Quantitative Real-Time PCR Assays of Bacterial DNA in Sediments of the Flint-Mammoth Cave System with Evidence for Nitrospira Spp. At Sites Undergoing Limestone Dissolution and Karst Aquifer Evolution

Rick Fowler
Eli Roberson
Shivendra Sahi
Biotechnology Center
Department of Biology
Western Kentucky University
Bowling Green, KY 42101

Abstract

Quantitative Real-Time PCR (qRT-PCR) is used to compare the densities of eubacterial 16S rDNA in sediments from the Flint-Mammoth Cave System while DNA sequence information and restriction fragment biomarkers demonstrate the presence of *Nitrospira spp*. in bacterial communities. Thirteen samples were collected from sites with a range of hydrologic conditions and compared with respect to nanograms of DNA per gram of sediment by amplification of environmental DNA relative to *E. coli* genomic DNA with universal primers for eubacterial 16S rDNA. Saturated clastic stream sediments where the process of limestone dissolution and cavern enlargement processes were active displayed high DNA levels with the associated presence of a eubacterial clone closely related to *Nitrospira spp*. Two sediment samples from an inactive and dry cave environment were assayed and shown to contain 45 ppm nitrate, but were negative for detectible bacterial DNA. It is concluded that *Nitrospira spp*. are associated with bacterial communities in actively evolving karst aquifers and may contribute to cavern enlargement by consumption of carbonate minerals as chemolithoautotrophs in the absence of other carbon sources in the cave environment. Also, nitrates in dry sediment do not appear to be attributable to active bacterial nitrification.

1. Introduction

Nitrifying bacteria are capable of colonizing biofilms in karst environments including limestone surfaces, terrestrial and subterranean streams and their sediments, sinkholes, bedrock walls, ceilings, and floors of caverns, in addition to being found within the limestone matrix itself (5, 7, 20, 22). *Nitrobacter* found in cave biofilms are more abundant (10^5 cells/gram of sediment) and of a different species (*N. agilis*) than those found in the surface soil and rhizosphere (*N. winogradsky*, 10^3 cells/gram of soil) as shown by classical microbiological techniques of culturing and morphological identification of species (7, 17). An extensive study in Catherines and Olivias Domes in Mammoth Cave and another at Charons Cascade were successful in culturing and identifying a diversity of bacterial species, but many could not be identified (24, 26).

Modern DNA analysis techniques have revealed the existence of previously unknown bacterial species in the environment that cannot be grown under laboratory conditions in pure culture, and revolutionized our understanding of biogeochemical community diversity. It is now known that environmental bacteria are two orders of magnitude more diverse than previously revealed by culturing

techniques, and that bacteria thrive under conditions previously thought to be unfit for life. Many have evolved specialized genetic systems and strategies for survival (1, 10, 12, 18, 19, 22, 28).

Among the bacterial species now identified are chemolithoautotrophs such as *Nitrospira spp.* and relatives capable of deriving energy by the oxidation of nitrite ions (NO_2^-) to NO_3^- using only the inorganic carbon found in carbonate minerals (CO_3^{2-}) as a food source. Nitrifying bacterial communities consisting of structured biofilms with *Nitrosomonas, Nitrobacter, Nitrosospina,* and *Nitrospira spp* are responsible for the operation of municipal wastewater treatment plants and detoxifying bioreactors (3, 7, 10, 11, 15, 27).

Such bacteria are particularly interesting in the context of speleology as important ecological modulators, but also they may contribute to NO_3^- production and distribution by deriving energy from nitrification while consuming carbon in the form of inorganic CO_3^{2-} in lieu of organic carbon sources, thus contributing to karst aquifer evolution, cave formation, and cavern enlargement, and sinkhole collapse. The same processes may be accomplished by nitrifying bacteria flushed into the cave from the surface with the genetic potential to switch their genetic machinery and adapt as troglophilic species utilizing CO_3^{2-} as a food source and deriving energy by the oxidation of NO_2^- in the absence of consumption of NO_3^- by plants, thus leading to the accumulation of nitrates in sediments where hydrological conditions have altered over time so that no flow or other movement of water displaces nitrate while evaporation concentrates it in place.

