

## Wide Distribution and Diversity of Members of the Bacterial Kingdom *Acidobacterium* in the Environment

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**To assess the distribution and diversity of members of the recently identified bacterial kingdom *Acidobacterium*, members of this kingdom present in 43 environmental samples were surveyed by PCR amplification. A primer designed to amplify rRNA gene sequences (ribosomal DNAs [rDNAs]) from most known members of the kingdom was used to interrogate bulk DNA extracted from the samples. Positive PCR results were obtained with all temperate soil and sediment samples tested, as well as some hot spring samples, indicating that members of this kingdom are very widespread in terrestrial environments. PCR primers specific for four phylogenetic subgroups within the kingdom were used in similar surveys. All four subgroups were detected in most neutral soils and some sediments, while only two of the groups were seen in most low-pH environments. The combined use of these primers allowed identification of a novel lineage within the kingdom in a hot spring environment. Phylogenetic analysis of rDNA sequences from our survey and the literature outlines at least six major subgroups within the kingdom. Taken together, these data suggest that members of the *Acidobacterium* kingdom are as genetically and metabolically diverse, environmentally widespread and perhaps as ecologically important as the well-known *Proteobacteria* and gram-positive bacterial kingdoms.**

The use of rRNA sequence-based analysis of microbial populations has allowed study of complex communities in the environment without the requirement for laboratory cultivation of organisms. This approach has revealed astonishing diversity in many environments (3, 7, 12, 13, 17, 21, 23, 24, 30, 39). Several studies have analyzed over 100 sequences from a single environmental sample, and yet, remarkably, very little repetition has been seen among sequences, and virtually all previous studies have revealed novel groups with few or no known cultivated members. Most of these studies have identified new genera and even kingdoms (also termed “divisions” or “phyla” by other authors; “kingdoms” is here used in accordance with Woese [42]) of microorganisms, strongly suggesting that we as yet understand little about the total diversity of microbes in the environment (30).

One approach to studying complex ecosystems is to study subsets of the taxa present in hopes of better understanding the roles of individual groups within the larger community. During a recent survey of soil bacteria associated with pinyon-juniper woodlands in the southwestern United States, we recovered numerous 16S ribosomal DNA (rDNA) sequences that clustered into a diverse, novel group having only a single known cultivated member, *Acidobacterium capsulatum* (21). Analysis of “novel” sequences reported in studies of other soil samples (4, 23, 35, 37) revealed that many of them also clustered within this large group, suggesting that it was likely to be widespread in the environment. In addition, three extensive rDNA sequence analyses have indicated that the group is probably as phylogenetically diverse and distinct as previously recognized bacterial kingdoms and constitutes a previously unknown, major bacterial lineage (17, 21, 24). Such phylogenetically coherent groups are amenable to study by rDNA sequence-based techniques, because their relationships facilitate the design of

group- or species-specific PCR primers and oligonucleotide probes for determination of their diversity, distribution, abundance, and other properties.

We have undertaken a PCR-based survey of a wide variety of samples representing terrestrial, marine, aerosol, subsurface, hot spring, and animal environments in order to understand more about the distribution and diversity of members of the kingdom *Acidobacterium*. Based on available 16S rDNA sequences, a PCR primer was designed and tested that specifically amplifies rDNAs from this group when used with a conserved reverse primer. Four additional primers, targeting four phylogenetic subgroups identified in our original study (21), also were designed and evaluated. These five primers were then used to interrogate bulk community DNA extracted from diverse environments. Concurrently, an analysis of sequences reported in the literature was performed in order to map the known phylogenetic diversity of the group, facilitate primer evaluation, and further investigate the environmental distribution of members of the four subgroups targeted in the PCR survey.

### MATERIALS AND METHODS

**Environmental sample collection and extraction of nucleic acids.** Marine snow and marine picoplankton (collected and purified by Ed DeLong, Monterey Bay Aquarium Research Institute, Monterey, Calif.) (34) and animal fecal DNAs (horse, pig and ostrich feces; extracted by Connie Gebhart, University of Minnesota) were provided as purified nucleic acids. Hot spring and temperate sediment samples were collected with sterile spatulas and corers into 50-ml polypropylene tubes, frozen immediately on dry ice, and stored at  $-70^{\circ}\text{C}$ . Soil samples (approximately 1 kg) were collected into clean plastic bags for shipment to the laboratory. Upon receipt, soil samples were stored under refrigeration or frozen on dry ice until transferred to a freezer ( $-70^{\circ}\text{C}$ ) for long-term storage. Sample sources and descriptions are listed in Table 1. Chemical analysis of soil samples was performed by the Soil, Water and Air Testing Laboratory at New Mexico State University.

