A DNA Fingerprinting Technique to Survey Microbial Diversity in Caves

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Abstract

A comprehensive survey of microbial species from cave sediments and karst aquifers is needed in order to appreciate their role in cavern formation, aquifer evolution, and cave ecology. The time consuming practice of culturing organisms from the environment has had limited success for only a few species, and those organisms that cannot be grown in the lab are omitted. We extract DNA directly from cave sediments and amplify bacterial, fungal, or algal 16S rDNA using the polymerase chain reaction and selected primers labeled with fluorescent dyes. Genetic libraries of bacterial 16S rDNA have been generated from cave sediments at selected sites in Mammoth Cave, and hundreds of cloned 16S rDNA sequences from cave bacteria have been analyzed. Species are being identified or taxonomically classified by phylogenetic sequence analysis and comparison to electronic nucleic acid databases, and characteristic fragment lengths have been tabulated for cloned or cultured cave bacterial 16S rDNA and standards. The 16S rDNA sequence and fragment database constitutes a reference to which DNA profiles of cave sediment bacterial communities can be compared.

Introduction

Mammoth Cave in Kentucky, with over 500 kilometers of surveyed passages, is the longest known cave system in the world. It has been the focus of much research into the formation and evolution of limestone caves and karst aquifers and it harbors a unique subterranean ecosytem. Earlier studies in our laboratories have examined the rate of limestone dissolution in stream sediments at the lowest level of Mammoth Cave where carbon dioxide partial pressures are an order of magnitude higher than in the stream itself. The higher levels of carbon dioxide presumably result from the action on organic materials by microorganisms in the sediment. Some of the microorganisms may be producing other acids that accelerate limestone dissolution and thus contribute to cavern enlargement and aquifer evolution (Vaughn, 1998; Vaughn *et al.*,1998)

Before the impact of microbial action on cave formation and cave ecology can be assessed, a thorough census of microorganisms of caves and karst aquifers is required. Some attempts have been made to survey and identify bacteria associated with Mammoth Cave sediments by selective culturing and morphologic characterization; but, of the strains that could be isolated and grown on a dish in the laboratory, the majority could not be identified (Rusterholz and Mallory, 1994). Current efforts in our group are addressing bacterial involvement in limestone dissolution by growing cave bacteria in liquid culture (Elliott *et al.*, 2000).

There are difficulties in using direct culturing methods for the study of microbial ecology in environmental settings. Traditional methods rely on the ability to culture any bacterial species present under laboratory conditions using classical microbiological techniques. In natural environments bacteria do not live alone in isolated culture, but instead they form interdependent communities of bacterial species called biofilms. Environmental strains have unknown nutritional requirements and less than 1% of those actually present are ever isolated in the laboratory (Amann *et al.*, 1992; Siering, 1998). The unknown factors are magnified greatly when attempting to culture microorganisms from extreme environments such as hydrothermal springs or volcanic vents (Moyer

et al., 1994; Hugenholz *et al*., 1998), deep-sea sediments (Vetriani *et al*., 1999), salt lake beds (Minz *et al*., 1999), and subterranean ecosystems (Rusterholtz and Mallory, 1994; Elliott *et al*., 2000).

However, modern DNA analysis techniques are revolutionizing our understanding of bacterial diversity in the environment and have been applied to extreme environments including particular caves known to harbor bacterial communities in isolated and unusual geochemical conditions. New genera of bacteria capable of expressing genes with medical and practical applications have been discovered and are now the focus of many cave microbial studies (Angert *et al*., 1998; Holmes *et al*., 2001; Northtup *et al*., 2000).

We have begun a survey of microorganisms inside Mammoth Cave using modern DNA analysis techniques for the first widespread inventory of its microbial communities. The method described below is suitable for a broad survey of bacterial communities throughout the vast cave system and is applicable to a large number of samples. Our technique relies upon comparison of bacterial DNA fingerprints to a cave bacterial database of detailed genetic information derived from cultured and cloned organisms from selected cave sediments. We invite collaborations with other caves nationwide to contribute to the growth of our cave biomarker database.

Description of the Technique

Genetic identification of environmental strains.