Cave sediments have been an important source of potassium nitrate, or saltpeter, since the Middle Ages via the process of leaching soluble nitrate minerals from sediment and conversion to the potassium salt by filtration through wood ash. The potassium nitrate (KNO_3) thus produced is recovered for use after evaporation of the water used for the leaching process, and serves as the primary ingredient of gunpowder when combined with sulfur and charcoal. During the eighteenth and nineteenth centuries throughout the eastern parts of North America, significant industrial operations were installed in the more accessible entrance passages within limestone caves where mining, leaching, and pumping processes could be accomplished using nearby sources of water and manual labor. Mammoth Cave in Kentucky was extensively mined for dry sediments during the war of 1812 and the saltpeter produced there eventually contributed to the success of the E. I. DuPont deNemoirs and Company (DuPont) as the principal producer of gunpowder for the United States (6, 16, 17, 23, 25).

Nitrate minerals are minor constituents (less than 1% by weight) of some sulfate speleothems, but the high solubility of all nitrate salts limits their persistence in speleothems to extremely dry cave passages where they cannot be transported by drainage, dripping, or movement of water. Nitrates are found primarily in dry, porous, aerated floor sediments with low carbon content, relatively little phosphorus (0.1 to 1.4% by weight), and low total nitrogen (0.08 to 0.13% by weight). Distribution of nitrates in floor sediments is topographically evenly distributed and is concentrated within the upper one meter of depth, and sediments depleted of nitrates can be regenerated within several years. Cave sediments protected from surface drainage have concentrated nitrates in the range of thousands of parts per million (ppm) due to evaporation of moisture from the sediment into the cave atmosphere, while surface and sinkhole limestones subject to rainfall and subsurface drainage are depleted of nitrates to a concentration of 1 or 2 ppm as solution processes leach the salts into the subsurface. Intermediate levels of 10 to 100 ppm nitrates are found where surface drainage exchanges with the subsurface and nitrate concentration increases dramatically at the boundary of the subsurface-cave atmosphere independently of limestone type or stratigraphy (16).

Early observations recognized that nitrates are produced by electrochemical oxidation of atmospheric nitrogen gases by lightning discharges. More importantly, atmospheric nitrogen cycling by rhizospheric and other topsoil bacteria is a major global biogeochemical process widely recognized as a dominant production mechanism of nitrates that are primarily utilized by plants, particularly in regions with lush vegetation. Some of these nitrates not utilized by plants can percolate through porous karst vadose zones and be rapidly discharged and distributed in cave sediments consistently with the observed spatial distribution and concentration of nitrates in cave sediments. Evaporation of water in the cave atmosphere leaves behind pockets of solutes including nitrates throughout the cave system in varying concentrations, further distributed by hydrological drainages containing soluble nitrates. Theories about the origin of nitrates in cave sediments include decomposition of nitrogenous organic compounds in bat guano, deposition of nitrates by evaporation of leachates from surface

sources, or in situ oxidation of ammonium ion (NH_4^+) to nitrate ion (NO_3^-) by nitrifying bacteria such as *Nitrobacter* and *Nitrosomonas spp*. (7, 17, 23).

Typical saltpeter caves in the southeastern United States have temperatures between 10 and 18°C and humidities between 90 and 99%. Geochemical analysis has shown that nitrate minerals can be found at various concentrations throughout networks of cave passages and they are not limited to the entrance zones where historical mining operations were installed. Nitrate minerals are minor constituents (less than 1% by weight) of some sulfate speleothems, but the high solubility of all nitrate salts limits their persistence in speleothems to extremely dry cave passages where they cannot be transported by drainage, dripping, or movement of water (7, 17, 23).

