J. Microbiol. Biotechnol. (2003), **13**(2), 236–242

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Analysis of Bacterial Community Structure in Bulk Soil, Rhizosphere Soil, and Root Samples of Hot Pepper Plants Using FAME and 16S rDNA Clone Libraries

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Received: September 5, 2002
Accepted: December 30, 2002

Abstract A culture-independent and -dependent survey of the bacterial community structure in the rhizosphere and soil samples from hot pepper plants was conducted using 16S rDNA clone library and FAME analyses. Out of the 78 clones sequenced, 56% belonged to *Proteobacteria*, 4% to high G+C Gram-positive group, 3% to *Cytophyga-Flexibacter-Bacteroides*, and 32% could not be grouped with any known taxonomic division. Among the 127 FAME isolates identified, 66% belonged to low G+C Gram-positive bacteria (*Bacillus* spp.) and 26% to high G+C Gram-positive bacteria. In a cluster analysis, the results for both methods were found to be strikingly dissimilar. The current study is the first comparative study of FAME and 16S rDNA clonal analyses performed on the same set of soil, rhizosphere soil, and root samples.

Key words: 16S rDNA, soil, rhizosphere, sequencing, FAME

For decades, soil microbiologists have tried to gain a better understanding of soil microbial ecology by identifying the microbial composition/diversity within a variety of environmental samples, yet there has always been a lack of coordinated effort between culture-dependent and independent methods.

FAME (Fatty Acid Methyl Ester) analysis is one culture-dependent method that allows for the rapid identification of environmental isolates. Membrane-bound methylated fatty acids from each isolate are extracted and analyzed by gas liquid chromatography. FAME analysis generates a unique microbial fingerprint or profile, which is queried against microbial libraries or databases for identification purposes. The method can also be subjected to quantitative analysis, but requires considerable biomass collection from environmental samples. The greatest inherent limitation of culture-dependent techniques such as FAME is to recover a representative number of microorganisms from a sample. It is assumed that less than 1% of the viable microbial community from terrestrial samples is culturable *in-vitro*, making quantitative analysis extremely difficult [2, 10, 15, 27, 31]. Although a variety of media have been developed to optimize culture conditions of various heterotrophic microorganisms from environmental samples, it is still estimated that only 0.001–4% of the total is typically recovered with any one type of media employed [10].

In order to obtain a more balanced estimate of the microbial diversity within a sample, the 16S rDNA PCR amplification, a representative culture-independent method, is typically employed [2]. Based on the 16S rDNA sequence diversity, the composition of microorganisms within a sample can be estimated using a variety of techniques including, but not limited to, cloning and sequencing, denaturant/temperature gradient gel electrophoresis, and terminal restriction length fragment polymorphisms (T-RFLP). There are a number of reports where D(T)GGE are used to compare bacterial communities in the soil-root systems [5, 22, 30]. They showed that the number of Gram-positive bacteria might be higher in the rhizosphere than previously realized [5, 18, 22]. For example, *Bacillus* species were found to dominate rhizosphere samples of chrysanthemum, grass, and barley plants [5, 7, 18]. While the bacterial communities among the soil samples could be compared on the basis of the phylogenetic analyses of 16S rDNA sequences, the possible functionality of the microbial population can only be inferred using this method. The major problem with this or any other PCR-based approach is the biases associated with DNA amplification of mixed samples [25]. Another problem of this approach to study microbial diversity and community structure is the rarity of sequences matching with previously characterized

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organisms [15] which can be used to further characterize the organisms of interests.

Accordingly, a parallel approach combining 16S rDNA clone library construction (a culture-independent approach) and FAME analysis (a culture-dependent approach) should greatly strengthen the field of microbial ecology by allowing for a more complete understanding of both the diversity and the potential functionality of the community. However, there are only a few studies for the comparisons between microbial compositions obtained by culture-dependent and culture-independent methods [4, 6, 19, 23].

The main objective of this study was to gain a better understanding of how representative the culturable fraction of the microbial community is to that obtained from a typical culture-independent technique. In so doing, we compared the parallel 16S rDNA clone library and FAME results obtained from different soil samples associated with hot pepper plants, using a cluster analysis. The second more minor objective was to describe the phylogenetic assignment of unknown rhizosphere bacteria.