Nucleic acids were extracted and purified from approximately 0.5-g (or 0.5-ml) aliquots of each sample by using procedures described by Kuske et al. (20). Each sample was placed in a 2-ml screw-cap tube, and 0.3 g of each of three sizes of glass beads (710 to 1,180, 425 to 600, or 106  $\mu\text{m}$  in diameter; Sigma, St. Louis, Mo.) was added to each sample. TENS buffer (50 mM Tris [pH 8.0], 20 mM disodium EDTA, 100 mM NaCl, 1% [wt/vol] sodium dodecyl sulfate) was added to fill each tube, vortexed to mix thoroughly, and incubated in a  $70^{\circ}\text{C}$  water bath

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TABLE 1. Presence of members of subgroups in environmental samples

Source location <sup>a</sup>	Source type	Sample type <sup>b</sup>	pH	Reaction with primer <sup>c</sup> :			
				Y	O	G	A
<b>Soils</b>							
Sunset Crater, Ariz.	Volcanic	Cinders	7.1	+	+	+	+
Sunset Crater, Ariz.	Pinyon rhizosphere	Cinders	7.1	+	+	+	+
Cosnino, Ariz.	Pinyon woodland	Sandy loam	7.6	+	+	+	+
Cosnino, Ariz.	Pinyon rhizosphere	Sandy loam	7.3	+	+	+	+
Las Cruces, N. Mex.	Chile field	Sandy loam	7.4	+	+	+	+
Raleigh, N.C.	Meadow	Clay loam	4.5	+	+	+	+
Elk River, Minn.	Garden "black soil"	Organic	6.5	+	+	+	+
Paineville, Ohio	Agricultural	Sandy loam	6.8	+	+	+	+
Elk River, Minn.	Aged cow manure	Organic	6.1	+	+	+	+
Oslo, Norway	Boreal forest	Organic	ND <sup>d</sup>	+	+/-	+	+
Antelope Valley, Calif.	Citrus grove	Sandy loam	6.1	+	-	+	+
Dugway, Utah	Semi-arid desert	Sandy clay loam	7.7	+	-	+	+
Elk River, Minn.	Gravel pit "white peat"	Silt organic	7.2	+	-	+	+/-
Niceville, Fla.	Pine forest	Sandy	3.1	-	-	+	+
Poncha Pass, Colo.	Roadside fill	Sandy	2.5	-	-	+	+
Berkeley Heights, N.J.	Hardwood forest	Organic	4.0	-	-	+	+
Ohio	Cranberry bog	Muck; rifle peat	5.4	-	-	+/-	+
Wilmington, N.C.	Coastal pine forest	Sandy loam	3.2	-	-	-	+
<b>Sediments and mats</b>							
Los Alamos, N. Mex.	Marsh (anaerobic)	Organic	6.5	+	+	+	+
Abiquiu, N. Mex.	River (anaerobic)	Organic	ND	+	+/-	+	+/-
Los Alamos, N. Mex.	Shallow lake	Organic	5.8	+	-	+	+
Santa Rosa I., Fla.	Shallow marine	Sandy	7.4	-	-	+	+/-
Pine Barrens, N.J.	Cedar swamp	Organic	Acidic	-	-	-	+
Pine Barrens, N.J.	Hardwood forest	Streambed	Acidic	-	-	-	+
YNP	71°C hot spring	Sediment	7	-	+	-	+
YNP	67°C hot spring	Mat	7	-	+	-	-
YNP	64°C hot spring	Mat	7	-	+	-	-
YNP	67°C hot spring	Mat	9	-	-	-	-
YNP	77°C hot spring	Sediment	8	-	-	-	-
YNP	75°C hot spring	Sediment	7	-	-	-	-
YNP	72°C hot spring	Sediment	1	-	-	-	-
YNP	70°C hot spring	Mat	4	-	-	-	-
YNP	18°C spring	Mat	1.5	-	-	-	-
<b>Other samples</b>							
Minneapolis, Minn.	Horse, pig, ostrich	Feces	ND	-	-	-	-
North Central Pacific Ocean	Water	Marine snow	ND	-	-	-	-
North Central Pacific Ocean	Water	Picoplankton	ND	-	-	-	-
Dugway, Utah	Aerosol	Filtrate	ND	-	-	-	-
Los Alamos, N. Mex.	Aerosol	Filtrate	ND	-	-	-	-
Albuquerque, N. Mex.	Aerosol	Filtrate	ND	-	-	-	-
Artesia, N. Mex.	Aerosol	Filtrate	ND	-	-	-	-
White Sands, N. Mex.	Aerosol	Filtrate	ND	-	-	-	-
Lechugilla Cave, N. Mex.	Cave wall rock	Corrosion residue	ND	-	-	-	-