Nitrates are found primarily in dry, porous, aerated floor sediments with low carbon content, relatively little phosphorus (0.1 to 1.4% by weight), and low total nitrogen (0.08 to 0.13% by weight). Distribution of nitrates in floor sediments is topographically evenly distributed and is concentrated within the upper one meter of depth, and sediments depleted of nitrates can be regenerated within several years. Cave sediments protected from surface drainage have concentrated nitrates in the range of thousands of parts per million (ppm) due to evaporation of moisture from the sediment into the cave atmosphere, while surface and sinkhole limestones subject to rainfall and subsurface drainage are depleted of nitrates. Historical saltpeter mining operations were usually associated with bat hibernacula thus giving rise to the theory that nitrates result from decomposition of guano. This theory is inconsistent with volumes of more recent evidence showing chemical composition, distribution, and regeneration rate of nitrates in cave sediments where bats did not deposit guano or where bats had abandoned hibernacula (16, 17, 23).

Samples of saturated clastic sediments and moist deposits from 13 sites in the Flint-Mammoth Cave System are shown in this study to contain eubacterial 16S rDNA genes in cave sediment DNA relative to the 16S rDNA gene of *E. coli* genomic DNA at levels from zero to over 5000 nanograms of 16S rDNA genes in environmental bacterial genomic DNA per gram of sediment using a quantitative Real-Time Polymerase Chain Reaction technique (qRT-PCR). This quantitative data is a useful indicator of bacterial density in the sediments. Furthermore, *Nitrospira spp*. are identified in some bacterial communities using a combination of DNA fragment analysis and correlation with a Mammoth Cave bacterial 16S rDNA clone database. The particular *Nitrospira sp.* associated with Mammoth Cave is represented by clone CCU23. The entire nucleotide sequence of CCU23 eubacterial 16S rDNA has been determined and posted on GenBank (accession AY221079) allowing phylogenetic classification with restriction enzyme mapping by computer analyses. Fluorescent DNA fragment standards for BssHII and HhaI cleavage fragments have been measured experimentally for CCU23 and other clones in the database. The combination of fragment and DNA sequence data provides a confident genetic biomarker for CCU23 also shared by other members of the genus *Nitrospira* as determined by alignment of CCU23 with multiple closely related *Nitrospira spp*. 16S rDNA sequences downloaded from GenBank (http://ncbi.nlm.nih.gov) and the Ribosomal Database Project (RDP; http://rdp.cme.msu.edu) genetic databases.

In contrast to the saturated clastic sediments from hydrologically active sites, a comparison is made with Turner Avenue on Level 4 in the Flint-Mammoth Cave System where it has been evidently inactive with respect to limestone dissolution and hydrological evolution for millions of years (25). Duplicate samples of very fine, dry protected sediments were collected from the upper 5 cm in a virgin deposit of silt under a rock overhang recessed approximately 1 m and 0.5 m in height at Albright Junction. These dry sediments show no evidence for the presence of bacteria using quantitative DNA analysis, however low levels of NO_3^- (45 ppm) were detected using ion chromatography. Thus the nitrates at Albright Junction do not appear to be attributable to *in situ* production by chemolithoautotrophic *Nitrospira* bacteria like those found in sediment samples collected from saturated clastic sediments in hydrologicaly active cave passages where limestone dissolution is contributing to karst aquifer evolution and cavern enlargement.

2. Materials and Methods

2.1 Site description and sample collection methods

Samples were collected using aseptic technique wearing latex gloves. Sterile centrifuge tubes were opened at the time of collection and used to scoop sediment. The closed tubes were enclosed within the inverted latex gloves used for collection by tying the wrists in a knot and sealing in a Ziploc plastic bag. Samples were kept on ice upon exiting the cave and until DNA was extracted from the sediment within 24 hrs.