Using modern DNA technology, bacteria can be identified and classified according to the sequences of their genes encoding 16S ribosomal RNA (16S rDNA). Different species of bacteria possess characteristic 16S rDNA sequences. Bacterial 16S rDNA sequences may be selectively amplified from the mixture of DNA fragments extracted from the environment to create many copies for more detailed studies (Figure 1). With this technique, bacterial species can be identified and their genetic relationships can be determined without the need to culture individual strains in the laboratory. Furthermore, environmental bacteria that cannot be grown in the laboratory can still be detected by the presence of 16S rDNA (Siering, 1998; Angert *et al*., 1998; Holmes *et al*., 2001).

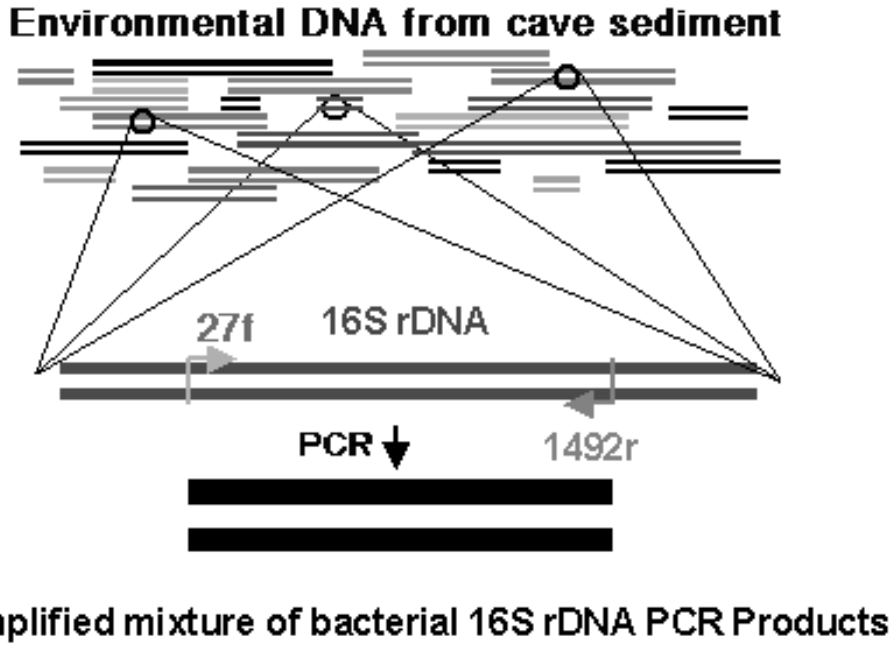

Figure 1. *Diagram showing how specific DNA sequences extracted from cave sediment can be targeted for analysis using the Polymerase Chain Reaction (polymerase chain reaction).* Some of the many different fragments of environmental DNA encode bacterial 16S rDNA (top). The 27f and 1492r short DNA sequences are conserved among the bacteria (middle), and they can be used as primers to amplify a mixture of bacterial 16S DNA sequences (bottom) while incorporating fluorescent dyes for analysis.

Sampling and DNA extraction

Sediment samples were collected from upstream, middle, and downstream points within Charons Cascade, along Echo River at the lowest level of Mammoth Cave. Sediment was scooped wearing latex gloves into sterile centrifuge tubes (Figure 2A) and kept on ice until DNA was extracted. DNA was extracted from one gram of cave sediment using a simplified procedure, and the mixed environmental nucleic acids were visualized by agarose gel electrophoresis (Figure 3). Cave sediment contains many microorganisms, including bacteria, fungi, protozoans, and even larger cave invertebrates (Figure 2B) with small particles of dead plant and animal material. All of these things contribute to the mixture of DNA fragments that can be extracted directly from cave sediment.

Figure 2*. Sample collection in Mammoth Cave.* Sediment was scooped wearing latex gloves into sterile tubes (A) and kept on ice until DNA was extracted. In addition to bacteria, environmental DNA contains sequences from fungi, protozoans, and even cave invertebrates (B) with decomposed plant and animal material.

