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Distribution Patterns of the Members of Phylum *Acidobacteria* in Global Soil Samples

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The distribution pattern of the phylum *Acidobacteria*, a previously uncultured bacterial group, was investigated by molecular ecological analyses of global soil samples collected from pristine ecosystems across five continents. Acidobacterial 16S rDNAs were observed in almost all soil samples, and members of acidobacterial primer group A were detected in all samples that harbored the phylum *Acidobacteria*. Other primer groups, Y, G, and O, showed limited distribution patterns. We further divided the primer groups into acidobacterial subdivisions (class-level). Subdivisional distribution patterns were determined by comparing the observed T-RFs with theoretical T-RFs predicted by *in silico* digestion of acidobacterial 16S rDNAs. Consistent with the PCR results obtained with subgroup-specific primers, T-RFLP analyses showed that acidobacterial subdivision 1 belonging to primer group A was present in the majority of the soil samples. This study revealed that the phylum *Acidobacteria* could be globally distributed. At the subdivisional level, acidobacterial subdivision 1 might be the most widely distributed group in this phylum, indicating that members of subdivision 1 might be adapted to various soil environments, and members belonging to other subdivisions might be restricted to certain geographic regions or habitats.

Keywords: *Acidobacteria*, distribution, PCR, T-RFLP

Bacteria belonging to the phylum *Acidobacteria* are a bacterial group that has not been successfully cultured in a laboratory environment. They have been identified using molecular surveys based on 16S rRNA gene sequences in community DNAs directly extracted from the environment, typically *via* PCR, cloning, and sequencing [1, 16, 25, 31, 36, 41]. To date, more than 20,000 sequences represent this phylum in public databases and almost all *Acidobacteria*-related sequences have been recovered from uncultivated microorganisms [5, 17]. Despite the ubiquity and abundance of bacteria belonging to the phylum *Acidobacteria*, our knowledge of the phylum *Acidobacteria* is very limited because of the lack of described species in this phylum. "Bergey's Manual of Systematic Bacteriology" [12] and the "Taxonomic Outline of *Bacteria* and *Archaea*" [13] describe the phylum *Acidobacteria* with only three species (*Acidobacterium capsulatum*, *Geothrix fermentans*, and *Holophaga foetida*), whereas there are five recently recognized species (*Acanthopleuribacter pedis*, *Edaphobacter aggregans*, *Edaphobacter modestus*, *Chloracidobacterium thermophilum*, and *Terriglobus roseus*) [2, 8, 10, 24]. Although many investigators are actively involved in elucidating the niche of this phylum, the ecological role of *Acidobacteria* remains largely unknown.

Members of the phylum *Acidobacteria* are considered to be ubiquitous and abundant, particularly in soil environments. The phylum *Acidobacteria* has been reported to make up an average of 20% (range, 5–46%) of sequences obtained in 16S rDNA clone libraries constructed from various soils [17]. In a study conducted by Kuske *et al.* [25], the phylum *Acidobacteria* was found to be the most dominant bacterial group in 16S rDNA clone libraries constructed from soil samples taken from the Sunset Crater National Monument (53.6%) and Coconino National Forest (57.1%) in Arizona, U.S.A. Later, Dunbar *et al.* [7] observed that *Acidobacteria*-related sequences were most abundant in clone libraries constructed from soil samples collected from the same geographic location. Similarly, we found in a previous study that *Acidobacteria*-related sequences represented a large proportion (>50%) of both 16S rDNA- and rRNA-derived clone libraries constructed from the rhizosphere of a chestnut tree (*Castanea crenata*) [28], thus suggesting that the phylum *Acidobacteria* could be numerically dominant and metabolically active in the soil. However, the majority of studies, including those mentioned above, sampled over

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a limited geographic scale and revealed phylum-level distribution patterns. Because the phylogenetic breadth of the phylum *Acidobacteria* is considered nearly as great as that of the phylum *Proteobacteria* [17], distribution patterns determined at the level lower than phylum could help to explain the ecological niche of *Acidobacteria*. Here, we describe the subdivisional (and subgroup-specific) distribution patterns of the phylum *Acidobacteria* in soil samples collected from around the world. We analyzed community DNAs extracted from 33 global soil samples using PCR assays with acidobacterial subgroup-specific primers, and conducted terminal restriction fragment length polymorphism (T-RFLP) analysis of acidobacterial 16S rDNAs amplified from the soil samples. Our results indicated that some groups of the phylum *Acidobacteria* could be distributed widely and others might be restricted to particular geographic regions or habitats.

