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

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Abstract:

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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 asses 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

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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

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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 (Grotenhuis 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 is 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 pisolitic 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_2O_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.

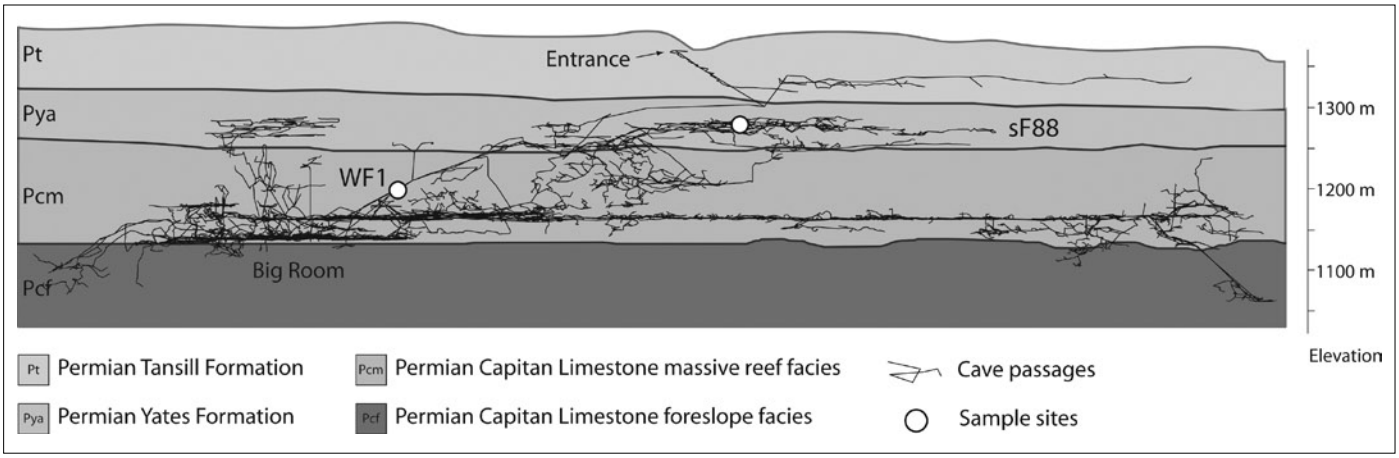

Fig. 1. Profile (line plot facing north) of the Carlsbad Cavern cave system (approximately 48 km of passage is represented) with the corresponding geologic units of the Capitan Reef complex overlain. The two sample locations (WF1 and sF88) are indicated by the filled circles. The entrance and 'Big Room' 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 dH_2O 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 quadrapole 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 than 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 MS 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^{-1} 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 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 Silde-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 $MgCl_2$, 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 Mastercycle 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 30 s, 70°C 1 min 30 s, and a final extension period of 70°C 2 min. PCR products were quantified by electrophoresis using a 1.2% 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 manufacturers 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 ReadIR 4200 DNA sequencer (Li-Cor, Lincoln, NE), which achieved approximately an 800 base rRNA gene insert in both the forward and reverse directions.

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 phylotype 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 parsimonious (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). The sequences obtained from the 16S rRNA gene clones in this study were deposited in the Genbank database, accession numbers DQ066600-DQ066618 and DQ228711-DQ228720.

Statistical Approaches

The statistical analyses were performed using EstimateS version 7.5.0 (Colwell, 2005). Each clone represented a separate sample without replacement, and 100 randomizations were performed to obtain the Chao2 estimator for each sample size (Chao et al., 2005; Gotelli & Colwell, 2001; Hughes et al., 2001). Using the singletons and doubletons calculated for each sample collection by EstimateS, we used the log transformation of Chao to calculate the 95% confidence intervals (Chao, 1987; Chao et al., 2005). Rarefaction curves were plotted using SigmaPlot for Windows Version 7.0 (SPSS Inc., Point Richmond, CA).

Scanning Electron Microscopy (SEM)

Samples were fixed prior to analysis in 4% paraformaldehyde/PBS. Biological samples were washed in 70% ethanol, and dehydrated in an ethanol series to 100%. Samples were dried in a critical point dryer using liquid CO_2 before examination under environmental SEM conditions using a FEI Quanta 200 ESEM with a Princeton Gamma Tech Avalon Microanalysis system and environmental secondary as well as backscatter electron detectors. Bulk chemistry of the host rock was determined by crushing samples and examining them by SEM-EDX using and EDAX brand EDX mounted on a Philips XL30 TMP scanning electron microscope.