For quantitative data on a variety of sites throughout the Flint-Mammoth system, qRT-PCR was performed on an archived set of DNA samples (Figure 2) collected and extracted in association with expeditions in June 2002 and October 2002 including saturated clastic sediments in the Flint-Mammoth System from Unknown Cave in Pohl Avenue at Three Spoons Inn (POHL3SP) and saturated limestone paste collected from the ceiling in Turner Avenue near Brucker Breakdown (TURNPST); sediments in Colossal Cave at Grand Avenue (COLGRND), at Dyer's Dome (COLDYER), Colossal River and along the Colossal-Salts link passage (COLSALT); Salts Cave dry wall deposits in the main chamber containing mirabilite crystals (SALTMIN); along with four samples of sediment from Charons Cascade (MCCC1, MCCC2, MCCC3, and MCCC4) and two from Cathedral Domes (CATHDM2, CATHDM3) collected in historic Mammoth Cave. Albright Junction samples (ALBRJCT1, ALBRJCT2) were collected in June 2003 from protected, dry, extremely fine-grained virgin sediments under a rock ledge approximately 10 m into a side passage leading north from Albright Junction. Samples other than ALBRJCT1, ALBRJCT2, TURNPST, and SALTMIN were in the upper 5 centimeters of saturated clastic sediments where persistent flowing water or standing pools were predominant (an individual *Oronectes sp.* crayfish was observed in a 10-centimeter-deep pool at the time POHL3SP was collected, and smaller invertebrates such as isopods and arthropods were observed at many sites).

2.2 Extraction and quantification of nitrates from ALBRJCT sediments

Water-soluble nitrate salts were leached by addition of 10 milliliters sterile deionized water (Barnstead Nanopure) to 10 gam sediment in a sterile 50 milliliters polypropylene centrifuge tube. Samples were placed horizontally and agitated in a rotary shaker at 250 rpm at a constant temperature of 25C for 18 hours. Sediment particles were removed from the extract by centrifugation at 7000 x g at 4 for 30 minutes. in an IEC PR-7000M centrifuge with a number 766 swinging bucket rotor. Aqueous supernatants were removed with a sterile serological pipette, transferred to new sterile 50-milliliter centrifuge tubes, and centrifuged again. The second supernatant was decanted and stored in a sterile 15-milliliter centrifuge tube. The leachate contained the soluble nitrates from 1 g sediment per ml of H_2O. Both leachate samples were submitted to the Materials Characterization Center at Western Kentucky University for detection and quantification of nitrate using ion chromatography. The official results returned for the duplicate samples were 47.79 ppm and 42.64 ppm for an average of 45.22 ppm.

2.3 Extraction of DNA from sediment samples

All procedures with DNA were carried out using aseptic technique with sterile reagents and materials. Saturated clastic sediment samples were filtered to remove water before weighing by centrifugation in a CoStar centrifugal microfiltration device (number?) through a 0.2 m membrane for retention of bacteria. DNA was extracted directly from sediments using the Ultraclean Soil DNA Kit (MO Bio Laboratories, Solana Beach, CA) by adding a known mass of 0.5–1.0 grams of sediment to the kit using aseptic technique and following the manufacturer's instructions. The sediment DNA was recovered in a volume of 50 uL and was visualized by loading 5 L (10% of total yield) on a 1.5% agarose gel (10 cm, Tris-Acetate-EDTA buffer) run at 6 V/cm stained with 1 mg/ml ethidium bromide for 16 hrs at 4°C, rinsed with deionized H_2O, and photoanalyzed using a Kodak EDAS 290 gel documentation system. Yields and molecular weights of environmental DNA samples were estimated by comparison to a

known amount of size standard (1 kb ladder, New England Biolabs, Beverly, MA).