Materials and Methods

Soil Samples and Nucleic Acid Extraction

Global soil samples were collected from the surface soil in pristine ecosystems across five continents (Table 1). All soils were collected in the spring (moist season) of the respective hemisphere. Details of sampling methods and soil characterization were described previously by Fulthorpe *et al.* [11]. Korean soil samples were collected from about 5 to 10 cm below the surface soil from grass lands (DT, YJ, and MS), mountain (WS), and beach (IC). Community DNAs of each sample were extracted directly using a PowerSoil DNA isolation kit (MoBio, Solana Beach, CA, U.S.A.) according to the manufacturer's protocol.

Table 1. Presence of members of acidobacterial primer groups in global soil samples.

Sampling location[a]			PCR with subgroup-specific primer[b]			
Continent	Region	Site (soil code)	A	O	G	Y
Africa	South Africa	Helshoogte (HH)	+	–	–	–
		Mooreesburg (MB)	+	–	–	+
		Mamreweg (MR)	–	–	–	–
		Welgevallen (WG)	+	–	–	–
		Paarl Mountain (PM)	+	–	–	+
Australia	Southwestern Australia	Bridge town (BN)	+	–	–	+
		Geraldton (GE)	+	–	–	+
		Jarrahdale (JD)	+	+	+	+
		Kelleberrin (KE)	+	–	–	+
		Merredin (ME)	–	–	–	–
Eurasia	Korea	Dong-tan (DT)	+	–	+	+
		Incheon (IC)	+	–	+	+
		Yeo-ju (YJ)	+	–	+	+
		Mi-shi (MS)	+	+	+	+
		Wang-San (WS)	+	+	+	+
	Russia	Russian site I (R-I)	+	+	+	–
North America	California	Chabot (CH)	+	–	–	+
		Cloverdale (CL)	+	+	+	+
		Hillgate (HG)	+	+	+	+
		Murrieta (MU)	+	+	+	+
		Venice Hills (VH)	+	+	+	+
	Saskatchewan	Bittern (BT)	–	–	–	–
		Porcupine (PC)	+	+	+	+
		Waitville (WV)	+	–	+	+
		Waskesieu (WK)	+	+	+	+
South America	Central Chile	La Campana (LC)	+	–	–	+
		Lago Penuelas (LP)	+	–	–	–
		Fray Jorge (FJ)	+	+	+	+

[a]Geographic Positioning System codes and other details for the sampling site are described in papers by Fulthorpe *et al.* [11] and Cho and Tiedje [3].
[b]Subgroup-specific primers designed by Barns *et al.* [1].
+, Visible PCR product band of correct size on ethidium bromide-stained agarose gels; –, no PCR product of correct size visible.