<sup>a</sup> YNP, Yellowstone National Park, Wyo. Additional source information available from the authors upon request.

<sup>b</sup> Mat, photosynthetic microbial mat. Marine snow and picoplankton samples are described in reference 34; the cave corrosion residue is described in reference 6.

<sup>c</sup> +, visible PCR product band present on ethidium bromide-stained agarose gel; +/-, faint band present, or visible band present only in reamplification reactions; -, no product band of correct size visible in any reaction.

<sup>d</sup> ND, not determined.

for 40 min. Samples were mixed well at 10-min intervals during the incubation. After incubation, samples were homogenized at 5,000 rpm for 3 min in a mini bead beater cell disrupter (type BX-4; Bio-Spec Products, Bartlesville, Okla.) and then centrifuged at 12,000 × g for 10 min to pellet the bead mix. The supernatant was transferred, and the bead pellet was washed once with 1 ml of TE buffer (10 mM Tris [pH 8.0], 1 mM EDTA) and centrifuged again. The wash supernatant was pooled with the original supernatant. Nucleic acids were precipitated from the solution by using 0.1 volume of 3 M sodium acetate (pH 5.2) and 2.5 volumes of ethanol, incubation on ice, and centrifugation for 30 min at 12,000 × g. Precipitated nucleic acids were suspended in TE. DNA concentration was estimated in ethidium bromide-stained 3% SeaKem agarose gels (FMC Bioproducts, Rockland, Maine) by using lambda DNA as a calibration standard or by solution quantitation with PicoGreen dye (Molecular Probes, Eugene, Oreg.) (20). DNA was purified away from contaminants by using Sephadex G-200 spin columns (in 96-well coarse polypropylene filter plates; Advanced

Genetic Technology Corp., Gaithersburg, Md.) equilibrated in TE, as described previously (20). The clear column eluate containing DNA was precipitated and suspended in TE buffer, and the DNA concentration was determined as described above. Negative control samples were prepared with TENS buffer alone, with no sample addition, and proceeding with all extraction and purification steps described above.

**PCR amplification of small subunit rRNA genes from plasmids and environmental DNAs.** Preparation of plasmid DNAs from rDNA clones from two soils of the arid Southwest United States is described by Kuske et al. (21). The forward primer sequences used in amplification reactions are listed in Table 2, together with optimum annealing temperatures used in thermal cycling reactions. Annealing temperatures were optimized by amplification of *56 Acidobacterium* kingdom and other bacterial kingdom rDNA clones characterized in our previous study (21). The optimum temperature was determined to be the lowest temperature at which amplification of target group clones, and no amplification of nontarget

TABLE 2. Forward rDNA PCR primers used in the survey of environmental DNA samples

Phylogenetic group	Primer (reference) <sup>a</sup>	Nucleotide sequence (5'→3') <sup>b</sup>	Target region <sup>c</sup>	Optimum annealing temp (°C) <sup>d</sup>
All bacteria	pA (10)	AGAGTTTGATCCTGGCTCAG	8–27	42
Kingdom <i>Acidobacterium</i>	31F	GATCCTGGCTCAGAATC	15–31	42
Subgroups <sup>e</sup>				
A	A	GCCTGAGAGGGCRC	293–306	50
G	G	CGCAAGCCTGACGAC	379–393	60
O	O	CGACGGTACCTTGCGT	480–497	57
Y	Y	GGTACYGTTTGTAAAGSTC	484–503	57

<sup>a</sup> All primers, except pA, were designed in this study.