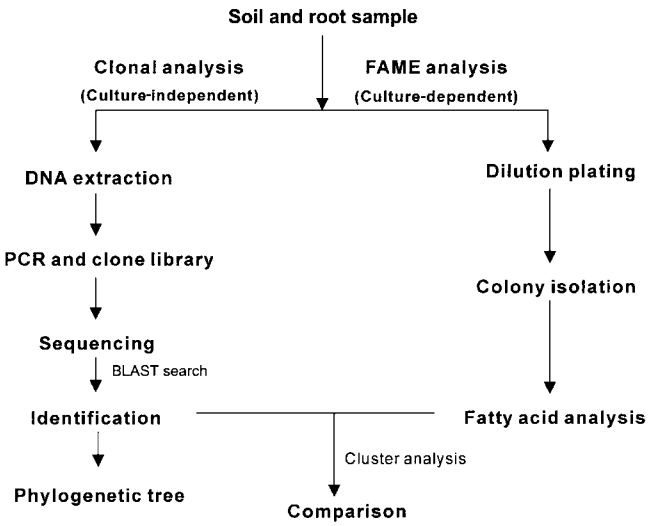

Fig. 1. A flow chart representing the simultaneous culture-independent and culture-dependent techniques used to process samples in the current study.

MATERIALS AND METHODS

Soil and Root Sampling

From hot pepper (*Capsicum annuum* L.) plants grown in greenhouse fields in Jinju, Korea, the root, rhizosphere, and bulk soil samples were collected in April, 1999. The loose bulk soil was removed by shaking the roots of 3 different plants. The rhizosphere soil and root samples were taken as follows: roots were carefully isolated from the soil by hand, using sterile tweezers. Roots were scraped and washed twice in 10 mM potassium phosphate buffer for 20 min to remove any microorganisms. The removed soil was combined and defined as rhizosphere soil. The soil chemical properties are presented in Table 1 [24]. One gram samples of washed root were combined and stored at 4°C prior to analysis.

Fatty Acid Analysis for Identification of Bacteria from Plant Root, Rhizosphere, and Bulk Soil Samples

One gram of the root material was placed into tubes (13× 100 mm) with Teflon-lined screw caps, and 3 ml of 10 mM potassium phosphate buffer and glass beads (1.0 mm dia, Biospec Product, Inc.) were added. Soil samples were resuspended in phosphate buffer minus glass beads. The tubes were vortexed for 3 min before serially diluting and evenly plating onto three separate 10% TSB (Tryptic Soy Broth) solid agar plates. Plates were incubated at 28°C for 5 days. Forty-five, 42, and 40 colonies were randomly chosen for the soil (ks), rhizosphere soil (krs), and root (kr) samples, respectively. To obtain pure cultures, each colony was transferred to new 10% TSBA plates three times.

FAME analysis was conducted using the recommended procedure described by the manufacturer (Microbial ID, Inc., Newark, DW, U.S.A.). The extracted samples were analyzed by a Sherlock Microbial Identification System with a Hewlett-Packard 6890A gas chromatograph (Palo Alto, CA, U.S.A.) [11, 17, 23].

DNA Extraction and 16S rDNA PCR Amplification

The total genomic DNA extractions from the bulk soil, rhizosphere soil, and root samples were conducted by soft lysis using freeze-thawing according to Tsai and Olsen [28]. The crude nucleic acid extracts were subjected to a further purification step using a Wizard Genomic DNA Purification (Promega Co., Madison, WI, U.S.A.). Fifty μl of the PCR reaction mixture contained the following final concentrations or total amounts: 1 μl of the template DNA, 50 mM Tris (pH 8.3), 250 μg of bovine serum albumin (BSA) per ml, 2.5 mM $MgCl_2$, 200 nM deoxynucleoside triphosphates, 200 nM of each universal SSU rRNA primer, and 3 U of *Taq* DNA polymerase. All reagents

Table 1. Chemical properties of hot pepper plant soils.