Geologic analysis

Whole rock samples were separated into an outer residue layer and interior bedrock, powdered using a Spex Certiprep 8515 shatterbox and analyzed using a Rigaku Ultima III XRD under air-dried conditions. Samples were run from 2 (theta) to 70 (theta) with a step size of 0.05 (theta) and a count time of 2 seconds. For petrographic analysis, whole rock samples were sectioned, embedded in resin and cut and ground to 0.03mm for thin section analysis by Vancouver Petrographic Ltd. Samples were analyzed using a Nikon E400Pol polarizing microscope.

RESULTS

We began by identifying two distinct areas in Carlsbad Cavern that appeared to have limited organic carbon input, but quite different geologic settings (Fig. 1). WF1 is considered a chemically simple environment predominately calcium carbonate in chemistry, while sF88 is chemically complex, reflecting shoal and eolian deposition process that interlayered pisolitic dolomite with crystalline volcanic and metamorphic rock fragments. As a result, the Yates contains various accessory minerals, including hematite, magnetite (Fe_3O_4), tourmaline [$(Ca,Na)(Li,Mg,Al)(Al,Fe,Mn)_6(BO_3)_3(Si_6O_{18})(OH)_4$], zircon ($ZrSiO_4$), rutile ($TiO_2$), apatite [$Ca_5(PO_4)_3(F,Cl,OH)$] and epidote [$Ca_2(Al,Fe)_3(SiO_4)_3(OH)$]. To confirm these chemistries, we carried out EDX analysis of the host rock (Table 1), which confirmed the limestone nature of WF1 and the presence of quartz siltstone (SiO), magnesium, aluminum, potassium and iron at sF88. The other elements previously identified in the Yates (Li, Mn, B, etc.) were below the minimal detectable limits of this technique. At WF1 (Capitan), the rock on which microbial species are growing does not appear to have undergone any significant rock fabric changes, while at sF88 (Yates) varied colored corrosion residues were seen on the surface. We counted the number of microbial cells at each location by fluorescent microscopy (Barton et al., 2006). At WF1, there were 1.75×10^6 cells g^{-1} of wall rock, while at sF88 we observed 6.48×10^5 cells g^{-1} of material, which match the cell numbers seen in similarly oligotrophic cave environments (Barton et al., 2006). No eukaryotic predators were observed by microscopy from either location, ruling out predation as a driver of diversity (Hahn & Hofle, 2001).

Organic Chemistry and LC/MS analysis

Given the different lithology and mineralogy at each site, for example the Yates in known to be rich in organics and is a major reservoir for oil and gas in the Delaware Basin (Borer & Harris, 1991), we carried out a qualitative analysis to determine if there was a difference in the type of organic material present at each location. This would also rule out the presence of organics as a result of surface spills from the commercial facilities (sewage tanks, fuel tanks, etc.) above the cave. Analysis was done of each of

