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A phylogenetic microarray targeting 16S rRNA genes from the bacterial division *Acidobacteria* reveals a lineage-specific distribution in a soil clay fraction

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ABSTRACT

We designed an oligonucleotide microarray using probe sequences based upon a phylogenetic analysis of 16S rRNA genes recovered from members of the bacterial division *Acidobacteria*. A total of 42,194 oligonucleotide probes targeting members of the *Acidobacteria* division at multiple phylogenetic levels were included on a high-density microarray. Positive control hybridizations revealed a linear relationship between hybridization signal and template concentration, and a substantial decrease in non-specific hybridization was achieved through the addition of 2.5 M betaine to the hybridization buffer. A mean hybridization signal value was calculated for each *Acidobacteria* lineage, with the resultant lineage-specific hybridization data revealing strong predictive value for the positive control hybridizations. The *Acidobacteria* phylochip was then used to evaluate *Acidobacteria* rRNA genes from a Wisconsin soil and within a soil clay fraction. The *Acidobacteria* hybridization profile revealed the predominance of *Acidobacteria* subdivisions four and six, and also suggested a decrease in the abundance of subdivision six relative to subdivision four in the soil clay fraction. The change in relative abundance of these subdivisions in a soil clay fraction was supported by data from quantitative PCR. These results support the utility of a phylogenetic microarray in revealing changes in microbial population-level distributions in a complex soil microbial assemblage.

1. Introduction

The vast majority of prokaryotes live within oligotrophic natural environments and are poorly represented in culture collections (Amann et al., 1995; Torsvik et al., 1990, 1994, 1996). This is especially true of soil microorganisms, which have contributed greatly to our arsenal of antimicrobial agents, yet every census of soil microorganisms to date has revealed only fragmentary evidence of the extant phylogenetic and metabolic diversity present in any soil. Efforts to understand the distribution and ecological roles of environmental microorganisms have been aided by the molecular suite of tools now available, particularly those based on analysis of the small subunit ribosomal RNA gene (16S rRNA). Polymerase chain reaction (PCR) amplification of 16S rRNA genes from natural environments reveals many 16S rRNA gene sequences that are highly divergent from known cultured phyla (Woese, 1987; Hugenholtz et al., 1998a,b; Meier et al., 1999; Dojka et al., 2000; Wilson et al., 2002). Many of the highly divergent 16S rRNA gene sequences together comprise entirely new monophyletic prokaryotic lineages, forming newly recognized divisions (Barns et al., 1994; Head et al., 1998; Hugenholtz et al., 1998a,b; Pace et al., 1986; Ward et al., 1990). Of the bacterial divisions revealed primarily by rRNA gene sequence data, the division *Acidobacteria* is ubiquitous in soils and sediments (Liesack et al., 1994; Kuske et al., 1997; Barns et al., 1999). All of the cultured *Acidobacteria* isolates to date fall into four of the proposed subdivisions (Kishimoto et al., 1991; Ludwig et al., 1997; Hugenholtz et al., 1998a,b; Barns et al., 1999; Janssen et al., 2002), the number of which has recently been expanded to include up to 26 subdivisions (Zimmermann et al., 2005; Barns et al., 2007). A phylogenetic analysis of 16S rRNA gene sequences

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recovered from a soil at the West Madison Agricultural Research Station (WMARS) revealed that 25% of the rRNA genes were affiliated with the *Acidobacteria* division (Liles et al., 2003).

Phylogenetic microarrays, or "phylochips", have been used to discriminate rapidly between diverse 16S rRNA genes present within cultured or environmental microorganisms (Wilson et al., 2002; Brodie et al., 2006; Palmer et al., 2006; Huyghe et al., 2008). Compared to the labor- and resource-intensive efforts to clone and sequence a representative number of clones from a 16S rRNA gene clone library, phylogenetic microarrays can provide an efficient readout of the phylogenetic diversity present in an environmental sample. Furthermore, a hierarchical design allows probing for microbial taxa at different phylogenetic levels (Huyghe et al., 2008), giving information on the presence or absence of the branches and the twigs on the tree of life. In the present study, the division *Acidobacteria* was targeted by a phylogenetic microarray approach, with oligonucleotide probes targeting multiple phylogenetic levels. A series of positive control hybridizations were used to validate the phylogenetic microarray design and hybridization conditions, followed by experiments to determine the distribution of *Acidobacteria* taxa in a soil sample and a clay fraction from this same soil sample. Results from the *Acidobacteria* phylogenetic microarray were then tested by an independent molecular analysis to validate microarray hybridization results.

2. Materials and methods

2.1. Soil collection

Soil cores were collected from an undisturbed site at the West Madison Agricultural Research Station (WMARS) (Bintrim et al., 1997; Rondon et al., 2000). The ecosystem is a turfgrass understory without organic amendments. The soil type is a Plano silt loam containing 61% sand, 23% silt, and 16% clay, with 1.7% organic matter (pH 7.0). The top 10 cm of soil were sampled and sieving was used to remove roots and debris. Genomic DNA was isolated immediately after soil collection. The remainder of the soil sample was frozen at −80 °C.