Crop/Soil samples	pH (1:5)	EC (dSm^{-1})	OM (gkg^{-1})	av. P_2O_5 ($mgkg^{-1}$)	Exchangeable cations ($cmol+kg^{-1}$)			
					K	Ca	Mg	Na
Hot pepper	5.3	7.76	33	1044	1.725	10.05	2.7	0.75

were mixed and heated at 95°C for 5 min. Thirty cycles were run at 95°C for 1 min, 50°C for 1 min, and 72°C for 1 min, followed by 10 min elongation at 72°C. The primers used for amplification of the 16S ribosomal genes were 968f, 5'-AACGCGAAGAACCTTAC-3' forward and 1492r, 5'-TACGGYTACCTTGTTACGACTT-3' reverse [14]. PCR products were then purified using a Wizard PCR Preps DNA Purification System (Promega).

Clone Library Construction, Sequencing, and Phylogenetic Analysis

The purified 16S rDNA PCR products were ligated into a pGEM-T Easy Vector System II (Promega) according to the manufacturer's instructions. White recombinant transformants were selected and screened for the correct insert size with the restriction enzyme *Eco*RI [21]. The plasmid DNA was prepared for sequencing using a Wizard Plus SV Minipreps DNA Purification System (Promega). PCR was used for the sequencing templates. Ten μl reaction volumes contained the following final concentrations or total amounts: 1 μl of DNA, 2 μl Terminator Ready Reaction Mix, and 200 nM each of T7 and SP6 primers. The reagents were combined and heated at 96°C for 1 min. A twenty-five cycle PCR was run at 96°C for 10 s, 50°C for 10 s, and 60°C for 4 min. Approximately 500 bases (position 968 to position 1,492, *Escherichia coli* position) of the 78 total clones were sequenced using an ABI 377 sequencer (Applied Biosystems, Inc., Foster City, CA, U.S.A.).

The clone 16S rDNA sequences and representative sequences were aligned using Clustal W [26]. Reference sequences were extracted from GenBank. The evolutionary distances were calculated by the method of Kimura 2-parameter and a phylogenetic tree was constructed by the neighbor-joining method [20] with MEGA2 for Windows, including a bootstrap analysis [13]. Taxonomic assignments were conducted by comparing the rDNA clone sequences to the GenBank nucleotide database using BLAST [1]. Those clones whose identities were corroborated by BLAST were given an assignment, while all others were classified as unknown organisms. A phylogenetic tree (Fig. 4) was constructed using the 78 sequences, including 25 unknown sequences showing less than 95% homology to any known rDNA sequence. The nucleotide sequence data reported in the current paper will appear in the GenBank/EMBL/DDBJ nucleotide sequence databases under accession number(s) AB075049-AB075126.

Cluster Analysis

Based on the frequency of each clone or isolate, community similarities were analyzed by cluster analysis (Minitab, State College, PA, U.S.A.). Similarities in the community structure quantified by the cluster analysis were determined based on the single linkage method using Euclidean distance measurements to determine the differences between clusters (Fig. 3).

Results and Discussion

Genomic DNA was isolated from bulk soil, rhizosphere soil, and root samples of hot pepper plants sown in a greenhouse soil. The 16S rDNA was amplified with eubacterial primers from the total genomic community DNA and cloned into plasmid vectors. A 500 bp fragment, the expected size of the cloned 16S rDNA insert, was amplified and sequenced from positive (white) colonies. In total, seventy eight 16S rDNA clones were obtained (Fig. 2).

The phylogenetic analysis of the 78 partial 16S rDNA clone sequences revealed the presence of two major and several minor groups which fell into several established lines of eubacteria (Fig. 2). Most clones belonged to the gamma *Proteobacteria* subdivision (41%, 32 clones), CFB (*Cytophaga/Flexibacter/Bacteroides* group) phyla (3%, 2 clones), the alpha *Proteobacteria* subdivision (6%, 5 clones), the beta *Proteobacteria* subdivision (9%, 7 clones), high G+C Gram-positive bacteria (4%, 3 clones), and Eukaryota group (5%, 4 clones). Twenty-five clones (32%) could not be assigned to any described taxa.