2.4 Quantitative Real-Time PCR (qRT-PCR)

Reaction mixtures with a final volume of 50 L consisted of 25 L SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA), 5 L of 5M eubacterial universal primer 27f (5'-AGAGTTTGATCMtggctcag-3'), 5 L of 5M eubacterial universal primer 1492r (5'-TACGGYTACCTTGTTACGACTT-3'), and sediment DNA extract plus sterile nanopure water containing up to 100 ng template (estimated by agarose gel photoanalysis) in 15 L template volume. Pprimers were custom synthesized by Sigma-Genosys Biotechnologies, The Woodlands, TX. Thermocycling and optical monitoring during qRT-PCR were performed on an iCycler Real-Time PCR machine (Bio-Rad Laboratories) with fluorescent monitoring at 490 nm during the extension step of a thermocycling program consisting of an initial denaturation at 95°C; 50 cycles of 1 min. at 95°C (denaturation), 1 minute at 55°C (annealing), and 1 minute at 72°C (extension, monitor SYBR Green fluorescence at 490 nm); followed by a final extension step of 72°C for 10 minutes.

Graphs showing SYBR Green fluorescence as a function of cycle number are shown in Figures 2A, 3A, and 3B, and quantitative assays with *E. coli* genomic DNA as a standard are shown in Figures 2B and 3C. The technique of qRT-PCR allows direct observation of the PCR amplification process, and the logarithmic accumulation of double stranded PCR products (amplicons) can be monitored using a variety of fluorescent techniques. In these experiments the increase in the fluorescent signal of SYBR Green, an intercalating dye specific for double stranded DNA, is monitored as increasing numbers of copies of double stranded eubacterial 16S rDNA (amplicons) are produced during PCR. Furthermore, there is a linear mathematical relationship between the logarithm of the amount of target DNA (template) in the reaction and the number of thermal cycles required to reach a calculated threshold level of fluorescence, called the threshold cycle (C_t). This relationship can be used to determine the concentration of DNA in the original sample with a high degree of accuracy and specificity over more than five orders of magnitude (refs). In these experiments DNA extracted from cave sediments and *E. coli* genomic DNA were amplified with 27f and 1492r universal primers. The standard curve of *E. coli* DNA displays a linear relationship extending beyond the range of 0.01 ng to 100 ng of genomic DNA as shown in Fig. 2B and Figure 3C. Quantitative results are summarized and normalized to represent ng DNA per gram of sediment in Figure 4.

2.5 DNA sequencing

Sequencing reactions contained approximately 100 ng cloned plasmid DNA with 4 uL Big Dye Terminator mix (Applied Biosystems) and 1.6 picomoles of sequencing primer per 10 uL final reaction volume. Thermocycling conditions were as recommended by the manufacturer with reaction cleanup by isopropanol precipitation. Fluorescent capillary electrophoresis was carried out on an Applied Biosystems Prism 310 Genetic Analyzer. Sequencing primers were T7 and SP6 located outside the cloned 16S rDNA in the plasmid vector sequences, 27f and 1492r universal bacterial primers (21) at the termini of the cloned sequences, and 530f and 1100r universal bacterial primers to provide overlapping complementary fragments internally in the cloned sequences (21).

2.6 Sequence alignments and genetic analysis

DNA sequences from eubacterial 16S rDNA genes cloned from DNA extracted from Charon's Cascade were assembled from raw data, stored, and manipulated within a database created using the Vector NTI Suite 8.0 (Informax). Vector NTI sequence database management and analysis tools included ContigExpress for editing raw data, AlignX for ClustalW alignments and similarity relationship trees, and BLAST searches against the GenBank nucleotide sequence database (http://ncbi.nlm.nih.gov). Sequences were also analyzed with software available from the Ribosomal Database Project (http://rdp.cme.msu.edu) for identification and alignment using the SEQ_MATCH and SEQ_ALIGN online software tools, and Check_Chimera to eliminate cloning artifacts from the database.