PCR Amplification of Acidobacterial 16S rDNA

16S rRNA genes of acidobacterial species and of their subgroups were amplified using *Acidobacteria*-specific primers (31F: GAT CCT GGC TCA GAA TC, 1518R: AAG GAG GTG ATC CAN CCR CA) and subgroup-specific primers (subgroup A: GCC TGA GAG GGC RC [*E. coli* position, 293–306]; subgroup G: CGC AAG CCT GAC GAC [*E. coli* position, 379–393] with 1518R primer; subgroup O: CGA CGG TAC CTT GCG T [*E. coli* position, 480–497]; subgroup Y: GGT ACY GTT TGT AAG STC [*E. coli* position, 484–503] with 1492R primer: AAG GAG GTG ATC CAG CCG CA) designed to anneal to a conserved position in the 3' and 5' regions of acidobacterial 16S rRNA genes [1]. The reaction mixture included 25 µl of RED *Taq* ReadyMix PCR Reaction mix with $MgCl_2$ (Sigma, St. Louis, MO, U.S.A.), 1 µl each of the forward and reverse primers (stock concentration, 20 µM), 200 ng of template DNA extracted from the soil sample, and sterilized distilled water to give a 50-µl final volume. The PCR thermal profile was as follows: initial denaturation at 95°C for 5 min and 30 cycles consisting of denaturation at 95°C for 1 min, primer annealing at 42°C (31 F), 50°C (subgroup A), 60°C (subgroup G), and 57°C (subgroups Y and O) for 1 min, and an extension at 72°C for 2 min. The final elongation step was extended to 20 min. PCR amplification was performed using a GeneAmp PCR system 9700 (Applied Biosystems, Foster City, CA, U.S.A.).

Terminal Restriction Fragment Length Polymorphism Analyses

For terminal restriction fragment length polymorphism (T-RFLP) analysis, acidobacterial 16S rDNAs were amplified with 6-carboxyfluorescein (6-Fam; Takara, Japan)-labeled acidobacterial specific primer 31F and universal reverse primer 1518R. Amplified acidobacterial 16S rDNA was purified using a QIAquick PCR purification kit (Qiagen) and digested with the tetrameric restriction enzyme HhaI (Promega) [9]. The enzyme reaction mixture included 50 ng of purified PCR product, 1 µl of 10 U HhaI restriction enzyme, 2 µl of 10× reaction buffer, and sterilized distilled water to give a final volume of 20 µl. The mixture was incubated for digestion at 37°C for 3 h. Later, digested fragments were purified using a QIAquick PCR purification kit and stored at −20°C until analyzed.

Two µl of digested fragments was mixed with 2 µl of internal size standard (GS1000-ROX; Applied Biosystems, Foster City, CA, U.S.A.) and 12 µl of deionized formamide and then denatured at 94°C for 2 min and chilled immediately on ice. Fragments were analyzed in GeneScan mode on a ABI Prism 3100 Genetic Analyzer (Applied Biosystems). Sizes of terminal restriction fragments (T-RFs) were determined using GeneScan analytical software 2.02 (Applied Biosystems). Peaks less than 1.5 bp apart from a larger peak (shoulder peaks) were eliminated. Peaks that were not present in both replicates (irreproducible peaks) were considered PCR artifacts and thus removed. According to the approach suggested by Luna *et al.* [32], the cut-off for the discrimination of each peak from baseline noise was calculated to be 0.16% of the total fluorescence.

Theoretical T-RF Size Estimation

For the calculation of theoretical sizes of the T-RFs, we collected 264 acidobacterial 16S rRNA gene sequences, which are described in papers published by Hugenholz *et al.* [16], Barns *et al.* [1], Sait *et al.* [37], and Lee *et al.* [28]. These sequences were cut at the position of 31F primer site and digested *in silico* by HhaI using BioEdit software [14]. The first 5' fragments from the digested sequence were grouped against each acidobacterial-subgroup and were regarded as theoretical T-RFs for the *Acidobacteria*.