The distribution of the clones from the individual samples (plant and soil) was as follows: 50% of the clones obtained from the hot pepper rhizosphere soil and root samples (14 each) demonstrated homology to the gamma

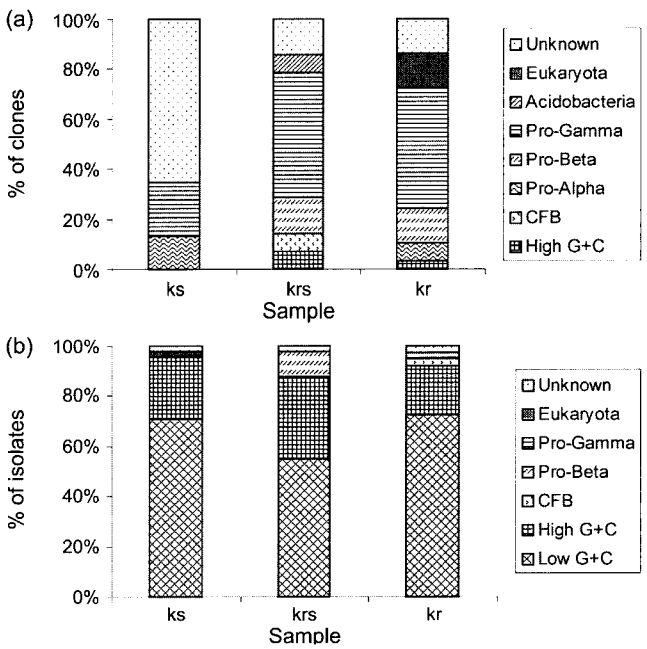

Fig. 2. A comparison of the bacterial division distribution among clones (a) and isolates (b) obtained from bulk soil (ks), rhizosphere soil (krs), and root (kr) in hot pepper samples.

Proteobacteria subdivision, while 65% (15 clones) of the clones obtained from the bulk soil samples fell into the unknown group.

One hundred and twenty seven isolates were identified by FAME analysis. Most isolates 66% (84 isolates) belonged to low G+C Gram-positive bacteria (*Bacillus* genus), 26% (33 isolates) to high G+C Gram-positive bacteria (*Actinobacter*), and 3% (4 isolates) to the beta *Proteobacteria* subdivision. Only three isolates (2%) fell into the unknown group by FAME analysis. The distribution of the isolates from individual samples (plant and soil) was as follows: 71, 44, and 72% of the isolates obtained from the bulk soil (32 isolates), rhizosphere soil (23 isolates) and root (29 isolates) samples, respectively, demonstrated homology to low G+C positive bacteria, while 24, 26, and 20% of the isolates obtained from the bulk soil (11 isolates), rhizosphere soil (14 isolates) and root (8 isolates) samples, respectively, demonstrated homology to high G+C positive bacteria (Fig. 2). FAME analysis did not identify any presence of the alpha *Proteobacteria* subdivision in either the bulk soil or root samples, whereas 4 isolates from the rhizosphere soil (krs) were grouped into the beta *Proteobacteria* subdivision. These results did not match those found by clonal analysis. None of the clones were identified as Gram-positive bacteria to which most of the cultured isolates belonged. Therefore, these results suggest that the culturable community represents only a small part of the community [23].

When FAME and clonal sequence data were compared by cluster analysis, both methods revealed different clustering. The clonal and FAME analyses grouped the root samples together (Fig. 3). Cluster analysis indicated 95% similarity between the rhizosphere soil and root cloned sequences. However, there was low similarity (60%) between the bulk soil and the other two samples. FAME analysis indicated a 100% similarity between the bulk soil and the root samples, and the isolates obtained from the rhizosphere demonstrated 95% similarity to the other two