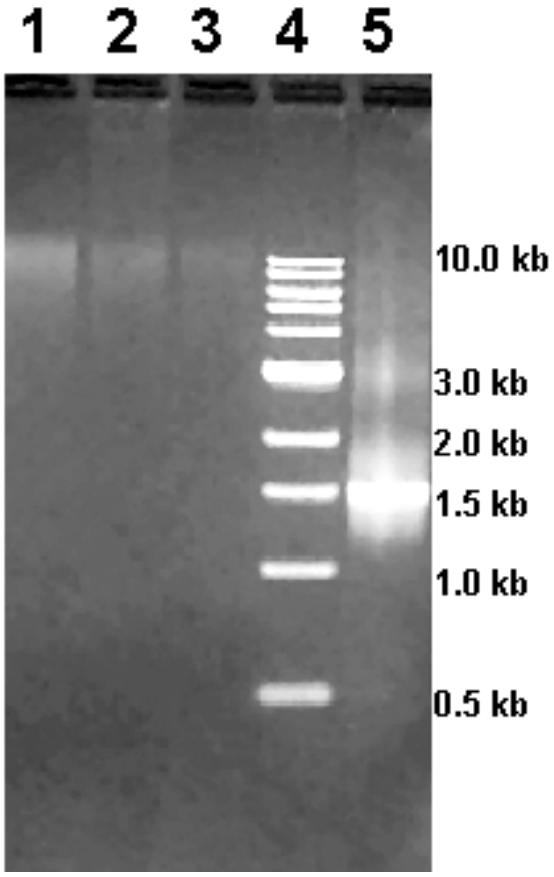

Figure 3. *Agarose gel showing cave sediment DNA and 16S rDNA polymerase chain reaction product.* DNA fragments at approximately 10.0 kb were extracted from 0.5 g sediment collected at upstream (lane 1), middle (lane 2), and downstream (lane 3) sites near Charons Cascade. DNA was amplified by polymerase chain reaction to

Amplification of 16S rDNA

In order to study the DNA of cave bacteria among all the DNA fragments present, specific DNA sequences were amplified out of the mixture using the polymerase chain reaction with specific bacterial primers. Our study focuses on the bacterial community in general thus we are using primers 27f and 1492r, short sequences that are conserved among a broad range of bacteria (Lane, 1991; Layton *et al*., 1994).
A polymerase chain reaction product from cave sediment representing the cave bacterial community was seen by agarose gel electrophoresis with the expected size of about 1.5kb (Figure 3). The environmental polymerase chain reaction product consists of a mixture of 16S rDNA from all bacterial species that have in common

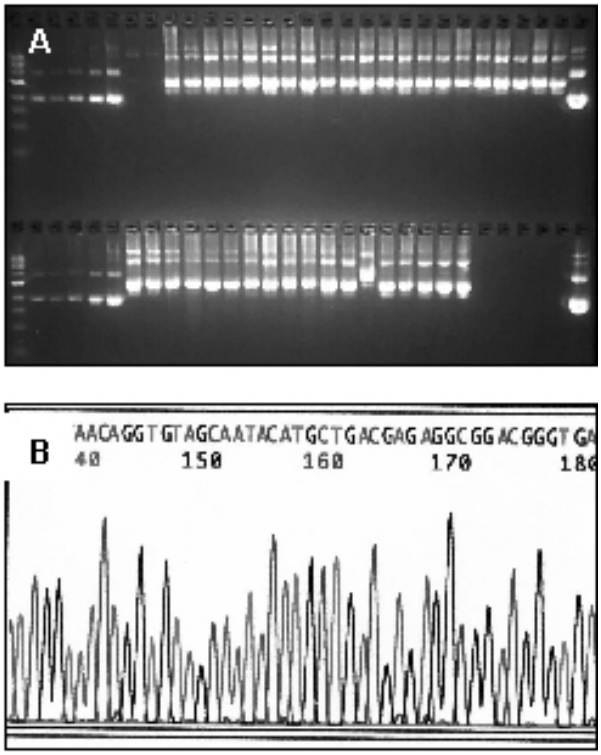

Figure 4. *Agarose gel (A) showing plasmid DNAs from a cave clone library and automated DNA sequencing data (B).* Each of the cloned plasmid DNA molecules shown on the gel carries one kind of bacterial 16S rDNA from the cave sediment (A). The DNA sequence of each cloned 16S rDNA was determined using automated fluorescent DNA sequencing with capillary electrophoresis (B) to generate data that was compared to online genetic databases. Table 1 shows a summary of the taxonomic groupsof the nearest genetic relatives using the Ribosomal Database Project online (http://rdp.cme.msu.edu).

the 27f and 1492r primer sequences. In order to differentiate among the many types of bacteria in the community, we must sort the amplified genes by molecular cloning and DNA sequencing or distinguish them by their terminal restriction fragment lengths.