2.7 Fluorescent DNA fragment analysis

A fluorescent-labeled 27f primer (6FAM-27f) was used to detect DNA fragments cleaved at a specific DNA sequence by restriction enzymes HhaI or BssHII. The lengths of these fragments can be measured accurately and are a function of the DNA sequence, determined by the number of nucleotides from the fluorescent terminus to the restriction enzyme recognition and cleavage sequence. Specific restriction fragment lengths are measured with internal standards by fluorescent detection and are correlated with cloned cave bacterial DNA sequences in a Mammoth Cave bacterial 16S rDNA database (WKU ref). For analyses specifically intended for fragment analysis, only 30 cycles of PCR were used to ensure product purity.

Samples were processed after the qRT-PCR reaction by transferring the contents of the qRT-PCR reaction to a sterile 1.5-milliliter microcentrifuge tube. Excess primers, nucleotides, and buffers were removed with the Ultraclean PCR Cleanup Kit (MO Bio Laboratories) following the manufactuer's instructions. From the total yield of 50 L from the PCR cleanup kit, 25 L was cleaved with HhaI at 37C and the other 25 L was cleaved with BssHII at 50°C. Each restriction digest was carried out in a total volume of 50 L with 20 units enzyme for 6 hours. in the manufacturer's recommended buffer (New England Biolabs, Bedford, MA). After digestion was complete, samples were ethanol precipitated by the addition of 5 L 3M NaOAc (0.1 volume) and 165 L ethanol (three volumes). Samples were chilled at -20°C and centrifuged at 13,000 x g for 15 minutes at 4C to precipitate the DNA. After decanting the supernatant, pellets were rinsed by the addi-

tion of 500 L cold 70% ethanol followed by centrifugation, decantation of the supernatant, then dried *in vacuo*. Dry DNA samples were dissolved in 25 L deionized formamide (Amresco) containing 1% (0.25 L) of the internal standard fluorescent marker ROX500 fragments (Applied Biosystems). Samples were denatured at 95C for 4 minutes and quick chilled to 4C in a themocycler (MJ Research, Cambridge, MA) in the sample rack of an ABI Prism 310 Genetic Analyzer. Fragment electrophoresis detection, and length measurement were performed an ABI 310 running GeneScan software (Applied Biosystems). Raw fragment peak data, diluted with formamide containing ROX500 if necessary not to overload the capillary and detector, was refined for presentation as histograms using the Genotyper DNA profiling software package (Applied Biosystems).

3. Results and discussion

3.1 Quantification of eubacterial 16S rDNA in sediments

Quantification of DNA by qRT-PCR is shown in Figures 2 and 3. The qRT-PCR data derives from the measurement of an increase in fluorescence of the intercalating dye SYBR Green, which is specific for double stranded DNA and produces a strong fluorescent signal proportional to DNA concentration. The increase in DNA concentration results from the specific amplification 16 rDNA extracted from eubacterial communities in the cave sediments. Fluorescence is plotted on the Y axis as a function of the number of thermal cycles, plotted on the X axis, in which the reactions are heated from 55C to 72C to 95C repeatedly to geometrically amplify the number of copies of a specific gene determined by the DNA sequence of the primers used in the reaction As the number of qRT-PCR cycles increases, fluorescence of SYBR Green increases in the reaction mixture as more copies of double-stranded copies of cave eubacterial 16S rDNA are generated.

The greater amounts of target DNA added to qRT-PCR reactions, fewer cycles are required to amplify the target DNA geometrically above a specified threshold. Therefore, the number of cycles required to reach the threshold, termed the threshold cycle or C_t, is inversely proportional to the amount of DNA added to the reaction. Figures 2B and 3C illustrate the linear relationship that exists between the log of the starting quantity (SQ) of target DNA (X axis) and the C_t (Y axis). The starting quantity was normalized to represent the amount of DNA in nanograns per gram of sediment as follows:

Highest normalized concentrations of eubacterial DNA (Figure 4) were measured at Charons Cascade in the historic section of Mammoth Cave. The site receives much input from upstream tributaries and empties into River Styx, and it is subject to periodic backflooding from the Green River that results in a rich sandy deposit punctuated by a waterfall approximately 10 meters in height creating a permanent pool at its base (12, 13, 14, 30, 31). Four samples collected at Charons Cascade at the base of the waterfall and around the pool (MCCC1, MCCC2, MCCC3, MCCC4) had 3480.00, 5506.67, 14.93, and 762.67 ng DNA per gram of sediment, respectively. High DNA levels were measured in POHL3SP, COLSALT and COLGRND sediments where persistent active drainages were occasionally dammed into pools, with 610.67, 409.33, and 14.93 ng DNA per gram of sediment respectively. Pools at the bottoms of vertical shafts and high drainage areas but little accumulated clastic sediment such as CATHDM2, CATHDM3, and COLDYER, had concentrations of bacterial DNA in the sediments ranging from 0.05, 0.06, to 69.87 ng DNA per gram of sediment, respectively. TURNPST contained 0.87 ng DNA per gram and consisted of paste on a ceiling found where capillary seepage into the ceiling is sufficient to dissolve limestone and leave behind mineral solutes after water has evaporated in the cave air producing a moist, gray punk rock material. SALTMIN contained 0.21 ng DNA per gram of sediment and consisted of fine, dry particles with visible crystals of mirabilite. No evidence for DNA was seen in either ALBRJCT1 or ALBJCT2, despite attempts to increase detection by increasing the amount of DNA added to the qRT-PCR reaction and magnifying the fluorescent signal. In the same experiment, CATHDM3 and CATHDM2, both known to give positive results but with very low levels of DNA, were used as positive controls and a qRT-PCR reaction without any added DNA was used as a negative control (Figure 3).

3.2 DNA sequence data

Environmental DNA from a samples collected in October, 2000 at Charons Cascade were used to create a clone library with a random sampling of individual copies of cave bacterial 16S rDNA spliced into a plasmid cloning vector. The recombinant DNA molecules thus produced were used to transform *E. coli* cells, and those *E coli* cells are frozen for storage and revived to prepare plasmids carrying individual molecular copies of cave 16S rDNA for detailed sequence analysis. A view of the identities and relationships among the bacte-

rial community has been developed by matching and aligning approximately 1,500 bp of cloned cave 16S rDNA genes to produce a molecular similarity tree with 62 representative individuals from the cave community (11). Among the clones of particular interest was CCU23 that carried a cave eubacterial 16S rDNA sequence closely related to the same gene among members of the genus *Nitrospira*.

Clone CCU23 (Charons Cascade, Upstream, clone 23) was identified using the BLAST search engine available on the GenBank website () and the SEARCH and MATCH tools on the Ribosomal Database project website () as a close relative to the nitrifying genus *Nitrospira*, a characteristic of which is autotrophic growth by utilizing inorganic carbonate or bicarbonate as a food source with chemical energy for growth supplied by the oxidation of nitrite to nitrate. An alignment of the first 100 nucleotides of the CCU23 and related sequences is shown in Figure 5A, and similarity between CCU23 and its closest genetic matches over the full length 16S rDNA gene sequence (~1500 bp) are illustrated in Figure5B.

A distinguishing feature of the sequences is shown in Figure 5A and is utilized in this study to survey for CCU23 and other *Nitrospira spp*. by the use of two restriction enzymes which cut DNA at specific nucleotide sequences (10; Section 3.3). The enzyme HhaI cleaves DNA at the four-nucleotide sequence 5'-GCGC-3' while the enzyme BssHII cleaves DNA at the six-nucleotide sequence 5'-GCGCGC-3' which is statistically less frequent and is further underrepresented in bacterial DNA, making the BssHII fragment of 34 nucleotides in length a very specific feature of CCU23 and other *Nitrospira spp.* 16S rDNA sequences. Hha I cleavage sites occur much more frequently and virtually every eubacterial 16S rDNA sequence is cleaved at some position within 1500 nucleotides from the fluorescent terminus, therefore a profile generated by HhaI cleavage displays many fragments representing the broad bacterial community. Note also, the HhaI cleaves twice within the outlined six nucleotide BssHII sequence and cleavage there results in a pair of DNA fragments differing in length by two nucleotides. The lengths of these fragments can be measured with accuracy and great sensitivity. This combination makes them useful as biomarkers for CCU23 and *Nitrospira spp.* in environmental DNA samples (10, 11).