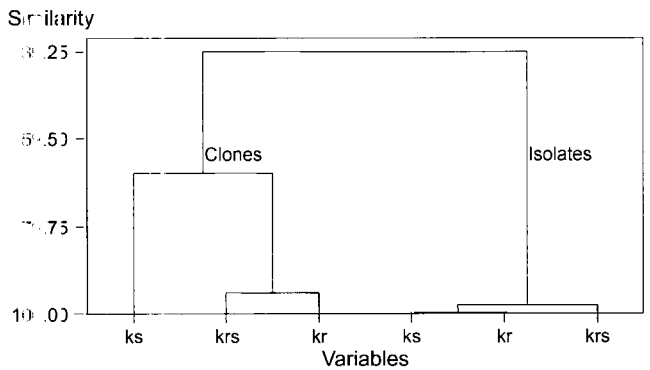

Fig. 3. Cluster analysis of clone sequences and FAME isolate identifications obtained from hot pepper soil (ks), rhizosphere soil (krs), and root (kr) samples.

samples. These FAME results are questionable in that the isolates derived from root samples should have greater similarity to those of the rhizosphere soil than to those of the bulk soil.

In the current study, strikingly different results between culture-independent (16S rDNA clone analysis) and culture-dependent (FAME) methods with the same sample are not uncommon. In particular, microbial community analysis results with FAME were totally distinct from those obtained with the culture-independent method. For example, clonal analysis of rhizosphere soil (krs) and root samples (kr) of hot pepper plants showed high numbers of the gamma *Proteobacteria* subdivision, whereas bacterial isolates identified by the FAME analysis showed a higher frequency of low G+C Gram-positive bacteria (*Bacillus* genus) for all samples. However, none of the clones sequenced revealed homology with *Bacillus*. The results are similar to those of Hugenholtz *et al.* [9] and Smit *et al.* [23], who found that *Proteobacteria* and *Actinobacteria* are richly represented in sequences from culture-independent environmental samples, yet not in culture-dependent samples. The genus *Bacillus* are fast-growing and heterotrophic bacteria that are readily culturable on TSBA media, yet when isolating soil DNA for clonal analysis, other researchers have commented on the resistance of Gram-positive bacteria to lysis [15, 17, 23], thereby possibly explaining why the results of the current FAME culturable analysis and the clonal analysis did not match.

Sixty percent of the clones sequenced from the bulk soil samples (Fig. 2) fell into the unknown group. The phylogenetic tree shown in Fig. 4 was used to ascribe the phylogenetic positions of these unknown clone sequences. The phylogenetic distribution of the clone clustering with reference sequences among the unclassified clone sequences is as follows: clone sequences KR28, KS28, KS18, and KS19 are most closely related to the alpha *Proteobacteria* subdivision, KR9 and KS9 to the beta *Proteobacteria* subdivision, KS5 and KS20 to the gamma *Proteobacteria* subdivision, and KS10 to the delta *Proteobacteria* subdivision. However, some of the unknown sequences (KS12, KS1, KS17, KS15, and KS16 from the bulk soil samples, KRS4, KRS13, KRS29, and KRS2 from the rhizosphere samples, and KR23 and KR29 from the root samples) were not related to any of the reference sequences. From the current results, it was inferred that many nonculturable bacteria could inhabit the rhizosphere as well as the soil [10, 27, 31].

This result proposes the need for a combined approach that can adequately analyze bacterial diversity in soil and the rhizosphere. In the current study, clonal analysis was more robust in revealing overall diversity, yet FAME analysis provided additional information that could not be ascertained from the sequence/clone data alone. Another advantage of FAME analysis is that soil isolates can be