<sup>b</sup> R, mixture of G and A (1:1); Y, mixture of C and T (1:1); S, mixture of C and G (1:1).

<sup>c</sup> Positions correspond to *E. coli* nucleotide numbering (14).

<sup>d</sup> Optimum PCR annealing temperature for specificity, when used with the 1492R reverse primer.

<sup>e</sup> Subgroups as indicated in Fig. 1.

clones, was observed. For all reactions, the reverse primer used was the “universal” 1492R primer (22). Amplification reaction mixtures contained 30 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl<sub>2</sub>, 5 μg of bovine serum albumin (Boehringer Mannheim), 200 μM (each) deoxynucleoside triphosphates, 25 pmol of each primer, and 1 U of *Taq* polymerase (AmpliTaq LD; Perkin-Elmer, Foster City, Calif.) in a final reaction volume of 25 μl. For primer testing, 100 pg of plasmid DNA per reaction was used. For amplification of environmental DNAs, approximately 0.1 to 1 ng of bulk DNA (where purified DNA concentrations were sufficient) was used. PCR was conducted with a Perkin-Elmer 9600 thermal cycler as follows: 2 min of denaturation at 94°C, followed by 30 cycles of 30 s at the optimized annealing temperature (Table 2), 60 s at 72°C (extension), and 5 s at 94°C (denaturation), with a final 5-min 72°C extension step after cycling was complete. For reamplification reactions, products from reactions with primer set 31F-1492R were diluted in serial 10-fold dilutions, and 1 μl each of the 10<sup>1</sup> and 10<sup>2</sup> dilutions was used to template reamplification reactions with one or more subgroup-specific primers. Five microliters of each reaction mixture was analyzed on 1 or 1.5% SeaKem agarose gels, and DNA was visualized by ethidium bromide staining and UV illumination. All samples were tested at least twice with each primer set to confirm results.

**rDNA gene clones from a Yellowstone hot spring sample.** A clone library of small subunit rRNA gene copies was generated from a photosynthetic mat sample from the unnamed hot spring designated “GFP.” PCR products (1.5 kb) from 31F-1492R amplification reactions were ligated into pGEM-T plasmid vectors (Promega, Madison, Wis.) using T4 DNA ligase and overnight incubation at 16°C, according to the manufacturer’s protocols. Recombinant plasmids were transformed into *Escherichia coli* JM109 competent cells (Promega), and colonies containing plasmids with inserts were identified by blue/white color selection on agar plates (32). Individual white colonies were subjected to PCR of insert sequences to select clones with the correct size insert (11). Clones were screened by restriction fragment length polymorphism (RFLP) analysis of PCR products, using the restriction enzymes *Hha*I and *Rsa*I (28). PCR products from seven clones, representative of each of seven RFLP types identified, were purified with PCR Prep columns (Qiagen, Inc., Chatsworth, Calif.). One microgram of purified DNA was used as a template in cycle sequencing reactions with fluorescent dye-labeled terminators (ABI PRISM dye terminator cycle sequencing kit; Perkin-Elmer). Primers used for sequencing (Table 2) were pA (9), 533FU (22), P3MOD (5'-ATTAGATACCTDGTAGTCC-3'; *E. coli* bases 787 to 806) (40), the reverse complement of P3MOD, EC910/931-RC (5'-CTCAAAGGAATTGACGGGGGC-3'; *E. coli* nucleotides [nt] 931 to 910), and 1492R. Electrophoresis of sequencing reactions was performed by using 4.0% polyacrylamide gels on a 373A Stretch DNA sequencer (Applied Biosystems, Inc., Foster City, Calif.). The nearly full-length sequence of clone GFP1 was analyzed for secondary structure anomalies and submitted to the CHECK\_CHIMERA program of the Ribosomal Database Project (RDP) (25) to detect the presence of possible chimeric artifacts. Sequence data for clone GFP1 have been deposited in the NCBI database under accession no. AF130858.

**Phylogenetic analysis of sequence data.** Sequences were obtained from GenBank, the RDP, and unpublished sources. In some cases, affiliation with the kingdom *Acidobacterium* was tested with SIMILARITY\_RANK (25) and FASTA (Wisconsin Package version 9.1; Genetics Computer Group [GCG], Madison, Wis.) analyses of the RDP and GenBank databases, respectively. Alignment of sequences was accomplished with Clustal W (36) and manually with the GDE multiple sequence editor (25), by using conserved primary sequence regions and secondary structure folding as guidelines. Sequence regions which could not be aligned with confidence were excluded from analyses.