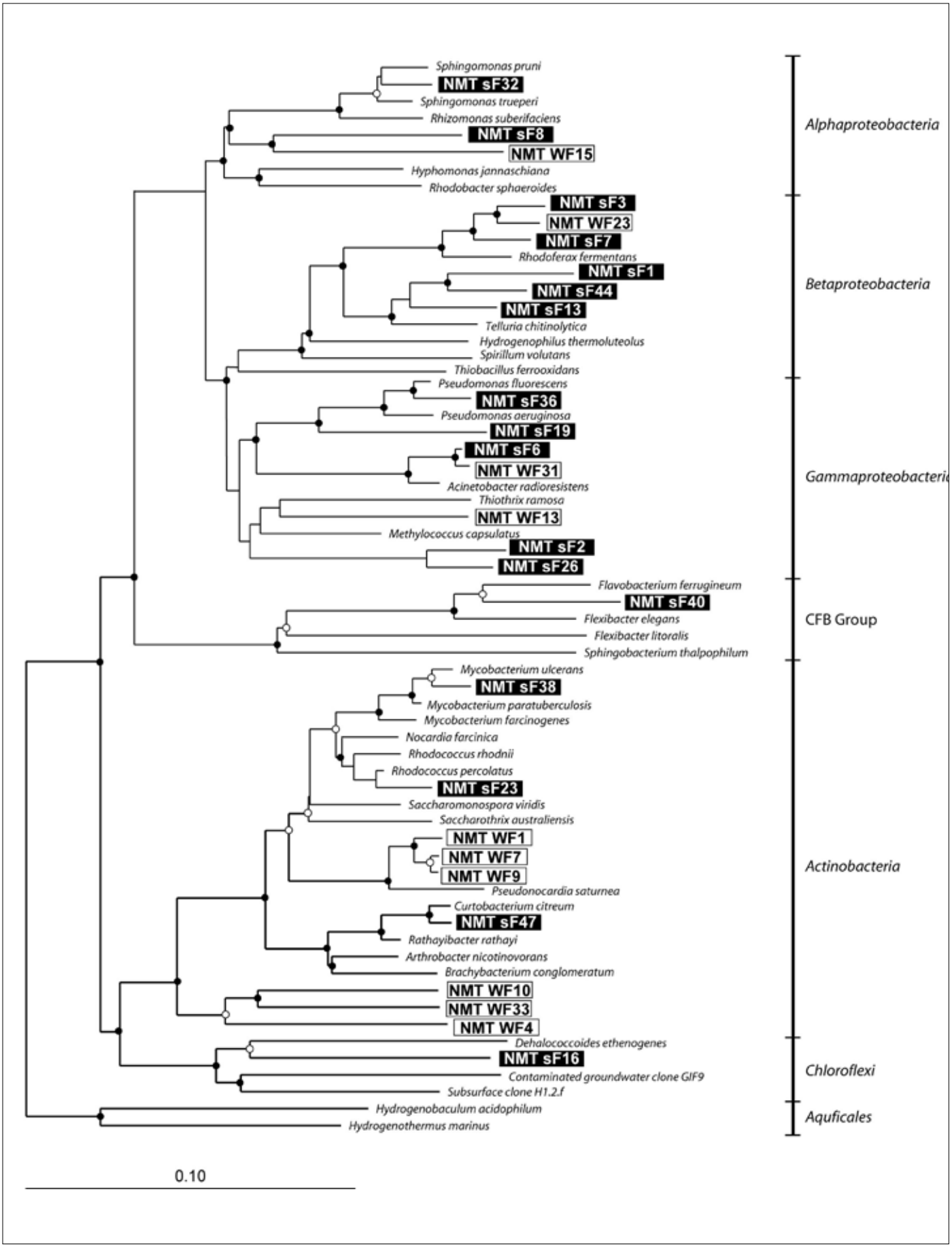

Fig. 2. Consensus dendogram of the 16S rRNA gene sequence phylotypes identified from the Carlsbad clone libraries WF1 (white boxes) and sF88 (black boxes). Phylogenetic analyses were carried out using both distance (Neighbor-Joining) and parsimonious (Heuristic) searches, with the robustness of inferred 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 μg 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 much 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).

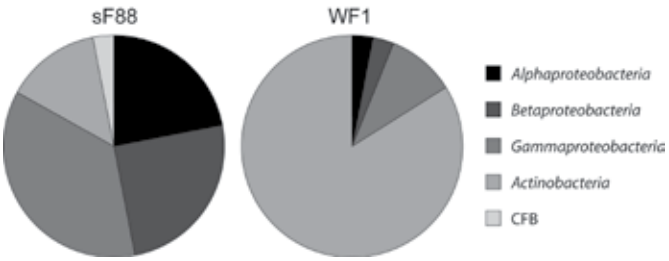

Fig.3. Pie-charts representing species distribution at each location within Carlsbad Cavern. The distribution of all divisions are represented by each cloned phylotype from the WF1 (88 clones) and sF88 (144 clones) libraries.

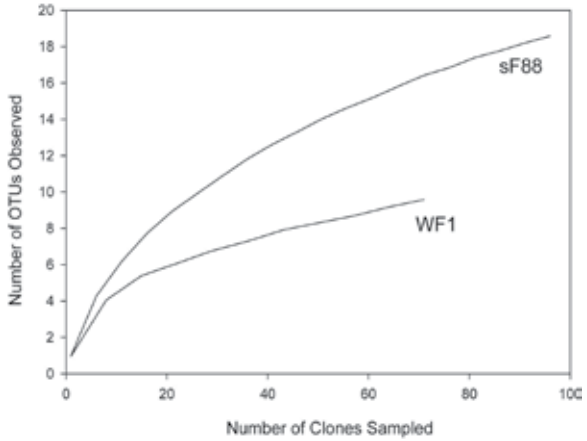

Fig.4. Rarefaction curves of observed operational taxonomic units (OTUs) represented by individual phylotypes identified within this study. Each rarefaction curve was calculated from the variance of the number of OTUs drawn in 100 randomizations at each sample size.

