Detection of Potentially Ubiquitous Taxa Using Culture-Independent Techniques

B. A. Rash* , Scott J. Ditch, and F. A. Rainey
Louisiana State University, Baton Rouge, LA

Louisiana State University
202 Life Sciences Building
Baton Rouge, LA 70803

Abstract

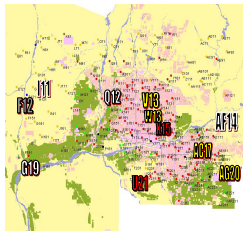
It is widely accepted that in many instances, the urban landscape can dramatically change various biological communities within a particular geographic area. However, little is known on how land use affects the diversity of the microorganisms. Recent advances in culture-independent techniques have demonstrated the presence of certain taxonomic groups in a variety of habitats. These taxa are considered by some to be ubiquitous and are represented by examples such as the uncultured members of the *Acidobacterium* and *Rubrobacter* lineages. The Central Arizona-Phoenix Long Term Ecological Research (CAP LTER) site is suitable for the study of (i) changes in bacterial community structure across land use gradients and (ii) the ubiquity of certain bacterial groups throughout a such a diverse environment. Culture-independent techniques including 16S rRNA gene clone libraries and specific 16S rRNA gene PCR primers have been applied to over 100 soil samples collected at the CAP LTER. From selected samples clone libraries were constructed and 4500 16S rRNA gene clones have been analyzed by ARDRA. 16S rRNA gene PCR primers specific for the *Acidobacterium* phylum have been designed using known sequence data. These primers have been used to determine the presence of members of these groups in all of the CAP LTER sites. We have demonstrated the presence of certain ARDRA patterns in soils from land under very different uses as well as composition. Such patterns can be considered to represent groups in the soil that are ubiquitous. Some ARDRA types are unique to individual soils. The use of 16S rRNA gene primers specific for the widespread *Acidobacterium* phylum has given a positive result for 87% of the soils tested. Interestingly many of the soils for which this taxon are considered absent are from urban areas (83% positive) while 98% of natural and urban desert soils gave a positive result. Comparisons have also been made between the soils from the pristine natural desert and desert remnants within the urban environment with the aim of determining the influence of urbanization on bacterial communities

Introduction

Through the widespread use of molecular applications of the 16S rRNA gene, some environtaxa sequences have been identified repeatedly from environments that are spatially and geographically diverse (4, 5). These studies suggest that within the domain *Bacteria* there lies certain "cosmopolitan" divisions that could be ubiquitous to terrestrial samples. For most examples of these taxa no cultured representatives exist. The study of these organisms in pure culture would provide powerful insight on bacterial diversity. Once such group, the members of the phylum *Acidobacterium*, have been demonstrated to be present in a wide range of environments (3).

The Central Arizona-Phoenix Long Term Ecological Research (CAP LTER) site is located in the heart of the Phoenix metropolitan area, an arid landscape (<50mm annual rainfall) that contains various relatively young urban settings as well as surrounding suburbs, agricultural regions, pristine open deserts outside the city, and other distinctly different land use types (Figure 1). Furthermore, patches of desert surrounded by the urban landscape, known as "desert remnants", could provide an estimate of degree of change in bacterial communities due to urbanization. The confinement of several diverse land use types within a small geographical region (2400 km²) makes the CAP LTER an ideal setting for studying whether ubiquitous taxa exist.

FIGURE 1. CAP LTER site and sample collection locations. Samples in large print have been used for 16S rRNA gene clone library construction. Color represents land use type.



- Desert Remnant
- Open Desert
- Urban
- Agricultural
- Riparian

TABLE 1. Background information on CAP LTER soils for which 16S rRNA gene clone libraries were constructed

Sample	Soil Description	clones collected
W13	Urban, highway	499
V13	Urban, industrial	503
AC17	Urban, residential	483
U21	Desert remnant	510
X15	Desert remnant	501
AF14	Open desert	496
AG20	Open Desert	448
I11	Open desert	514
F12	Open, riparian	493
G19	Agricultural	309
Q12	Agricultural	445

Objectives

1. **Determine if significant changes in bacterial communities occur when comparing soils from various land use types.** Approximately 500 16S rRNA gene clones from eleven CAP LTER soils were generated and subjected to Amplified Ribosomal DNA Restriction Analysis (ARDRA) and distinct banding pattern types have been partially sequenced.

2. **Search for taxonomic groups that are ubiquitous to all the CAP LTER samples collected.** Within the ARDRA database, there are banding pattern types that are present in all nine 16S rRNA gene clone libraries. Also, *Acidobacterium*-specific primers have been employed to test most of the CAP LTER soils for their presence in the environment.

