>NMT-WF23</td>
<td>1/1</td>
<td>Comamonas sp.</td>
<td>98%</td>
<td>DQ228718</td>
</tr>
<tr>
<td>Betaproteobacteria</td>
<td>NMT-WF13</td>
<td>11/13</td>
<td>Uncultured bacterial mud-clone</td>
<td>90%</td>
<td>DQ228716</td>
</tr>
<tr>
<td></td>
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<td>2/13</td>
<td>Acinetobacter johnsonii</td>
<td>99%</td>
<td>DQ228719</td>
</tr>
<tr>
<td>Actinobacteria</td>
<td>NMT-WF1</td>
<td>34/65</td>
<td>Pseudonocardia sp.</td>
<td>97%</td>
<td>DQ228711</td>
</tr>
<tr>
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<td>NMT-WF4</td>
<td>14/65</td>
<td>Bacterium Chibacore 1500</td>
<td>90%</td>
<td>DQ228712</td>
</tr>
<tr>
<td></td>
<td>NMT-WF7</td>
<td>4/65</td>
<td>Crossiella equi</td>
<td>97%</td>
<td>DQ228713</td>
</tr>
<tr>
<td></td>
<td>NMT-WF9</td>
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<td>Saccharothrix crythophilis</td>
<td>97%</td>
<td>DQ228714</td>
</tr>
<tr>
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<td>11/65</td>
<td>Actinomyces sp.</td>
<td>91%</td>
<td>DQ228715</td>
</tr>
<tr>
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<td>NMT-WF33</td>
<td>1/65</td>
<td>Actinobacterium sp.</td>
<td>93%</td>
<td>DQ228720</td>
</tr>
</tbody>
</table>

*Sequences were compared against the NCBI GenBank database using a standard BLAST search (08/04; Altschul et al. 1997)

Table 2. Summary of the unique phylotype groups identified in the sF88 and WF1 clone libraries.


Nei M., Kumar S. & Takahashi K., 1998 - The optimization principle in phylogenetic analysis tends to give incorrect topologies when the number of nucleotide or amino acids used is small. Proc. Ntl. Acad. Sci. USA., 95: 12390-12397.