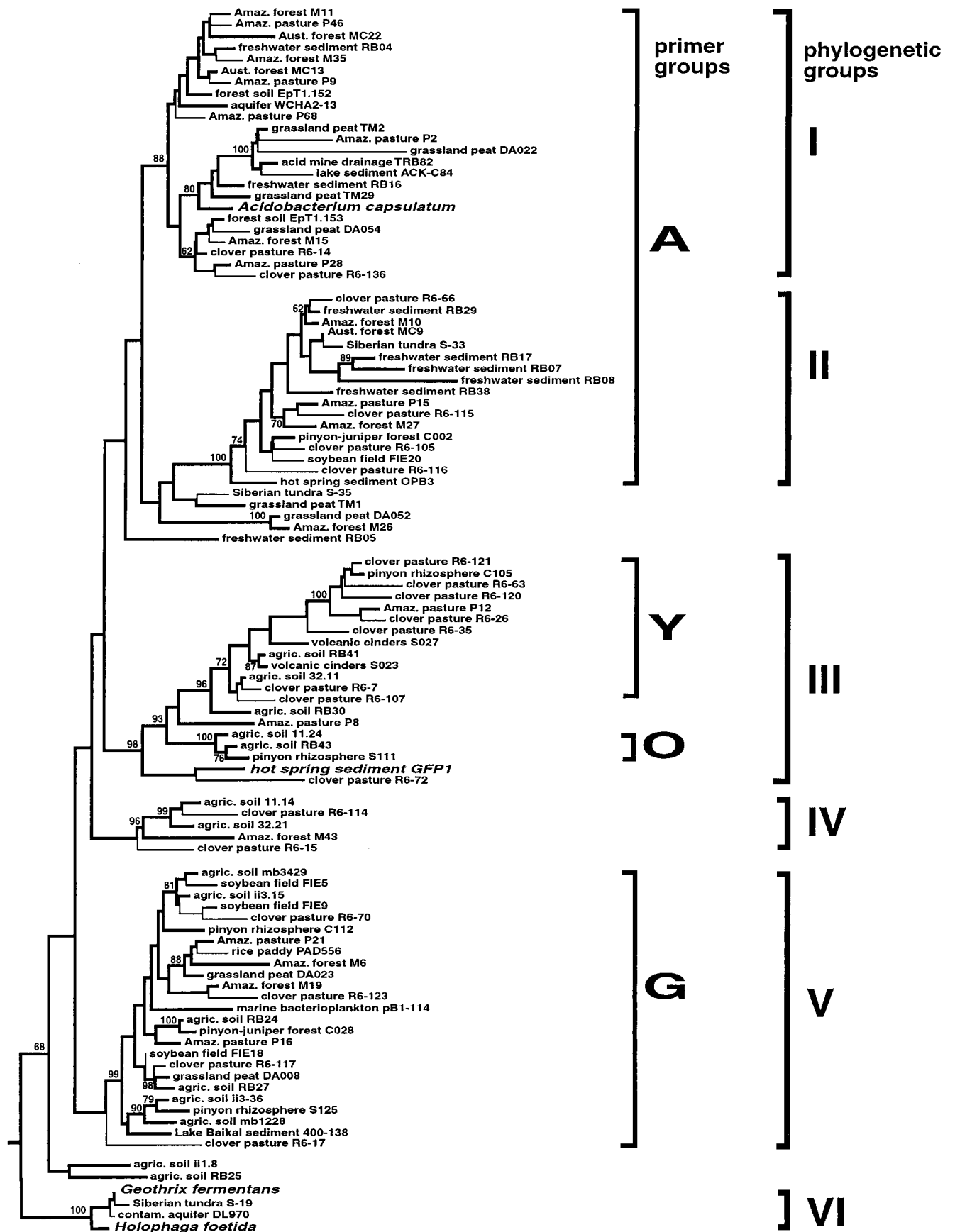
Maximum likelihood (ML) analyses were performed by using fastDNAmI (version 1.1; distributed by the RDP [29]) with empirical base frequencies, and a transition/transversion ratio (*T*) of 1.5, optimized by comparing likelihoods