* Brian A. Rash, Department of Biological Sciences, Louisiana State University, Room 202, Life Sciences Building, Baton Rouge, LA 70803. e-mail: brash@lsu.edu

Methods

Sample Collection and DNA Extraction. Soil samples were collected from the upper 2 in. of 200 sites within the CAP LTER area. DNA was extracted from samples using the technique of Smalla et al.(8) Nucleic acids were purified using a Prep-A-Gene kit (BioRad Industries).

PCR and cloning . Community ribosomal DNAs (rDNAs) were amplified by PCR from 20 to 200 ng of DNA in reaction mixtures totaling 50-µl containing (as final concentrations) 1X PCR Buffer (Roche), 50 µM each deoxynucleoside triphosphate, 200nM each forward and reverse primer, and 0.025OU of *Taq* polymerase (Roche) per ml. Reaction mixtures for universal primers were incubated in a Geneamp PCR system (Perkin-Elmer) at 98^{0}C for 6 minutes (for initial denaturation and activation of AmpliTaq); followed by 28 cycles at 52^{0}C for 30 s, 54^{0}C for 1 min, and 94^{0}C for 30 s; followed by a final extension period of 7 min at 72^{0}C. For all clone libraries, rDNAs were amplified with the forward primer 27F (specific for *Bacteria*) (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3'). PCR products were cloned with a TOPO XL cloning kit in accordance with the manufacturer's instructions (Invitrogen Corp.).

ARDRA and Sequencing. A 10µl aliquot of each M13 PCR product mixture was digested with *Taq*I restriction endonuclease for 1 hr at 65^{0}C or *Rsa*I restriction endonuclease for 1 hr at 37^{0}C. The reaction products were analyzed by Nuseive agarose (BioWhittaker Molecular Applications) (3% wt/vol) gel electrophoresis in 1X TBE buffer. The gel was then stained with 10ml of ethidium bromide and visualized by UV illumination. Restriction fragment profiles were subjected to cluster analysis with UPGMA to identify similar groups using Bionumerics software (Applied Maths). Restriction banding patterns of interest were sequenced.

Specific PCR amplification of 16S rRNA genes from environmental DNAs. To test for the presence of members of *Acidobacterium* were performed with *Acidobacterium*-specific primer 31F (5'-GATCCTGGTCAGAATC-3') designed by Barns et al. (3) and 1492R . The forward primer sequence used amplification reaction mixtures were identical to above, except the DNA template concentrations of 20 and 200 ng were tested. The cycling conditions for the 31F-1492R reactions follow those described above except for a final extension period of 15 minutes... For reamplification reactions, 5µl from the reactions with primer set 31F-1492R were used to template reamplification reactions Reaction mixtures were incubated in 3µl of each reaction mixture was analyzed on 1% agarose gels , and DNA was visualized by ethidium bromide and UV illumination. All samples were tested at least twice with the primer set to confirm results.

Phylogenetic Analyses. Sequences were compared to those in available databases using the BLAST (Basic Local Alignment Search Tool) network service (1) to determine their approximate phylogenetic affiliations. Compiled sequences were aligned by use of the ARB database (O. Strunk and W. Ludwig, ARB: a software environment for sequence data, 1999 [http://www.mikro.biologie.tu-muenchen.de])).

Results

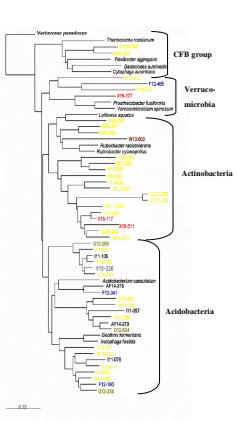


FIGURE 3. Phylogenetic tree generated by maximum likelihood method from a mask of 680 nucleotide positions, showing the broad phylogenetic diversity among selected CAP LTER 16S rRNA gene clones. Font color of clones represent land use type as shown in Figure 1. The scale bar indicates the number of substitutions per nucleotide position.

Distribution of ARDRA restriction banding patterns present in all 16S rRNA gene clone libraries constructed. Eleven different environmental samples, as shown in Figure 1, were chosen for 16S rRNA gene clone library construction at present. For each library construct, 309 to 510 clones were digested using *Taq*I restriction enzyme and screened by ARDRA. Dominant banding patterns from this analysis were further resolved by using *Rsa*I restriction enzyme. ARDRA group types for all clones (n=5,201) were then collected and compared via a UPGMA dendrogram. The number of unique ARDRA patterns between samples was significantly different for three of the soils (Figure 2). Chi square tests demonstrate that the amount of unique ARDRA patterns significantly differs from agricultural to urban to desert samples (data not shown). BLAST searches of representatives of ubiquitous ARDRA groups were used to assign each ARDRA group to a major taxonomic group. Table 1 lists the broad phylogenetic distribution of ARDRA group types found in all eleven libraries. These group types account for 48.1% of all of the clones. Mass sequencing of clones demonstrated a diverse collection of phylotypes in lineages that contain few or no cultured representative taxa (Figure 3).