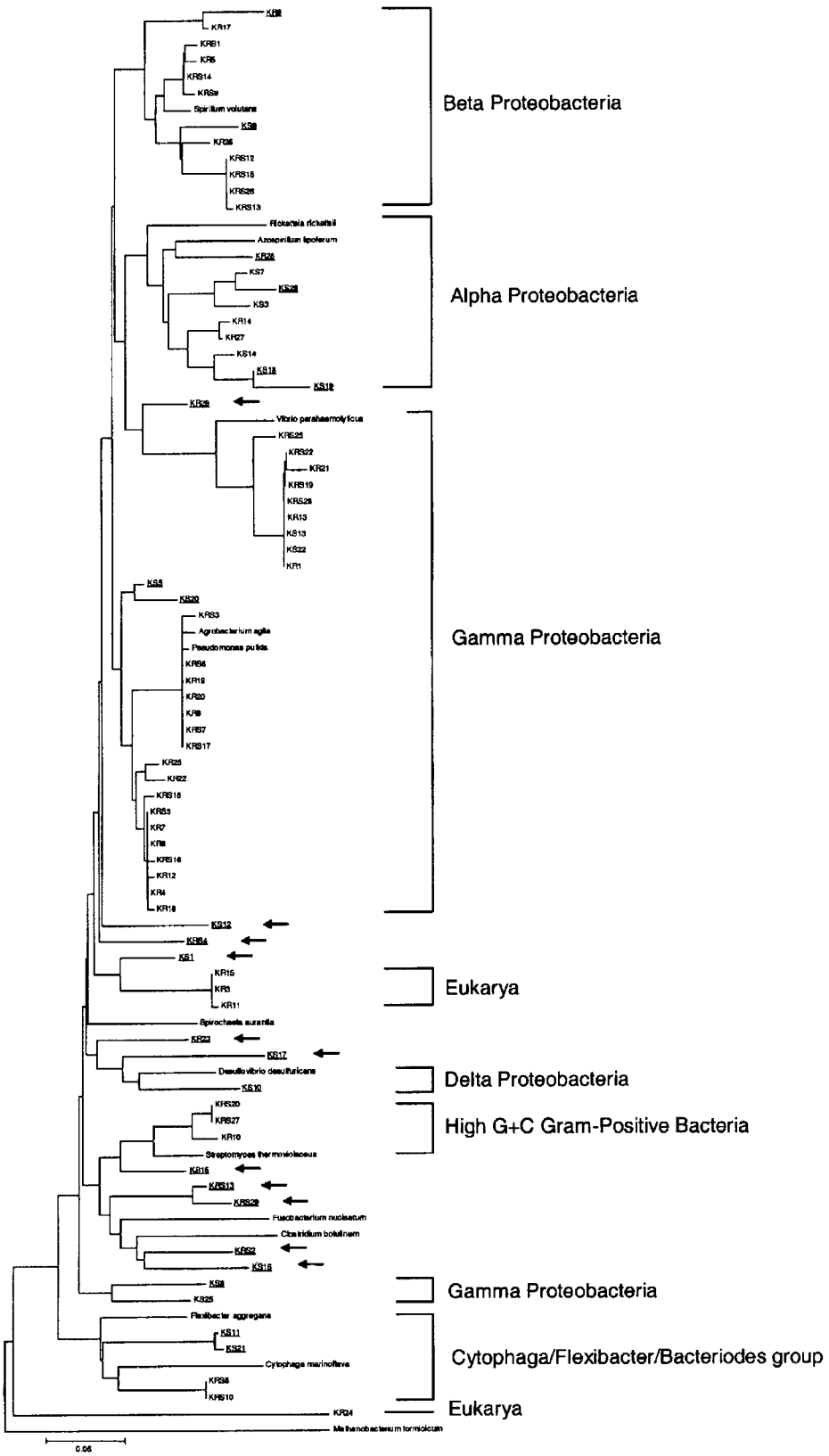

Fig. 4. Phylogenetic tree of bacterial sequences obtained from hot pepper root (kr), rhizosphere (krs), and soil (ks) samples.
Underlined clones have less than 95% homology with known GenBank sequences and arrows indicate sequences that did not group with any known bacterial division.

cultured, thereby allowing for their potential physicochemical function in a sample to be determined [32]. Furthermore, it is also possible to inoculate these organisms as a biocontrol or PGPB (plant growth promoting bacteria) [8, 12, 16]. Consequently, a combined approach of culture-dependent/independent methods provides a more complete bacterial classification of a sample and facilitates a better understanding of soil-root related microbial systems.

Acknowledgments

We thank B.-J. Park for performing the phylogenetic assignments of the clone sequences. J.-S. Kim was supported by a postdoctoral fellowship from RDA (Korea).