3.3 Fragment analysis data

DNA samples from clastic sediments at sites where limestone dissolution is an ongoing process were subjected to PCR with 6FAM-27f forward primer and 1492r reverse primer to create amplicons of eubacterial cave 16S rDNA labeled at the 5' end with the 6FAM fluorescent reporter. After cleavage with restriction enzymes, fragments were separated by length. Cleavage with BssHII produced the unique 34 bp fragment diagnostic for *Nitrospira* while HhaI produced a mixture of fragments including a pair of 36 and 38 bp in length as shown in Figure 6. The fluorescent fragment cleaved at the site most proximal to the 6FAM reporter is measured for each cleaved amplicon.

The specific fragments characteristic of CCU23 are evident in the fragment profiles shown in Figure 6, constituting strong evidence that *Nitrospira spp.* are present at these sites. The HhaI digests of the same samples show the HhaI biomarker fragment pair associated with the outlined sequences, and also display community diversity by revealing many other fragments generated by cleavage of 16S rDNA amplicons from other eubacteria.

3.4 Determination of NO_3^- in Albright Junction samples

Nitrate concentrations were determined for extracts of sediments collected at Albright Junction by ion chromatography. The average between two duplicate sediment samples was 45.22 ppm. The site of collection in Turner Avenue at Albright Junction was an undisturbed site beneath a rock overhang, although the sediments may have been subject to deposition of nitrates by evaporation or bacterial nitrification in the past. Our data and site observations suggest no current mechanism for regeneration of nitrates *in situ*.

4. Conclusions

The data show that greater numbers of bacteria are present in communities where processes of limestone dissolution and cavern enlargement are ongoing (2, 4, 8, 9, 12, 13, 14, 29, 30, 31, 32), and that restriction fragment biomarkers diagnostic for *Nitrospira spp.* are present in active communities of the Flint-Mammoth Cave System. Also, two sediments from an inactive and relatively dry cave passage in Turner Avenue (ALBRJCT1, ALBRJCT2) did not show evidence of any bacterial DNA but were shown to contain 45 ppm nitrate. Thus, nitrate in this particular sediment does not appear to be attributable to active bacterial nitrification.

Quantitative DNA measurements using Real-Time PCR amplification of 16S rDNA were made in reference to *E. coli* genomic DNA as a standard and stated as nanograms of DNA per

gram of sediment (dry weight). It is known that the *E. coli* genome (all chromosomal DNA in one cell) consists of 5.2×10^6 base pairs (bp) in length with seven 16S rDNA copies per genome. Other groups and individual genomic sequences are being added to a growing database showing a wide range of 16S rDNA copy numbers per genome across many taxa, ranging from one to 15 with an average of four copies per genome among the eubacteria (rrndb database ref). One copy of 16S rDNA is approximately 1,500 bp, thus it is possible to estimate the number of genomes or cell number (assuming genome size the same as *E. coli*) and four 16S rDNA copies per genome on average.

About the Author

Rick Fowler was trained in chemistry and biology before attending graduate school at the University of Tennessee. He received a Master's Degree in Biomedical Science while employed as a research associate in the Biology Division of Oak Ridge National Laboratory, and later pursued doctoral studies in environmental toxicology while working as a research associate at the Center for Environmental Biotechnology. Currently he is Laboratory Coordinator in the Biotechnology Center at Western Kentucky University and is assembling a multidisciplinary team to arrive at applied solutions for cave and karst management and environmental monitoring using the tools of biotechnology. He has 13 publications covering a variety of topics in biotechnology, and he has managed core labs in industry, academia, medical schools, and hospitals. He is a member of the National Speleological Society and the Cave Research Foundation.

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