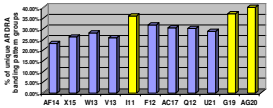


FIGURE 2. The percentage of unique ARDRA groups within the 16S rRNA clone libraries constructed. Bar graphs represent the number of unique ARDRA banding patterns divided by total number of clones generated. Yellow bars represent significantly different percentages.

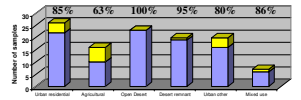


FIGURE 4. Bar graph showing the distribution of representatives of the phylum *Acidobacterium* in CAP LTER soils. The blue bars represent the number of soils that demonstrated a visible Acido-specific PCR product band present on an ethidium bromide-stained agarose gel. The red bars represent the number of soils with no visible PCR product. The percentages represent the proportion of each land use type that tested positive for the presence of *Acidobacterium*.

Distribution of *Acidobacterium* in CAP LTER samples. To map the distribution of members of *Acidobacterium*, 111 environmental samples representing 6 distinct land use types were screened with the 31F-1492R specific PCR primer pair (Figure 3). Those that gave no visible product were reamplified to confirm negative results. Three soils produced faint products when reamplified with the specific primers and were counted as positives. The two desert categories (open desert and desert remnants) were extremely widespread in *Acidobacterium* distribution, and a comparison of the two show no significant difference in abundance. Furthermore, the Type I error incorporated in each proportion extends to 100%, suggesting that the *Acidobacterium* phylum may be ubiquitous in CAP LTER desert samples. Using a Fisher's exact test, the combined desert samples proportions are significantly different to the combined urban residential and other categories (P=.0388) as well as the agricultural soils (P=.0066). Urban soils and agricultural samples show no significant difference (P>.05). The mixed category samples were not used in the statistical analysis.

TABLE 2. Similarity and abundance of taxonomic groups found in all 16S rRNA gene libraries of CAP LTER soils constructed

Taxonomic Group	Closest relative (accession no.)	Similarity* (%)	Overall abundance in all 16S rRNA gene clone libraries (%)
Alpha Proteobacteria	*Methylobacterium* Clone 1035-2 (AF423207)	98.0	3.0
	Methylobacterium Clone SJA-9 (AJ009451)	94.9	2.2
	Aminobacter Clone SM1E02 (AF445680)	95.5	2.7
	Sphingomonas Clone 768-2 (AF423293)	96.8	10.4
	Sphingomonas Clone 367-2 (AF423253)	98.9	1.3
	Sphingomonas Clone 739-2 (AF423289)	94.6	13.8
Beta Proteobacteria	*Massilia* Clone FTL254 (AF529109)	97.1	2.4
	Variovorax paradoxus (AF078758)	96.6	0.7
	Zoogloea Clone Wuba68 (AF336359)	99.0	2.0
	Xylophilus ampelinus (AF078758)	96.0	0.8
Actinobacteria	*Rubrobacter* Clone C027 (AF013526)	91.6	2.8
Acidobacteria	*Acidobacteria* Clone S009 (AF4037561)	95.0	0.8
Verrucomicrobiales	*Verrucomicrobium* Clone C019 (AF015522)	98.7	0.6
Low G/C Gram +	*Clostridium* Clone S0134 (AF507712)	97.2	2.5
	Bacillus Clone 1448-1 (AF423230)	97.0	0.1
	Bacillus niacini (AB021194)	99.0	2.0

* Based on 600-750 base pairs

Discussion

Since there is no wide range study on the dominant taxonomic groups in the environment, there is little perspective on the degree of change in bacterial communities within such a confined and diverse region. In the past, 16S rRNA gene clone libraries have been constructed for numerous habitats, some of which have been located in the same geographic area. However, due to the limited range of cloning technologies and sequencing capabilities, the amount of clones collected and sequenced have provided an inconclusive view on total bacterial diversity. This study begins the process of addressing this problem.

Constructing 16S rRNA gene libraries with a large collection of rDNA clones allows for more sufficient analysis of CAP LTER bacterial communities and searching for ubiquitous taxa. Results to date indicate that most rDNA libraries contain similar amounts of ARDRA patterns, suggesting that the bacterial diversity in these soils are similar. Information on taxonomic groups found in all rDNA libraries has also been assembled, demonstrating the possibility certain taxonomic groups being ubiquitous throughout the CAP LTER region. More rDNA libraries are being constructed to further validate these observations.

Coupled with clone libraries, the design and use of specific PCR primers can further validate the ubiquitous presence of taxa in the environment in a more efficient manner. The first group examined using this protocol is the *Acidobacterium* phylum. It seems apparent from the results that the distribution of *Acidobacterium* changes between desert and urban and agricultural areas in the CAP LTER. This would suggest that there are in fact terrestrial environments in which *Acidobacterium* may not exist. In the future , specific primers for other widespread taxa will be examined.

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