REFERENCES

1. Altschul, S. F., W. Gish, W. Iller, E. W. Myersand, and D. J. Lipman. 1990. Basic local alignment search tool. *J. Mol. Biol.* **215:** 403-410.
2. Amann, R. I., W. Ludwig, and K.-H. Schleifer. 1995. Phylogenetic identification and *in situ* detection of individual microbial cells without cultivation. *Microbiol. Rev.* **59:** 143-169
3. Borneman, J. and E. W. Triplett. 1997. Molecular microbial diversity in soils from eastern Amazonia: Evidence for unusual microorganisms and microbial population shifts associated with deforestation. *Appl. Environ. Microbiol.* **63:** 2647-2653.
4. de Lipthay, J. R., N. Tuxen, K. Johnsen, L. H. Hansen, E.-J. Albrechtsen, P. L. Bjerg, and J. Aamand. 2003. *In situ* exposure to low herbicide concentrations affects microbial population composition and catabolic gene frequency in an aerobic shallow aquifer. *Appl. Environ. Microbiol.* **69:** 461-467
5. Duineveld, B. M., G. A. Kowalchuk, A. Keijzer, J. D. van Elsas, and J. A. van Veen. 2001. Analysis of bacterial communities in the rhizosphere of chrysanthemum via denaturing gradient gel electrophoresis of PCR-amplified 16S rRNA as well as DNA fragments coding for 16S rRNA. *Appl. Environ. Microbiol.* **67:** 172-178.
6. Durbar, J., S. Takala, S. M. Barns, J. A. Davis, and C. R. Kuske. 1999. Levels of bacterial community diversity in four arid soils compared by cultivation and 16S rRNA gene cloning. *Appl. Environ. Microbiol.* **65:** 1662-1669.
7. Felske, A., A. Wolterink, R. van Lis, and A. D. L. Akkermans. 1998. Phylogeny of the main bacterial 16S rRNA sequences in Drentse A grassland soils (The Netherlands). *Appl. Environ. Microbiol.* **64:** 871-879.
8. Glick, B. R., C. L. Patten, G. Holguin, and D. M. Penrose. 1999. *Biochemical and Genetic Mechanisms Used by Plant Growth Promoting Bacteria.* Imperial College Press, London.
9. Hugenholtz, P., B. M. Goebel, and N. R. Pace. 1998. Impact of culture-independent studies on the emerging phylogenetic view of bacterial diversity. *J. Bacteriol.* **180:** 4765-4774.
10. Kieft, T. L. 2000. Size matters: dwarf cells in soil and subsurface terrestrial environments, pp. 19-46. *In* Colwell, R. R. and D. J. Grimes (eds.), *Nonculturable Microorganisms in the Environment*, ASM Press, Washington, DC, U.S.A.
11. Kim, J.-S., J.-B. Joo, H.-Y. Weon, C.-S. Kang, S.-K. Lee, and C.-S. Yahng. 2002. FAME analysis to monitor impact of organic matter on soil bacterial populations. *J. Microbiol. Biotechnol.* **12:** 382-388.
12. Kim, J.-S., M. Sakai, S.-K. Lee, C.-S. Yahng, and T. Matsuguchi. 2001. Comparison of the chemotaxis potential of bacteria isolated from spinach roots and nonrhizosphere soil. *J. Microbiol. Biotechnol.* **11:** 160-163.
13. Kumar, S., K. Tamura, I. B. Jakobsen, and M. Nei. 2001. MEGA2: Molecular Evolutionary Genetics Analysis software. Arizona State University, Tempe, Arizona, U.S.A.
14. Lane, D. J. 1991. 16S/23S rRNA sequencing, pp. 115-175. *In* Stackebrandt, E. and M. Goodfellow (eds.). *Nucleic Acid Techniques in Bacterial Systematics*. John Wiley and Sons, Inc., New York, U.S.A.
15. Liesack, W., H. J. Peter, F. A. Rainey, N. L. Ward-Rainey, and E. Stackebrandt. 1997. Microbial diversity in soil: The need for a combined approach using molecular and cultivation techniques, pp. 375-439. *In* van Elsas, J. D., J. T. Trevors, and E. M. H. Wellington (eds.). *Modern Soil Microbiology*, Marcel Dekker. Inc. New York, U.S.A.
16. Lim, H.-S., J.-M. Lee, and S.-D. Kim. 2002. A plant growth-promoting *Pseudomonas fluorescens* GL20 - mechanism for disease suppression, outer membrane receptors for ferric siderophore, and genetic improvement for increased biocontrol efficacy. *J. Microbiol. Biotechnol.* **12:** 240-249.
17. Mahaffee, W. F. and J. W. Kloepper. 1997. Temporal changes in the bacterial communities of soil, rhizosphere, and endorhiza associated with field-grown cucumber (*Cucumis sativus* L.). *Microb. Ecol.* **34:** 210-223.
18. Normander, B. and J. I. Prosser. 2000. Bacterial origin and community composition in the barley phytosphere as a function of habitat and presowing conditions. *Appl. Environ. Microbiol.* **66:** 4372-4377.
19. Orphan, V. J., L. T. Taylor, D. Hafenbradl, and E. F. Delong. 2000. Culture-dependent and culture-independent characterization of microbial assemblages associated with high-temperature petroleum reservoirs. *Appl. Environ. Microbiol.* **66:** 700-711.
20. Saitou, N. and M. Nei. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic tree. *Mol. Biol. Evol.* **4:** 406-425.
21. Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. *Molecular Cloning: A Laboratory Manual*. 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, U.S.A.
22. Smalla, K., G. Wieland, A. Buchner, A. Zock, J. Parzy, S. Kaiser, N. Roskot, H. Heuer, and G. Berg. 2001. Bulk and rhizosphere soil bacterial communities studied by denaturing gradient gel electrophoresis: Plant-dependent enrichment and seasonal shifts revealed. *Appl. Environ. Microbiol.* **67:** 4742-4751.
23. Smit, E., P. Leeflang, S. Gommans, J. van den Broek, S. van Mil, and K. Wernars. 2001. Diversity and seasonal fluctuations of the dominant members of the bacterial soil