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 groups into operational taxanomic 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-parametic 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 diffractrometry (XRD)

One of the most striking differences between WF1 and sF88 sites was the presence of a corrosion residue

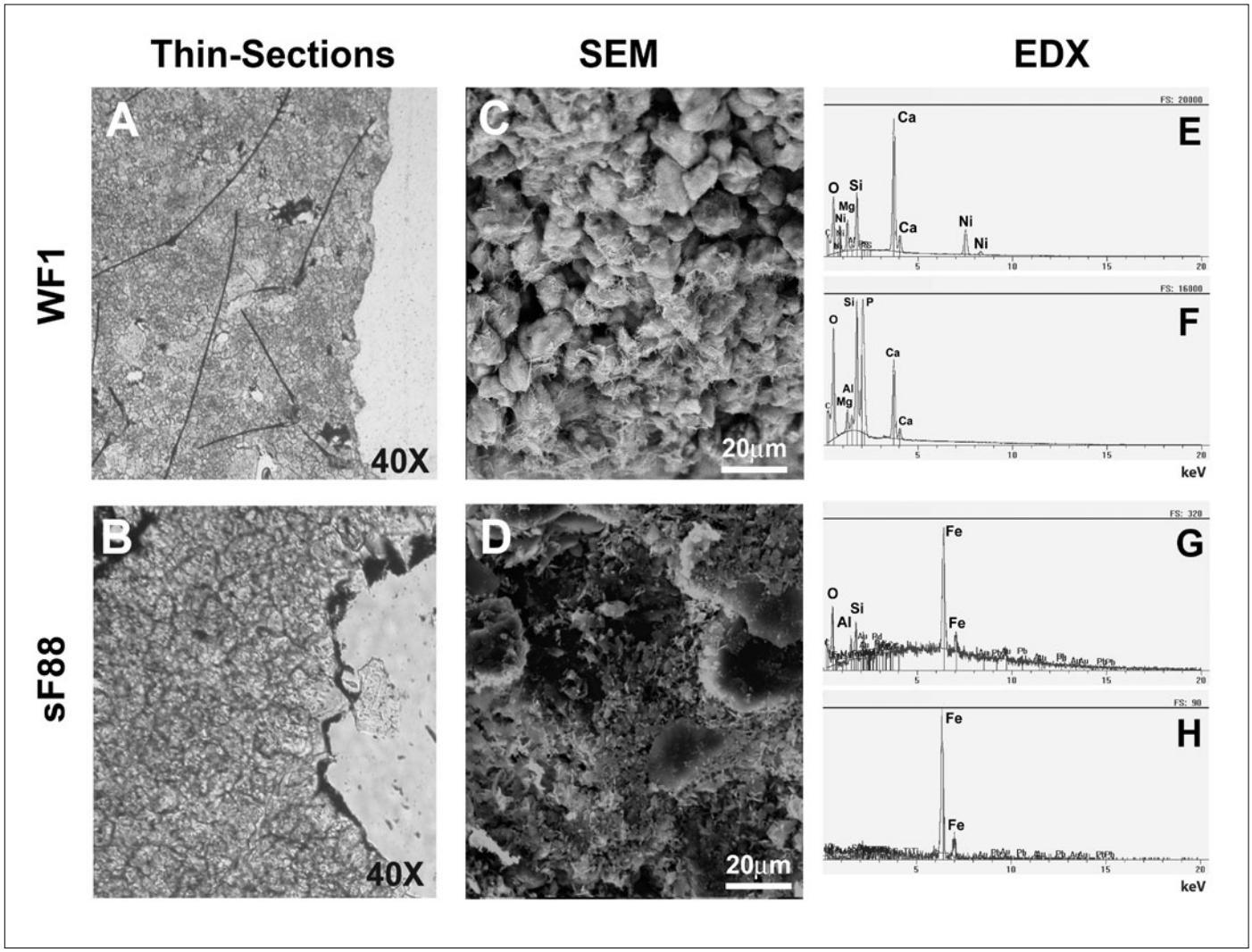

Fig.5. Petrographic thin-sections, SEM and EDX analysis of rock samples from WF1 and sF88. Samples were collected and subjected to thin sectioning and petrographic analysis at WF1 (A) and sF88 (B). The interior bedrock is mineralogically similar to the outer layer at WF1 (A), while the corrosion residue fills the entire field-of-view of the sF88 sample (B). SEM analysis reveals a similar surface mineralogy at WF1 (C), where individual calcite crystals covered with a biofilm material are seen on the surface. A fine, powdery residue predominates on the surface at sF88 (D). EDX analyses of acid extracts at each location demonstrate the predominance of clay particles at WF1 (E and F) and iron oxides and elemental iron at sF88 (G and H)