under *T* = 1.0 to 2.0. The tree of highest likelihood was found by repeated tree building with random sequence input orders and optimized by global rearrangement of branches. Distance matrix (DM) analysis of sequences was performed with PAUPSearch\* (a version of D. Swofford’s PAUP, distributed with the Wisconsin Package version 9.1), by using the hky85 distance correction. Unweighted maximum parsimony (MP) analyses were also performed with PAUPSearch\*. DM and MP trees were constructed by using 10 rounds of random sequence addition order and optimized by tree bisection-reconstruction branch swapping. Bootstrap analysis by MP and DM methods was performed with 100 resampled data sets. Insertion of partial sequences into an optimized ML tree of longer sequences was performed 100 times with the “restart” script of fastDNAmI (29), with random addition of taxa, without rearrangement of branches in the starting tree. The tree of highest likelihood from this analysis is shown in Fig. 1. The alignment used to generate the tree of Fig. 1 is available online (23a).

## RESULTS AND DISCUSSION

**Design and specificity of *Acidobacterium* kingdom- and subgroup-specific primers.** The initial design of the PCR primers used in this study was guided by available kingdom *Acidobacterium* rDNA sequences. A signature nucleotide (G) for the *Acidobacterium* kingdom was identified, corresponding to nt 31 of the *E. coli* sequence (14). This specificity was confirmed by using the CHECK\_PROBE function of the RDP (25). Amplification of archaeal or eukaryotic rDNA sequences by a primer targeting this region is very unlikely, due to numerous mismatches with sequences in these groups. A forward PCR primer (designated 31F) was designed, utilizing position 31 at its 3' end. Using 1492R as the reverse primer, 31F was tested against 56 rDNA clones representative of several bacterial kingdoms (21). The results (not shown) indicated that the primer is specific and inclusive for clones of the kingdom *Acidobacterium*, producing a single strong product in positive reactions and no product of any size with clones from outside the kingdom. In a similar fashion, primers for four subgroups of the kingdom identified in our original analysis (designated Y, O, A, and G) (21) were tested against the clone collection and found to be specific (data not shown). Sequences and optimized annealing temperatures for kingdom and subgroup primers are given in Table 2.

**Distribution of members of the kingdom *Acidobacterium*.** Our PCR-based survey of diverse environmental samples demonstrated that members of the kingdom *Acidobacterium* are widespread in the environment. Environmental DNAs were first tested for their ability to support PCR. All extracted and purified DNAs were found to support PCR in positive control reactions using primers pA and 1492R, with the exception of a red clay which also yielded no visible DNA by agarose gel





analysis (data not shown), possibly due to binding of DNA to clay particles during extraction. Negative control extractions (no input sample) gave no product when amplified with any primer set.

Amplification of 43 environmental DNAs with the *Acidobacterium* kingdom primer set (31F-1492R) yielded either a single visible band of the anticipated size (approximately 1.5 kb) or no visible product when analyzed on agarose gels (not shown). All soils and freshwater and marine sediments tested gave positive results with this primer set. In addition, several hot spring sediments and mats, from springs of neutral to alkaline pH and up to 71°C in temperature, were also positive with the kingdom-specific primer set. These soils and sediments were of diverse lithology, chemistry, temperature, and location, from both pristine and agricultural settings (Table 1). Soils and sediments that were chemically analyzed varied considerably in their texture, pH (Table 1), percent organic matter (0.5 to 84%), and nitrate nitrogen (1.5 to 497 ppm). Concentrations of mono- and divalent cations also varied over a wide range (Mg, 0.2 to 534 meq/liter; Ca, 0.21 to 9,510 meq/liter; Na, 0 to 137 meq/liter). Samples which did not yield products with the kingdom-specific primer (listed in the bottom section of Table 1) include acidic hot spring sediments, hot spring sediments with temperatures over 71°C, aerosol samples from five locations in the Southwest, animal feces (horse, pig, and ostrich), Pacific Ocean marine snow and marine picoplankton (34), and cave "corrosion residues" (6).