Cloning and Sequencing

The amplified 16S rDNA was spliced into a cloning and sequencing vector plasmid DNA. The circular recombinant plasmid molecules thus produced were used to transform *E. coli* for studies of individual copies of the environmental genes. A cave clone library of *E. coli* host cells carrying cave DNA sequences was created and plasmid DNA was purified from each clone. Each clone harbors just one typeofrecombinantplasmidDNArepresenting one bacterial 16S rDNA sequence originating from the cave sediment (Figure 4A).

Table 1. Nearest genetic relatives within clone library of bacteria from Mammoth Cave.

Taxon:	No. of clones (%)
Nitrospina sub dv.	8 (18%)
Proteobacteria	
Alpha	6 (14%)
Beta	8 (18%)
Gamma	1 (2%)
Delta	3 (7%)
Gram-positive	4 (9%)
Environ. clone WCHB1-31 grp.	4 (9%)
Unclassified/Unaligned	4 (9%)
Planctomyces and relatives	3 (7%)
Environ. clone PAD1 grp.	1 (2%)
Green non-sulphur and relatives	1 (2%)
Flexibacter/Cytophaga/Batteroides	1 (2%)

Nucleotide sequences of bacterial 16S rRNA genes from the clone library and cultured bacteria have been determined (Figure 4B) and compared to DNA sequence databases to find the taxonomic classification of the nearest genetic relative (Table 1). Four subgroups of Proteobacteria representing a high degree of diversity corresponded to 41% of the clones sequenced. It is noteworthy that 18% of the clones were closely related to the Nitrospina subdivision with few species previously known. Nitrospina may contribute to cave geochemistry and acid production through nitrification reactions that accumulate nitrate, particularly in the absence of plants. Other clones were related to Gram positive species, Planctomycetes, and various uncharacterized bacteria commonly found in soil. Some of the matches raised ecological red flags by indicating the presence of bacteria that derive energy through biodegradation of petroleum, creosote, heavy metals, or sewage.

Fragment Analysis

Rather than commit to cloning and sequencing from every cave sample examined, a snapshot of bacterial diversity can be generated easily and quickly for a larger number of samples by terminal restriction fragment length polymorphism (TRFLP) analysis. Snapshots from environmental samples depict multiple types of bacteria within the community in a given sediment sample, and the profile generated is a "fingerprint" with information about the types of bacteria present and their relative abundance.

Environmental DNA from cave sediments, plasmid DNA from the cave clone library, and