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)

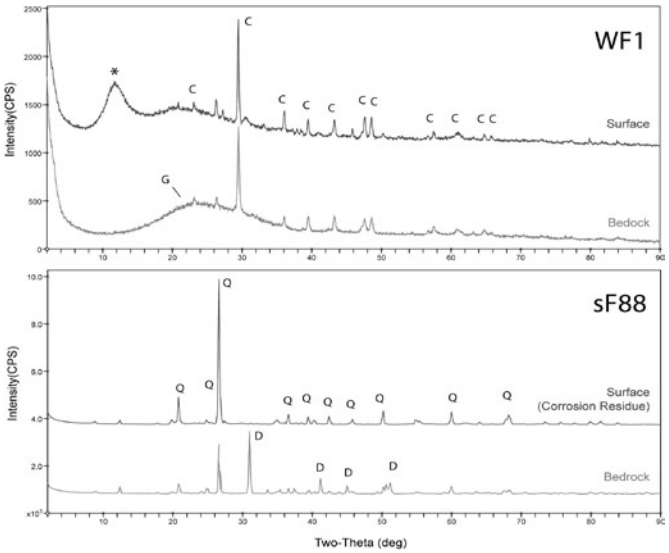

Fig.6. X-ray powder diffractometry (XRD) of the samples at WF1 and sF88. The samples were segregated into an interior (bedrock) and outer layer for a comparative analysis of mineralogical changes. The analysis at WF1 reveals the predominantly calcite nature of the bedrock, with an accumulation of clay particles in the surface layer. At sF88 there is a dramatic change in the mineral structure, with removal of the soluble dolomite and an enrichment of insoluble silicates. Peaks correspond to: C = calcite; Q = quartz; D = dolomite; * = unconsolidated endolite peak; and G = signature of glass support.

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 *Pseudonocardia* 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 *Pseudonocardia* 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 autoxidation, 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 phosphorous must be growth limiting factors at sF88. It is then hardly surprising that phylotypes related to the nitrogen assimilating species *Herbaspirillum frisingense* and *Janthinobacterium agaricidamnosusm* 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.

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REFERENCES

Achenbach L.A. & Coates J.D., 2000 - *Disparity between bacterial phylogeny and physiology.* ASM News, **66**: 714-715.

Altschul S.F., Madden T.L., Schaffer A.J., Zhang J., Zhang Z., Miller W. & Lipman D.J., 1997 - *Gapped BLAST and PSI-BLAST: a new generation of protein databae search programs.* Nuc. Acids Res., **25**: 3389-3402.

Angert E.R., Northup D.E., Reysenbach A.-L., Peek A.S., Goebel B.M. & Pace N.R., 1998 - *Molecular phylogenetic analysis of a bacterial community in Sulphur River, Parker Cave, Kentucky.* Am. Mineral., **83**: 1583-1592.

Banfield J.F. & Nealson K.H., 1997 - *Geomicrobiology: Interactions between microbes and minerals.* Rev. Mineral., Washington, D.C. Mineralogical Society of American, 448 p.

Barton H.A. & Jurado V., 2007 - *What's Up Down There: Microbial Diversity in Starved Cave Environments.* Microbe, **2**: 132-138.

Barton H.A. & Luiszer F., 2005 - *Microbial Metabolic Structure in a Sulfidic Cave Hot Spring: Potential Mechanisms of Biospeleogenesis.* J. Cave Karst Stud., **67**: 28-38.

Barton H.A., Taylor M.R. & Pace N.R., 2004 - *Molecular Phylogenetic Analysis of a Bacterial Community in an Oligotrophic Cave Environment.* Geomicrobiol. J., **21**: 11-20.