community in a wheat field as determined by cultivation and molecular methods. *Appl. Environ. Microbiol.* **67:** 2284-2291.

24. Sparks, D. L. 1996. *Methods of Soil Analysis*, Part 3. SSSA and ASA, Madison, U.S.A.
25. Suzuki, M., M. S. Rappé, and S. J. Giovannoni. 1998. Kinetic bias in estimates of coastal picoplankton community structure obtained by measurements of small-subunit rRNA gene PCR amplicon length heterogeneity. *Appl. Environ. Microbiol.* **64:** 4522-4529.
26. Thompson, J. D., D. G. Higgins, and T. J. Gibson. 1994. CLUSTAL W: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positions-specific gap penalties and weight matrix choice. *Nucleic Acids Res.* **22:** 4673-4680.
27. Torsvik, V., R. Sorhieim, and J. Goksoyr. 1996. Total bacterial diversity in soil and sediment communities - a review. *J. Ind. Microbiol.* **17:** 170-178.
28. Tsai, Y.-L. and B. H. Olsen. 1991. Rapid method for direct extraction of DNA from soil and sediments. *Appl. Environ. Microbiol.* **57:** 1070-1074.
29. Valinsky, L., G. D. Vedova, A. J. Scupham, S. Alvey, A. Figureroa, B. Yin, R. J. Hartin, M. Chrobak, D. E. Crowley, T. Jiang, and J. Borneman. 2002. Analysis of bacterial community composition by oligonucleotide fingerprinting of rRNA genes. *Appl. Environ. Microbiol.* **68:** 3243-3250.
30. Wieland, G., R. Neumann, and H. Backhaus. 2001. Variation of microbial communities in soil, rhizosphere, and root in response to crop species, soil type, and crop development. *Appl. Environ. Microbiol.* **67:** 5849-5854.
31. Wilson, M. and S. E. Lindow. 2000. Viable but non-culturable cells in plants-associated bacterial populations, pp. 229-241. *In* Colwell, R. R. and D. J. Grimes (eds.) *Nonculturable Microorganisms in the Environment.* ASM Press, Washington, DC, U.S.A.
32. Zuberer, D. A. 1994. Recovery and enumeration of viable bacteria, pp. 119-144. *In* Weaver, R. W. (ed.), *Methods of Soil Analysis*, Part 2. SSSA and ASA, Madison, U.S.A.