Members of this group have also been detected in several studies of soils and sediments by other researchers (4, 10, 15, 23, 24, 28, 35, 37, 41, 43). Additional environments reported in the literature from which *Acidobacterium* sequences have been obtained include lake snow and activated sludge (24), and benzene-contaminated aquifer material (1). This ubiquity supports the suggestion (24) that members of this kingdom are capable of growth in many different environments. Although it is conceivable that these organisms occupy the same micro-niche in many different habitats, the broad phylogenetic diversity of rDNA sequences in the *Acidobacterium* kingdom, comparable to that seen in members of the *Proteobacteria* and other bacterial kingdoms, indicates that members of this group are likely to be quite diverse physiologically and ecologically. Temperature gradient gel electrophoresis (10) and in situ hybridization with rRNA-targeted probes (24) have indicated that these organisms may be abundant and active in some environments as well.

**Distribution of phylogenetic subgroups in environmental samples.** To map the distribution of members of four phylogenetic subgroups identified in our original analysis (21), primers Y, O, G, and A were designed for these groups. All environmental DNAs, regardless of their reaction with the 31F primer, were screened with these primers (Table 1). Those which gave no product with the 31F-1492R primer pair were also negative with the subgroup primers, as anticipated. In order to confirm negative subgroup reactions, reamplification of products from positive 31F-1492R (kingdom-specific) reactions was performed with the subgroup primers. Three soils produced

faint products when reamplified with subgroup primers; however, in most cases, reamplification did not change the previous results.

The results given in Table 1 indicate that members of the A subgroup are possibly ubiquitous in soils and marine and freshwater sediments and are also present in some hot spring communities. Members of the G subgroup are also very widespread, but were not found in the hot spring samples analyzed. Fewer samples produced amplification products with the Y and O primers. These results concur with other cultivation and rDNA sequence-based surveys of microbial distribution, which have both found that phylogenetically related groups of organisms vary in their ubiquity (13, 33). As a result, some groups of organisms are widely distributed in the environment, while others are highly restricted to particular habitat types.

For those groups of more limited apparent distribution, as measured by our PCR survey, it may be possible to infer some physiological properties based on the nature of their habitats. For instance, members of the Y and O subgroups are largely absent from acidic soils and sediments (<pH 6); thus, neutrophily may be a common property of these groups. Some members of the O group are evidently thermophilic or thermotolerant, because they are present in hot spring environments up to 71°C. The observed distribution of members of these groups is probably determined to some extent by the phylogenetic breadth of PCR primer sequences as well. Mapping of the target sequence distribution onto the tree of Fig. 1 shows that the A group primer is likely to amplify rDNA sequences from a much more diverse (phylogenetically and probably physiologically) selection of organisms than the individual G, O, and Y primers. This is unlikely to explain all of the results observed, however, because the sequences of the combined Y and O groups appear as diverse as those of the G group, and yet are seen in fewer environments. In general, however, it is likely that the organisms detected by the primers used in this study were too diverse to be restricted to distinct habitat types.

The PCR approach to survey for the presence of specific groups of organisms is subject to the biases which may affect all rDNA-based studies of environmental samples (19, 38). Most relevant to this study is differential lysis of diverse cell types (20, 27), which may contribute to negative results in this survey. However, since members of the kingdom *Acidobacterium* are probably gram negative in cell type (18) and an aggressive lysis technique was used to obtain DNA (20), it seems likely that negative PCR results with environmental samples truly indicate the absence of members of the target group.

**Identification of a novel lineage in a hot spring sediment.** DNA from the 67°C hot spring GFP gave strong positive results in kingdom *Acidobacterium* primer set (31F-1492R) amplifications, but was negative with all four subgroup primers. To investigate this result further, PCR products from the 31F-1492R reaction were cloned, and seven clones with distinct RFLP patterns were partially sequenced and found to be >99% identical through the approximately 400 nt sequenced. The nearly complete (approximately 1,400 nt) sequence of one clone, GFP1, was generated. Phylogenetic analysis of GFP1