RESULTS AND DISCUSSION

PCR Surveys with Subgroup-Specific Primers

To map the distribution of the phylum *Acidobacteria*, we initially performed PCR assays on the community DNAs extracted from the 33 global soil samples with *Acidobacteria*-specific primers (31F and 1518R) [1] and universal primers (pA and pH) [34] for the amplification of 16S rRNA genes. Sequences belonging to the phylum *Acidobacteria* were observed in 25 of 28 samples of which 16S rDNA sequences were successfully amplified with the universal primer pair (Table 1). Five samples (R-II–R-IV, BT, RC) from Russia, Saskatchewan, and Central Chile produced no PCR products with both universal and *Acidobacteria*-specific primer pairs, possibly due to the presence of PCR inhibitors in the soil samples. These soil samples repeatedly showed negative results when re-amplifications were conducted. No acidobacterial amplicons were observed for the three samples MR, ME, and BT from South Africa, Southwestern Australia, and California, respectively. For the remaining *Acidobacteria*-positive samples, the distribution of acidobacterial subgroups (primer groups A, Y, O, and G) was surveyed with group-specific primer pairs previously designed by Barns *et al.* [1] (Table 1). Members of acidobacterial primer group A were detected in all samples that harbored the phylum *Acidobacteria.* Primer group Y was observed in 21 samples (84%) collected from all regions except for Russia. Members of this primer group were found as the second most widely distributed group in this study. Members of primer group G were observed in 15 samples (60%) mainly from Korea, California, and Saskatchewan. Primer group O showed the most limited amplification results (11 samples) in our study, and this group was detected only in samples that contained primer group G.

These results suggest that members of primer group A of the phylum *Acidobacteria* might be distributed widely. Similarly, Barns *et al.* [1] reported that members of primer groups A and G were present in almost all soil samples (100% and 94%, respectively) they collected from regions in the United States, and concluded that members of both primer groups were ubiquitous and widely distributed in soil. However, our results indicated that members of primer group G could be restricted to regions in the northern hemisphere, which covers the sampling regions used in the study of Barns *et al.* [1]. For primer groups Y and O, we could not find a distinct geographic distribution pattern, although members of these primer groups seemed to be absent from acidic soils in this study, which is consistent with results of the previous study of Barns *et al.* [1].

Although we observed certain distribution patterns indicating that some primer groups might be widely distributed and others might be restricted to particular geographic regions or habitats, each primer group could include phylogenetically diverse members [1, 25]. Therefore, to elucidate the distribution pattern of the phylum *Acidobacteria* at a more defined taxonomic level (monophyletic groups), we divided the primer groups into acidobacterial subdivisions [16], which may have the class-level taxonomic rank, by comparing the 16S rRNA gene (rDNA) sequence libraries of previous studies [1, 16, 28, 37]. We found that primer group A included acidobacterial subdivisions 1, 2, and 3, and primer group G included subdivision 6. Primer groups O and Y consisted of members belonging to acidobacterial subdivision 4. However, we failed to find subdivision-specific primer sequences from the libraries. Therefore, a T-RFLP approach was used to analyze the distribution patterns of the phylum *Acidobacteria* at the subdivisional level.

Subdivisional Distribution Pattern Determined by T-RFLP Analysis

For the T-RFLP approach, we performed *in silico* analyses with 264 acidobacterial 16S rDNA sequences in the libraries that have been published [1, 16, 28, 37]. After excluding partial sequences (*ca.* <600 bp from 5' end [*E. coli* position, 31]) that were not long enough to generate theoretical T-RFs, 199 sequences were digested *in silico* with the tetrameric restriction enzyme HhaI. Then, the sizes of the first 5' fragments were calculated and classified into acidobacterial subdivisions (Table 2). *In silico* digestion resulted in 41 theoretical T-RFs, which could reflect a taxonomic group (phylotype) at an under-subdivisional level [30, 33]. The majority of acidobacterial subdivisions

Table 2. Predicted size of theoretical terminal-restriction fragments (T-RFs) generated by *in silico* digestion of acidobacterial 16S rDNA sequences with the tetrameric endonuclease HhaI.

Subdivision[a]	Predicted size (bp)
1	85, 86, 88, 271–274, 288–290, 292, 339, 354
2	56
3	29, 30
4	87–90, 455, 574, 576
5	86, 88
6	148, 232–234, 371, 373, 375, 376, 574, 1103
7	182, 216
8	201, 352
Unclassified subdivision	60, 86, 97, 186, 193, 199, 216, 536

[a]1–8, as suggested by Hugenholtz *et al.* [16], unclassified, sequences unable to be classified into one of the 8 subdivisions.