	Sample Site			
	WF1		**sF88**	
Element	**Average Wt%[a]**	**SD**	**Average Wt%[a]**	**SD**
C	12.32	± 2.79	ND	-
O	41.25	± 0.72	49.11	± 12.32
Mg	9.43	± 0.31	1.18	± 0.62
Ca	36.16	± 4.44	ND	-
Si	0.84	± 0.64	33.55	± 15.21
Al	ND	-	7.74	± 3.98
K	ND	-	2.66	± 1.23
Fe	ND	-	5.77	± 4.25
Total	100%		100%	

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 1. EDX Bulk Elemental Analyses at WF1 and sF88

Phylogenetic Group	Clone	Clones Group	Closest identified relative	% Sequence ID	NCBI Accession Number
sF88					
Alphaproteobacteria	NMTsF8	26/32	*Brevundimonas nasdae*	99%	DQ066606
	NMTsF27	5/32	*Brevundimonas vesicularis*	94%	DQ066612
	NMTsF32	1/32	*Caulobacter subvibrioides*	99%	DQ066613
Betaproteobacteria	NMTsF1	18/29	*Massilia sp.*	98%	DQ066600
	NMTsF3	6/29	*Delfitia tsuruhatasis*	99%	DQ066602
	NMTsF7	2/29	*Acidovorax sp.*	98%	DQ066605
	NMTsF13	2/29	*Herbaspirillum frisingense*	100%	DQ066607
	NMTsF44	1/29	*Janthinobacterium agaricidamnosusm*	99%	DQ066617
Gammaproteobacteria	NMTsF2	17/31	*Stenotrophomonas sp.*	98%	DQ066601
	NMTsF6	1/31	*Uncultured Acinetobacter sp.*	99%	DQ066604
	NMTsF16	1/31	*Uncultured bacteria clone FS117-02*	89%	DQ066608
	NMTsF19	7/31	*Cellvibrio ostraviensis*	98%	DQ066609
	NMTsF26	4/31	*Xanthomas retroflexus*	99%	DQ066611
	NMTsF36	1/13	*Pseudomonas borealis*	99%	DQ066614
Cytophagales	NMTsF40	1/1	*Uncultured Bacteroidetes bacterium*	98%	DQ066616
Actinobacteria	NMTsF4	2/7	*Nocardioides sp.*	98%	DQ066603
	NMTsF23	2/7	*Rhodococcus sp.*	100%	DQ066610
	NMTsF38	1/7	*Mycobacterium gordonae*	99%	DQ066615
	NMTsF47	2/7	*Curtobacterium sp.*	99%	DQ066618
WF1 Library					
Alphaproteobacteria	NMT-WF15	1/1	*Methylobacterium aquaticum*	99%	DQ228717
Betaproteobacteria	NMT-WF23	1/1	*Comamonas sp.*	98%	DQ228718
Gammaproteobacteria	NMT-WF13	11/13	*Uncultured bacterial mud-clone*	90%	DQ228716
	NMT-WF31	2/13	*Acinetobacter johnsonii*	99%	DQ228719
Actinobacteria	NMT-WF1	34/65	*Pseudonocardia sp.*	97%	DQ228711
	NMT-WF4	14/65	*Bacterium Chibacore 1500*	90%	DQ228712
	NMT-WF7	4/65	*Crossiella equi*	97%	DQ228713
	NMT-WF9	1/65	*Saccharothrix cryophillis*	97%	DQ228714
	NMT-WF10	11/65	*Actinomyces sp.*	91%	DQ228715
	NMT-WF33	1/65	*Actinobacterium sp.*	93%	DQ228720

[a]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.

Barton H.A., Taylor N.M., Lubbers B.R. & Pemberton A.C., 2006 - *DNA extraction from low-biomass carbonate rock: an improved method with reduced contamination and the low-biomass contaminant database.* J. Microbiol. Meth., **66**: 21-31.

Begon M., Harper J.L. & Townsend C.R., 1998 - *Ecology: individuals, populations and communities.* Cambridge, Mass., Blackwell Scientific Publications, 1068 p.

Borer J.M. & Harris P.M., 1991 - *Lithofacies and Cyclicity of the Yates Formation, Permian Basin: Implications for Reservoir Heterogeneity.* Am. Assoc. Petrol. Geol. Bull., **75**: 726-779.

Boston P.J., Spilde M.N., Northup D.E., Melim L.A., Soroka D.S., Kleina L.G., Lavoie K.H., Hose L.D., Mallory L.M., Dahm C.N., Crossey L.J. & Schelble R.T., 2001 - *Cave Biosignature Suite: Microbes, Minerals and Mars.* Astrobiol., **1**: 25-55.

Bottrell S.H., Smart P.L., Whitaker F. & Raiswell R., 1991 - *Geochemistry and isotope systematics of sulphur in the mixing zone of Bahamian blue holes.* Appl. Geochem., **6**: 97-103.

