J. Microbiol. Biotechnol. (2004), **14**(3), 599–606

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Molecular Differentiation of *Bacillus* spp. Antagonistic Against Phytopathogenic Fungi Causing Damping-off Disease

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Received: November 17, 2003
Accepted: April 7, 2004

Abstract Gram-positive antagonistic bacilli were isolated from agricultural soils for possible use in biocontrol of plant pathogenic fungi, *Fusarium oxysporum*, *Rhizoctonia solani*, and/or *Pythium ultimum*. Among the 65 antagonistic Gram-positive soil isolates, 22 strains were identified as *Bacillus* species by 16S rDNA sequence analyses. Four strains, including DF14, especially exhibited multiple antagonistic properties against the three damping-off fungi. Genotypic properties of the *Bacillus* isolates were characterized by rapid molecular fingerprinting methods using repetitive extragenic palindromic-PCR (REP-PCR), ribosomal intergenic spacer-length polymorphisms (RIS-LP), 16S rDNA PCR-restriction fragment length polymorphisms (PCR-RFLP), and strain-specific PCR assays. The results indicated that the REP-PCR method was more valuable than the RIS-LP and 16S rDNA PCR-RFLP analyses as a rapid and reliable approach for bacilli typing and identification. The use of strain-specific primers designed based on 16S rDNA sequence comparisons enabled it to be possible to selectively detect a strain, DF14, which is being used as a biocontrol agent against damping-off fungi.

Key words: Antagonistic bacteria, *Bacillus* identification, 16S rDNA, REP-PCR, RIS-LP

In recent years, public concern over the persistence and long-term toxicity of man-made organic pesticides extensively used in agriculture has increased, and this has necessitated the re-evaluation of synthetic chemicals as a final solution to plant protection [9]. Biological control of plant disease using antagonistic rhizobacteria offers a new and powerful alternative to the use of synthetic pesticides which cause negative effects on the environment and human health [34]. Thus, there have been many studies on soil microorganisms with antagonistic activity against a variety of plant pathogens and various mechanisms have been proposed to explain their biocontrol [13, 19, 27]. However, the effectiveness of biopesticides is often unpredictable and too variable in the field environment. This is mainly due to difficulty in obtaining a stable formulation and the lack of enough ecological studies of biocontrol agents.

Bacillus species are characterized by their capability to form highly resistant endospores and to produce various antimicrobial substances [16, 28]. Many of the *Bacillus* antibiotics have antifungal activity and play a major role in biological control of phytopathogenic fungi [16, 28]. *Bacillus* species produce numerous resistant spores, which can be formulated into stable biocontrol products. The spore-forming *Bacillus* species have received less attention than the fluorescent pseudomonads in past studies on biocontrol, because less is known about the genetics of the Gram-positive bacteria and the mechanisms of their antagonistic activity [12]. However, recently, some Gram-positive antifungal bacteria have been intensively studied as biocontrol agents to solve formulation and persistence problems [6, 21, 23].

One difficulty in the field study of *Bacillus* is tracking of the bacteria in natural environments. Conventional standard methods, such as colony morphology [29] and fluorescent antibody techniques [2], are very obscure and time-consuming. Recently, PCR amplification of REP sequences has been proposed as a rapid and universal bacterial identification and typing procedure among strains of *E. coli* [8], *Rhizobium meliloti* [7], *Bradyrhizobium japonicum* [15], *Streptomyces* spp. [26], and *Xanthomonas* spp. [3]. However, most Gram-positive species, including bacilli, showed minimal REP-PCR amplification, leading to unsatisfactory outcomes [22, 32]. A rapid identification method using molecular fingerprint techniques is required for characterization of Gram-positive biocontrol strains for registration, patenting, and quality checking during production and storage, and tracking their fate in the field [22].

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The purposes of this study were to isolate potential antagonistic *Bacillus* species which suppress the growth of damping-off fungi, *F. oxysporum*, *R. solani*, and/or *P. ultimum*, from agricultural soils in Korea, analyze their phylogenetic relatedness based on 16S rDNA sequences, and identify and type them at the strain level using REP-PCR, RIS-LP, and rDNA-RFLP analyses. Strain-specific primers designed by comparisons of 16S rDNA sequences were also used to selectively recognize and detect an isolate, DF14, which was used as a biocontrol agent against damping-off fungi.