FIG. 1. Phylogenetic tree representing the known diversity of the kingdom *Acidobacterium*. Sequences (other than GFP1) were obtained from databases and unpublished sources and analyzed as described in the text. The source references for the sequences shown are as follows: Amaz. (Amazon) forest/Amaz. pasture, 5; Aust. (Australian) forest, 23 and 35; freshwater sediment, 41; forest soil, 26; grassland peat (TM sequences), 31; grassland peat (DA sequences), 10; acid mine drainage, 8; lake sediment, 15; clover pasture, 4; Siberian tundra, 43; pinyon-juniper forest/pinyon rhizosphere/volcanic cinders, 21; hot spring sediment (OP), 17; agricultural soil, 24; rice paddy, 37; and contaminated aquifer, 1. Others are unpublished sequences from GenBank. Bootstrap values for MP analysis are given for branches having >60% support. Phylogenetic groups are designated based on primary branching clades appearing in all (ML, MP, and DM) optimal trees and having >85% bootstrap support, based on long (>900 nt) sequences (heavy lines). Partial sequences (thin lines) represent sequences added to the optimized ML tree without global rearrangement. The tree was rooted with the rRNA sequence of *E. coli*. The primer groups are those sequences expected to amplify with the primers listed in Tables 1 and 2.

places it as a deep branch within the kingdom *Acidobacterium*, related to group IV. This subtractive PCR approach has thus enabled targeted identification of new phylogenetic diversity within the kingdom, without prior information on what form that diversity might take.

**Phylogenetic analysis of kingdom *Acidobacterium* rDNA sequences.** An analysis of sequences representative of the currently known phylogenetic and environmental diversity of the kingdom *Acidobacterium* is presented in Fig. 1. For initial analysis, sequences of greater than 900 nt were selected (represented by thick lines on Fig. 1). These were used to construct and optimize trees by the DM, MP, and ML methods and to perform bootstrap analyses. Groups that were moderately to well supported by bootstrap evaluations (>70% support) were also seen in all optimal trees. The tree of longer sequences was used as a backbone into which shorter sequences (thin lines in Fig. 1) were placed without rearrangement of the starting tree. These shorter sequences were chosen to span the phylogenetic diversity of sequences obtained in other studies. From this analysis, it can be seen that most of the partial sequences cluster within the primary groups outlined in the starting tree, although a few deeply branching sequences (Amazon pasture P68; Siberian tundra S-35; and clover pasture R6-15, R6-17, and R6-72) may constitute distinct lineages.

Our initial study of two arid soils (21) recovered 31 kingdom *Acidobacterium* rDNA sequences, all of which could be grouped into four phylogenetic subgroups having moderate to good bootstrap support. Most sequences in Fig. 1 still appear to cluster within the four groups originally identified, although the overall topology of the tree has changed somewhat. A minimum of six primary groups (I to VI in Fig. 1) within the kingdom *Acidobacterium* are now apparent. The branching order of these within the larger kingdom cannot be resolved from this analysis, except for the *Holophaga-Geothrix* lineage (group VI), which was consistently the deepest group. Several additional deeply branching lineages of variable placement were observed (grassland peat TM1, grassland peat DA052-Amazon forest M26, agriculture soil RB05, and agriculture soil RB25/ji 1.8) as well, and these may represent primary groups in their own right. Doubtless the number and diversity of such groups will continue to grow rapidly in the near future as rDNA sequences from additional environments accumulate. However, there is already evidence that the kingdom *Acidobacterium* harbors at least as much genetic, and probably metabolic, diversity as any previously identified bacterial kingdom.

Since our initial report (2) and the initiation of the studies described here, many additional sequences from the kingdom *Acidobacterium* have been obtained from diverse environments. Remarkably, over 250 such sequences are now known. Comparison of the sequence set used in construction of the tree of Fig. 1 with the sequences of the kingdom and subgroup primers indicates that several deeply branching groups would not be detected by these primers. Most significant of these is the *Holophaga-Geothrix* group. Although phylogenetic analyses have indicated membership of this group within the kingdom *Acidobacterium* (16, 17, 24), albeit as the deepest branch, these sequences lack the novel residue responsible for the specificity of the 31F primer. Addition of new sequences to the known kingdom diversity has not, however, changed the specificity of the four subgroup primers.

The kingdom *Acidobacterium* is an example of a vast group of organisms that were virtually unknown prior to rDNA sequence-based surveys. Suddenly, we are aware that they are diverse and widespread and may be very important in many environments. It has become evident that the microbial communities present in soils and sediments are too diverse to be

comprehensively described by general 16S rDNA cloning approaches. Yet it is equally evident that such communities may be dominated by organisms that are only distantly related to species which have been studied to any extent in the laboratory. Molecular sequence-based techniques, therefore, will likely play an important role in providing information about diversity and function in these communities.

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