consisted of more than one T-RF with some T-RFs (predicted sizes of 86, 88, 216, and 574) overlapping. Eight theoretical T-RFs were generated from sequences that were unable to be classified into one of the eight acidobacterial subdivisions suggested by Hugenholtz *et al.* [16]. After constructing the database of the theoretical T-RFs (Table 2), T-RFLP analyses were performed with 10 randomly selected soil samples. A total of 58 T-RFs with size ranges from 32 bp to 571 bp were observed for the selected soil samples (data not shown), and subjected to the identification of corresponding acidobacterial subdivisions (Table 3). If the size of an observed T-RF did not match one of the predicted sizes listed in Table 2, considering experimental errors in the T-RF size determination with capillary electrophoresis system [20, 21, 23], an offset value (+1 or −1) was added to the observed T-RF size to

Table 3. Presence of members of acidobacterial subdivisions in selected soil samples determined by T-RFLP analysis.

Soil sample	Acidobacterial subdivisions detected[a]						UK[c]
	1	4	5	6	7	UC[b]	
WG	–	–	–	–	+/– (100)	–	–
PM	–	–	–	–	+/– (45.5)	–	+ (54.5)
JD	+ (41.2)	–	–	–	+/– (21.5)	–	+ (37.3)
KE	+ (39.0)	–	+/– (19.5)	–	–	+/– (19.5)	+ (22.0)
DT	+ (31.7)	+/– (11.8)	+ (11.8)	–	–	+ (11.8)	+ (33.0)
WS	+ (12.4)	+/– (11.3)	+ (11.3)	+ (5.7)	+ (0.5)	+ (12.8)	+ (46.0)
R-I	–	–	–	–	+/– (58.1)	–	+ (41.9)
PC	+ (29.6)	+/– (0.7)	+ (0.7)	+ (10.1)	+ (0.7)	+ (13.2)	+ (46.0)
WK	+ (51.9)	–	–	–	+/– (14.7)	–	+ (33.4)
FJ	+ (41.9)	–	+/– (20.9)	–	–	+/– (20.9)	+ (16.3)

[a]+, Predicted sizes (bp) of theoretical T-RFs listed in Table 2 were matched to one of the estimated sizes of the observed T-RFs; –, not matched; +/–, matched after an offset value (+1 or −1) was added to the observed T-RF size to search for the corresponding subdivision. Numbers in parenthesis indicate the relative contribution (%) of the matched T-RFs to the total peak area of all observed T-RFs.

[b]Unclassified subdivision of the phylum *Acidobacteria* as suggested by Hugenholtz *et al.* [16].

[c]Unknown T-RF. Presence (+) or absence (–) of the observed T-RFs that were unable to match to any of the theoretical T-RFs even when an offset value was added to the observed T-RF size.

search for corresponding subdivisions. Results from eight such T-RFs are marked as "+/–" in Table 3. Thirty-nine (67.2%) T-RFs did not match any subdivisions despite use of the offset value, and they were considered to be unmatched T-RFs.