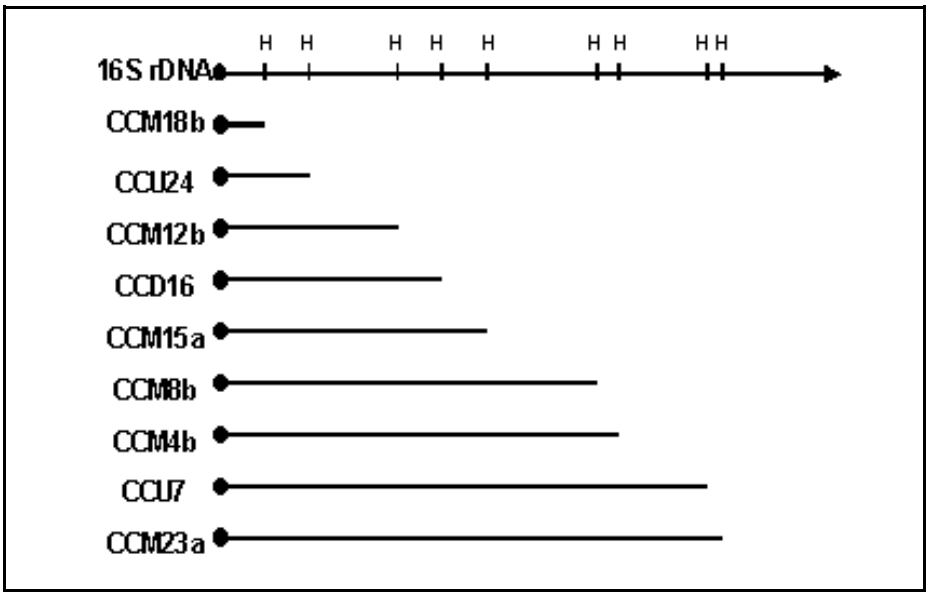

Figure 5. *Diagram showing fluorescent fragments that can be used as biomarkers for bacteria.* Fluorescent 27f primer was used to produce a mixture of labeled bacterial 16S rDNA sequences from cave sediment (top). Depending upon their individual DNA sequences, the fragments are cleaved by the enzyme HhaI (H) at some specific distance from the fluorescent terminus. A profile of the fragments derived from cave sediment is called a fingerprint. Fragment lengths with DNA sequence data from cloned and cultured bacterial 16S rDNA can be used to interpret the fingerprints.

genomic DNA from cultured organisms was amplified with fluorescent-labeled primer 27f and non-labeled 1492r. We obtained copies of 16S rDNA labeled at the 5' end of the 27f primer sequence with blue, green, or yellow fluorescent dyes. The end-labeled fluorescent polymerase chain reaction products were then digested with the restriction enzyme HhaI to generate fragments which were analyzed on a fluorescent genetic analyzer. Only the fragment from the fluorescent terminus up to the most proximal HhaI site is labeled and therefore observed by the fluorescence detector.

When TRFLP analysis is applied to the purified plasmid DNA samples in the clone library, each clone yields a single peak in the electropherogram with a characteristic defined fragment length determined by that particular DNA sequence. A total of 103 bacterial 16S rRNA genes have been analyzed by TRFLP including 87 from Charons Cascade in Mammoth Cave, along with nine cultured organisms from Mammoth Cave, four cultured from Lost River Cave, and three ATCC standard cultures. Their fragment sizes have been averaged over multiple determinations and tabulated in a database along with the corresponding DNA sequences and phylogenetic data.

Environmental DNA profiles are interpreted with the aid of the tabulated fragment data. DNA fingerprints of cave bacterial communities are labeled blue with 27f primer, while cloned or cultured standards are labeled green or yellow. Digestion of both environmental and cultured or plasmid DNA with HhaI followed by simultaneous capillary electrophoresis allows the corresponding peaks in the environmental profile to be directly superimposed with the 16S rDNA data from cloned and cultured bacteria (Figure 6).

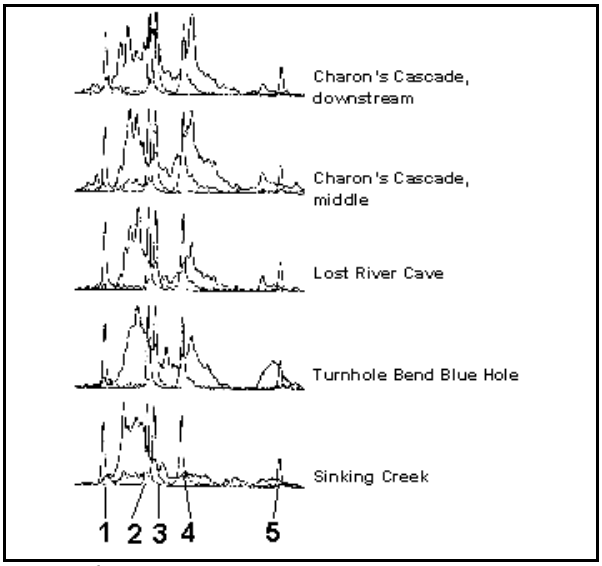

Figure 6. *Bacterial DNAfragment profiles from various cave and karst sediments superimposed with DNA fragments from the cave bacterial database. Bacterial 16S rDNA fingerprints labeled with blue fluorescent dye were mixed with yellow and green 16S rDNA fragments from cloned and cultured bacteria in the database. Standards are (1) MCNP clone CCU10, (2) Pseudomonas env. str. MCNP-CCCO12, (3) Pseudomonas env. str. MCNP-CCCO8, (4) MCNP clone CCU8, and (5) Staphylococcus aureus from a* standard depository (ATCC).