2.2. DNA isolation from soil or Escherichia coli cultures

A bead-beating method (Bio101, Inc., La Jolla, CA) was used to isolate genomic DNA from soil microorganisms. The genomic DNA isolated via a Bio101 kit is generally less than 20 kb in size, yet the harsh lysis conditions ensure that the genomic DNA is broadly representative of the soil microbial community (Burgmann et al., 2001). Samples were stored at −20 °C until further analysis.

A set of positive control *Acidobacteria* rRNA genes was available from a previous study, with recombinant clones containing *Acidobacteria* rRNA operons and associated genes cloned within a bacterial artificial chromosome (BAC) vector (Liles et al., 2003). BAC DNA was isolated from *E. coli* cultures grown in Luria Broth (BD Diagnostic Systems, Sparks, MD) overnight while shaking at 37 °C, using a Large-Construct DNA Isolation kit (Qiagen, Inc., Valencia, CA). The use of an *Acidobacteria*-specific primer set prevented *E. coli* 16S rRNA gene contamination of the amplicons.

2.3. PCR amplification of rRNA genes

An *Acidobacteria* 16S rRNA gene-specific primer (31F) was used to amplify 16S rRNA genes from soil genomic DNA or positive control 16S rRNA gene clones by PCR (Barns et al., 1999). It should be noted that although this division-level primer is not predicted to PCR amplify 16S rRNA genes from all of the extant *Acidobacteria* subdivisions (Barns et al., 2007), the *Acidobacteria* subdivisions present within WMARS soil were identified using a universal bacterial primer set (Liles et al., 2003) and each of the *Acidobacteria* subdivisions identified in WMARS may be PCR amplified using the *Acidobacteria* division-level primer 31F. Amplicons were produced using approximately 100 ng DNA template, 1 unit Taq polymerase (Promega, Madison, WI), 1 × Taq polymerase reaction buffer, 200 μM dNTPs, and 200 nM of each of the primers 31F (5′-GATTCTGAGCCAAGGATC, *Acidobacteria*-division specific)(Barns et al., 1999) and 1492R (5′-ACGGYTACCTTGTTACGACTT, universal Bacteria domain) (Medlin et al., 1988) in 50-μl. Reactions to be used for microarray hybridization directly incorporated the Cy3-dCTP dye (GE Healthcare, Piscataway, NJ) into the PCR product using a final concentration of 40 μM Cy3-dCTP (20% of total dCTP) according to manufacturer's instructions. The reaction was performed with 3 min denaturation at 95 °C, 30 cycles of 95 °C for 1 min, 55 °C annealing for 90 s, 72 °C extension for 150 s, followed by 7 min extension at 72 °C. All reactions were carried out in a Robocycler 96 (Stratagene, La Jolla, CA) with 50 μl mineral oil added to each tube. Reactions were analyzed by agarose gel electrophoresis to confirm production of a single (heterogeneous in the case of soil genomic DNA template) amplicon. The resulting PCR products were fragmented to an approximate average size of 200 bp using diluted DNAse I (final concentration 0.004 U/μl) for 30 min at 37 °C. Consistent DNA fragmentation was monitored by agarose gel electrophoresis. To remove unincorporated Cy3-dCTP, the fragmented PCR products were purified and concentrated over Centricon 3000 NMWL centrifuge concentrators (Millipore, Inc., Billerica, MA). The concentration of fragmented, labeled PCR product was determined using a Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE).

2.4. Phylogenetic analysis

Acidobacteria 16S rRNA gene sequences were aligned to a dataset of 6883 bacterial sequences (courtesy of Dr. Phillip Hugenholtz, http://rdp.cme.msu.edu/html/alignments.html) using the ARB software package (http://www.arb-home.de/) and refined manually to remove regions of ambiguous homology. Alignments used for phylogenetic analysis were minimized by the Lane mask (Lane et al., 1985) for bacterial data or an *Acidobacteria* filter (phylum specific 50% filter by base frequency) prepared in ARB. Phylogenetic trees for near full-length sequences (>1400 nt) were inferred within the ARB package using evolutionary distance (neighbor-joining algorithms with Felsenstein correction) and the PHYLIP program for maximum parsimony (Felsenstein, 1993). Partial sequences (<1400 nt) were inserted into trees without branch arrangement of full-length sequences using the parsimony insertion tool of ARB. The robustness of the tree topology was tested by bootstrap resampling with multiple outgroups.

2.5. Probe design

Acidobacteria-specific oligonucleotide probes were designed using the ARB software package and the ssujun02 dataset of sequences (http://www.arb-home.de/). Additional *Acidobacteria* sequences deposited in GenBank were also included. The dataset was limited to only *Acidobacteria* sequences larger than 500 bp and all potential chimeras were removed by partial treeing of the final alignment. Bulk probe design was accomplished through the ARB probe design function on two separate trees, one consisting of only full-length sequences (>1300 bp) and the other of full and partial sequences (>500 bp). Probes were designed for all clades supported by bootstrap values >= 85% (parsimony), supporting the presence of 10 monophyletic *Acidobacteria* subdivisions. Values used in the ARB probe design function were as follows: Length of Output = 100; Max. non-group hits = 0; Max. hairpin bonds = 4;