Brown A.A. & Loucks R.G., 1993 - *Influence of Sediment Type and Depositional Processes on Stratal Patterns in the Permian Basin-Margin Lamar Limestone, McKittrick Canyon, Texas.* Am. Assoc. Petrol. Geol. Bull., **57**: 435-474.

Canaveras J.C., Sanchez-Moral S., Soler V. & Saiz-Jimenez C., 2001 - *Microorganisms and microbially induced fabrics in cave walls.* Geomicrobiol. J., **18**: 223-240.

Chao A., 1987 - *Estimating the population size for capture-recapture data with unequal catchability.* Biometrics, **43**: 783-791.

Chao A., Chazdon R.L., Colwell R.K. & Shen T.-J., 2005 - *A new statistical approach for assembling similarity of species composition with incidence and abundance data.* Ecol. Lett., **8**: 148-159.

Chelius M.K. & Moore J.C., 2004 - *Molecular Phylogenetic Analysis of Archaea and Bacteria in Wind Cave, South Dakota.* Geomicrobiol. J., **21**: 123-134.

Colwell R.K., 2005, EstimateS: *Statistical estimation of species richness and shared species from samples.* Version 6.01b http://viceroy.eeb.uconn.edu/estimates

Conway de Macario E., Macario A.J. & Wolin M.J., 1982 - *Specific antisera and immunological procedures for characterization of methanogenic bacteria.* J. Bacteriol., **149**: 320-328.

Culver D.C., 1982 - *Cave Life*: *Evolution and Ecology.* Cambridge, MA, Harvard University Press, 200 p.

DuChene H.R., 2000 - *Bedrock Features of Lechuguilla Cave, Guadalupe Mountains, New Mexico.* J. Cave Karst Stud., **62**: 109-119.

Dworkin M., 2002 - *The Prokaryotes: An evolving electronic resource for the microbiological community.*, Springer-Verlag, New York.

Ehrlich H.L., 2002 - *Geomicrobiology*: New York, Marcel Dekker, Inc., 768 p.

Engel A.S., Stern L.A. & Bennett P.C., 2004 - *Microbial contributions to cave formation: New insights into sulfuric acid speleogenesis.* Geol., **32**: 369-372.

Forbes J.R., 2000 - *Geochemistry of Carlsbad Cavern Pool Waters, Guadalupe Mountains, New Mexico.* J. Cave Karst Stud., **62**: 127-134.

Gotelli N.J. & Colwell R.K., 2001 - *Quantifying biodiversity: proceedures and pitfalls in the measurement and comparison of species richness.* Ecol. Lett., **4**: 379-391.

Grotenhuis J.T.C., Smit M., Plugge C.M., Yuansheng X., Van Lammeren A.A.M., Stams A.J.M. & Zehnder A.J.B., 1991 - *Bacteriological composition and structure of granular sludge adapted to different substrates.* Appl. Environ. Microbiol., **57**: 1942-1949.

Groth I. & Saiz-Jimenez C., 1999 - *Actinomycetes in Hypogean Environments.* Geomicrobiol. J., **16**: 1-8.

Groth I., Schumann P., Laiz L., Moral-Sanchez S., Canaveras J.C. & Saiz-Jimenez C., 2001 - *Geomicrobiological study of the Grotta dei Cervi, Porto Badisco, Italy.* Geomicrobiol. J., **18**: 241-258.

Groth I., Vettermann R., Schuetze B., Schumann P. & Saiz-Jimenez C., 1999 - *Actinomycetes in Karstic caves of Northern Spain (Altamira and Tito Bustillo).* J. Microbiol. Meth., **36**: 115-122.

Hahn M.W. & Hofle M.G., 2001 - *Grazing of protozoa and its effect on populations of aquatic bacteria.* FEMS Microbiol. Ecol., **35**: 113-121.

Hill C.A., 1990 - *Sulfuric acid speleogenesis of Carlsbad Cavern and its relationship to hydrocarbons, Delaware Basin, New Mexico and Texas.* Am. Assoc. Petrol. Geol. Bull., **74**: 1685-1694.

Hughes J.B., Hellmann J.J., Ricketts T.H. & Bohannan B.J.M., 2001 - *Counting the uncountable: Statistical approaches to estimating microbial diversity.* Appl. Environ. Microbiol., **67**: 4399-4406.

Itoh H. & Shiba T., 2004 - *Polyphosphate synthetic activity of polyphosphate:AMP phosphotransferase in* Actinetobacter johnsonii *210A.* J. Bacteriol., **186**: 5178-5181.