Summary

Our technique, summarized in Figure 7, allows many different bacterial types to be surveyed in a single DNA test that can be applied to a larger number of cave sites. Of particular interest are those sites known to be undergoing limestone dissolution and cavern enlargement and where geochemical and hydrological data are being collected. The growing database of DNA sequence and phylogenetic information along with fragment sizes from the cave clone database provides a means for recognizing and monitoring bacterial species in cave sediments, without the need to isolate and culture the organisms.

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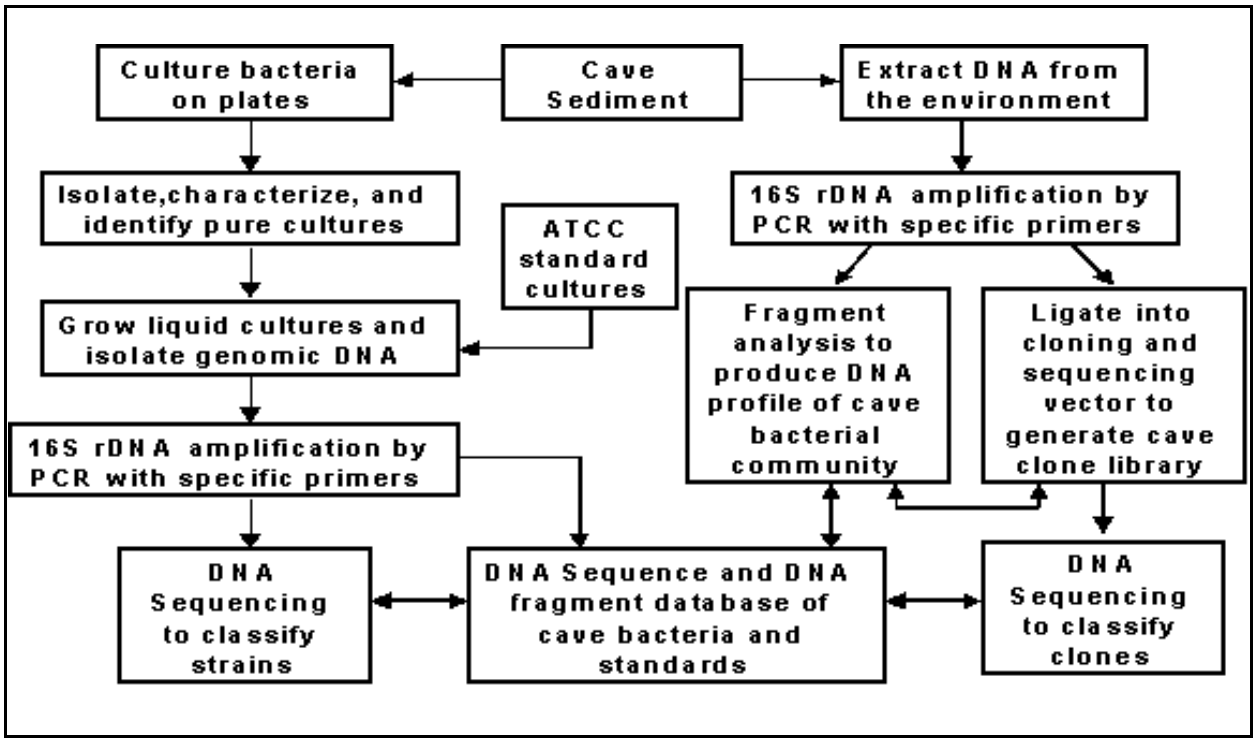

Figure 7. *Flow chart of information leading to the creation and growth of the database of genetic markers for cave bacteria.* On the left, cave sediment is the source for bacteria cultured and identified on plates. Isolated bacteria from the environment and from standard collections can be grown in liquid culture to yield DNA that can then be analyzed by DNA sequencing and fragment analysis to contribute to the database.On the right, environmental DNA can be extracted directly from cave sediment and subjected to polymerase chain reaction with specific bacterial primers. The amplified mixture of polymerase chain reaction products can be individually sorted and identified by cloning and sequencing the cave bacterial 16S rDNA. To survey a larger number of samples, the amplified polymerase chain reaction mixture is labeled with fluorescent dye and subjected to fragment analysis to produce a profile that is interpreted with the aid of the database.

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