Consistent with the PCR results obtained with subgroup (primer group)-specific primers, T-RFLP analysis showed that acidobacterial subdivision 1 belonging to primer group A was present in the majority of the soil samples. Peak areas of T-RFs corresponding to subdivision 1 contributed significantly to total T-RF peak areas (Table 3), indicating that members of acidobacterial subdivision 1 might be the dominant group of this phylum in these soil samples. However, subdivisions 2 and 3 belonging to primer group A were not detected in all samples, whereas subdivisions 4 (primer groups Y and O) and 6 (primer group G) were observed only in samples DT, WS, PC, and FJ. Acidobacterial subdivisions 5 and 7 as well as unclassified subdivisional groups (UC), which were not included in the primer groups, were detected in some of the samples. Although the T-RFLP analysis results differed slightly from those expected based on the PCR results, they confirmed our conclusion and those of Barns *et al.* [1] that members of primer group A could be widely distributed in various soils whereas others might have limited distribution. More specifically, T-RFLP analysis revealed that members of acidobacterial subdivision 1 could be globally distributed compared with other members of primer group A, indicating that bacteria in this subdivision might be well adapted to various soil environments. Members of subdivision 1 are considered to be aerobes [17] and have been frequently observed in high abundance in soil environments but also in other environments such as sewage sludge, marine hydrothermal sites, a hot spring microbial mat, and the human oral cavity [1, 7, 26, 27, 35, 39]. This subdivision includes the first isolate of the phylum *Acidobacteria*, *Acidobacterium capsulatum* [22], and most isolates cultured thus far [6, 15, 18, 19, 37, 38, 40]. In addition to subdivision 1 of primer group A, members of subdivision 7 (no primer group affiliation) also appeared to be widely distributed. However, most of the results for subdivision 7 were deduced from the offset-adjusted T-RFs (indicated as +/–) and the range of distribution of this subdivision was ambiguous. In contrast, acidobacterial subdivision 8 was absent in all soil samples used in this study. The only known members of subdivision 8 are *Holophaga foetida* and *Geothrix fermentens* – a homoacetogenic bacterium isolated from a black anoxic freshwater mud sample and an iron-reducing bacterium isolated from an aquifer sample, respectively [4, 29]. Considering that both isolates are obligate anaerobes and originated from anoxic sedimentary samples, members of this subdivision may possess a very different physiology than members of other subdivisions and might not be a normal constituent of the bacterial community in surface soil.

We attributed the partial discrepancy between the PCR and T-RFLP results and the many unmatched T-RFs in this study to the limited size of our theoretical T-RF database. Although derived from four published libraries, our T-RF database basically used the phylogenetic structure of the phylum *Acidobacteria* in the library of Hugenholtz *et al.* [16], in which eight subdivisions were originally suggested with 52 selected environmental representatives. The phylogenetic breadth of the phylum *Acidobacteria* has expanded as an increasing number of acidobacterial 16S rDNA sequences become available (>20,000 sequences in the RDP-II database). More recently, at least 26 subdivisions were suggested by Zimmermann *et al.* [41]. Considering that the phylum *Acidobacteria* may now include more subdivisions than originally suggested and that there could be more diverse sequences belonging to each of the subdivisions, our T-RF database likely missed many theoretical T-RFs that should have been identified properly as one of the previously suggested subdivisions or novel subdivisions as yet to be discovered.

Concluding Remarks

According to the literature published during 2002–2008 (111 papers in the Pubmed database), *Acidobacteria*-related sequences have been recovered mostly (85.5%) from soil samples collected from Europe, Asia, and North America. This biased distribution pattern of the phylum *Acidobacteria* in the literature might be attributed to the absence (or rarity) of research studying the phylum *Acidobacteria* and not to the absence (or rarity) of this phylum in other regions. Our study recovered acidobacterial 16S rDNA sequences from global soil samples collected from seven regions on five continents, suggesting that the phylum *Acidobacteria* is globally distributed. At the subdivisional level, our T-RFLP analysis revealed that members of acidobacterial subdivision 1 might be the most widely distributed group, whereas members belonging to other subdivisions might be restricted to certain geographic regions or habitats. We hypothesize that the limited distribution of some acidobacterial subdivisions might be due to their specialized physiological requirements rather than geographic separation (endemicity), as suggested by Cho and Tiedje [3]. Because bacterial endemicity was suggested to occur at the genotype level [3], it should not be observed at the subdivisional level even if members of the phylum *Acidobacteria* have a certain degree of endemicity. However, although we used soil samples collected from around the world, the geographic and habitat range of soil sampling in this study may not be adequate to explain the whole span of the distribution pattern of the phylum *Acidobacteria*. More comprehensive analyses of soil samples with a wide range of soil characteristics (*e.g.*, soil type, nutrients, vegetation) and geographic origin are required to fully understand the ecological (geographic as well as niche-specific) distribution patterns of the phylum

Acidobacteria. Quantitative analyses of acidobacterial rDNA and rRNA in soil samples collected from a range of environmental conditions is essential to gain an understanding of the correlation between the abundance and activity of the phylum *Acidobacteria* and their environmental factors.

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