Juttner F., 1984 - *Dynamics of the volatile organic substances associated with Cyanobacteria and Algae in a eutrophic shallow lake.* Appl. Environ. Microbiol., **47**: 814-820.

Klimchouk A.B., Ford D.C., Palmer A.N. & Dreybrodt W., 2000 - *Speleogenesis: Evolution of Karstic Aquifers.* Huntsville, AL., National Speleological Society, 528 p.

Laiz L., Groth I., Gonzalez I. & Saiz-Jimenez C., 1999 - *Microbiological study of the dripping water in Altamira Cave (Santillana del Mar, Spain).* J. Microbiol. Meth., **36**: 129-138.

Ley R.E., Harris J.K., Wilcox J., Spear J.R., Miller S.R., Bebout B.M., Maresca J.A., Bryant D.A., Sogin M.L. & Pace N.R., 2006 - *Unexpected diversity and complexity of the Guerrero Negro hypersaline microbial mat.* Appl. Environ. Microbiol., **72**: 3685-95.

Li Y., Kawamura Y., Fujiwara N., Naka T., Liu H., Huang X., Kobayashi K. & Ezaki T., 2004 - Sphingomonas yabuuchiae sp. nov. *and* Brevundimonas nasdae sp. nov.*, isolated from the Russian space laboratory Mir.* Int. J. Syst. Evol. Microbiol., **54**: 819-825.

Maidak B.L., Cole J.R., Lilburn T.G., Parker C.T., Saxman P.R., Stredwick J.M., Garrity G.M., Li B., Olsen G.J., Pramanik S., Schmidt T.M. & Tiedje J.M., 2000 - *The RDP (Ribosomal Database Project) continues.* Nuc. Acids Res., **28**: 173-174.

Mohn W.W. & Tiedje J.M., 1992 - *Microbial reductive dehalogenation*. Microbiol. Rev., **56**: 482-507.

Morgan J.F., 1958 - *Tissue Culture Nutrition*. Bacteriol. Rev., **22**: 20-45.

Mutti M. & Simo J.A., 1993 - *Stratigraphic Patterns and Cycle-Related Diagenesis of Upper Yates Formation, Permian, Guadalupe Mountains*. Am. Assoc. Petrol. Geol. Bull., **57**: 515-534.

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

Northup D.E., Barnes S.M., Yu L.E., Spilde M.N., Schelble R.T., Dano K.E., Crossey L.J., Connolly C.A., Boston P.J., Natvig D.O. & Dahm C.N., 2003 - *Diverse microbial communitiens inhabiting ferromanganese deposits in Lechuguilla and Spider Caves.* Environ. Microbiol., **5**: 1071-1086.

Osman S., Stuecker T., Newcombe D. & Venkateswaran K., 2005 - *Microbial burden and community profiles of the genesis curation laboratory*. Abstract 105th General Meeting, Am. Soc. Microbiol., Atlanta, GA.

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

Palmer A.N., 2000 - *Hydrochemical Interpretation of Cave Patterns in the Guadalupe Mountains, New Mexico*. J. Cave Karst Stud., **62**: 91-108.

Roth J.R., Lawrence J.G. & Bobik T.A., 1996 - *Cobalamin (coenzyme B12): synthesis and biological significance*. Ann. Rev. Microbiol., **50**: 137-181.

Saiz-Jimenez C. & Hermosin B., 1999 - *Thermally asisted hydrolysis and methylation of dissolved organic matter in dripping waters from the Altamira Cave*. J. Anal. Appl. Pyrol., **49**: 337-347.

Sarbu S.M., Kane T.C. & Kinkle B.K., 1996 - *A chemoautotrophically based cave ecosystem*. Science, **272**: 1953-1955.

Schink B., 2002 - *Synergistic interactions in the microbial world*. Anton. Leeuw., **81**: 257-261.

Sylvia D.M., Fuhrmann J.J., Hartel P.G. & Zuberer D.A., 1999 - *Principles and Applications of Soil Microbiology*. Upper Saddle River, NJ, Prentice Hall, 550 p.

Van Groenestijn J.W., Vlekke G.J.F.M., Anink D.M.E., Deinema M.H. & Zehnder A.J.B., 1988 - *Role of cations in accumulation and release of phosphate by* Acinetobacter *strain 210A*. Appl. Environ. Microbiol., **54**: 2894-2901.