MATERIALS AND METHODS

Isolation of Bacilli Antagonists

Agricultural soil samples were taken from upland fields in various locations in South Korea. The agricultural fields selected in this study have been under normal agricultural practices cultivating dry crops, such as legumes and barley, for more than 10 years. Samples from the top 15 cm of soil were taken, sifted through a 2-mm-pore-size sieve, and kept at 4°C prior to use. A 10-g soil sample from each site was homogenized with 95 ml of a sterile 0.85% saline solution by shaking at 200 rpm on a rotary shaker (Vision Co., Bucheon, Korea). The suspension was heated in a water-bath for 10 min at 80°C to select for spore-producing isolates and plated in serial dilutions on nutrient agar (Merck). The plates were incubated at 28°C for 7 days and then distinct single colonies were subcultured in nutrient broth for stocks and further characterization.

Bioassay for *In Vitro* Inhibition of Fungal Growth

The bacterial isolates were screened for *in vitro* antagonism against damping-off fungi, *F. oxysporum*, *R. solani*, and *P. ultimum*, using the plate test [24]. Fungi and the bacilli isolates were grown on potato dextrose agar (PDA) and nutrient broth, respectively. Then, the bacterial cells were harvested in a sterile saline to give a concentration of approximately 10^6-10^7 cells/ml. The fungal strain was inoculated by placing an agar plug from a PDA plate with mycelium onto the middle of the test plate, and a sterilized paper disc (6.35 mm diameter, Schleicher & Schuell Inc., Keene, U.S.A.) soaked into each cell suspension of the bacilli isolates was placed in the edges of the plate at four locations, approximately 3 cm from the center. The plates were incubated at 28°C and checked for zones of inhibition of mycelial growth after 3-5 days, when the fungal mycelium reached the border of the plate. The *in vitro* bioassay was tested on potato dextrose agar and each combination was replicated. The antagonistic interactions were analyzed by the determination of the size of the growth inhibition zone.

16S rDNA Sequencing

For initial identification of the antagonistic bacterial isolates, total genomic DNA was extracted from the isolates with a Wizard Genomic DNA Purification Kit (Promega, Madison, USA). PCR amplification of nearly full-length 16S rRNA genes was performed with 27f and 1492r (*E. coli* 16S rRNA gene sequence numbering) as previously described [18]. PCR products were purified from agarose gel slices by using a QIAquick Gel Extraction Kit (QIAGEN, Hilden, Germany). Sequencing was performed with an ABI Prism BigDye Terminator Cycle Sequencing Ready Kit (Applied Biosystems, Foster City, U.S.A.) according to the manufacturer's instructions with the sequencing primers 27f and 519r [14]. Approximately 400 unambiguous nucleotide positions were used for comparison to the data in GenBank using Basic Local Alignment Search Tool (BLAST) [1]. Then, for the selected bacilli isolates, complete 16S rDNA sequences were further determined. Sequences from nearest relatives were identified from the Ribosome Database Project (RDP) using the SIMILARITY-RANK program of the RDP [20]. An unrooted phylogenetic tree was constructed by using the neighbor-joining method and bootstrap analysis was performed, using PAUP 4.0 beta10, with 1000 replicate data sets [31].

Nucleotide Sequence Accession Numbers

The full 16S rDNA sequences of 22 bacilli isolates have been deposited in the GenBank under the following accession numbers: DF3, AY462196; DF7, AY462197; DF11, AY462198; DF12, AY462199; DF14, AY462200; DF15, AY462201; DF16, AY462202; DF18, AY462203; DF19, AY462202; DF20, AY462205; DF21, AY462206; DF27, AY462207; DF30, AY462208; DF36, AY462209; DF39, AY462210; DF41, AY462211; DF44, AY462212; DF48, AY462213; DF49, AY462214; DF56, AY462215; DF60, AY462216; and DF61, AY462217.

16S rDNA-RFLP Analysis

For rDNA-RFLP analysis, PCR amplification of nearly complete 16S rRNA genes was performed with 27f and 1492r as previously described [18]. PCR products were purified with Promega's Wizard PCR-Prep column (Promega, Madison, U.S.A.) and digested with the tetrameric restriction endonuclease *Cfo*I and *Msp*I (Boehringer Mannheim) as recommended by the manufacturer. The digested DNA fragments were separated by electrophoresis on horizontal 1.5% agarose gels. After the agarose gels were stained with ethidium bromide (0.5 μg/ml), images of the gels were analyzed by using an image analyzer BIO-PROFIL V.97 (Vilber Lourmat, France).

REP-PCR

REP-PCR mixtures were prepared as described previously using 100 ng of template DNA and primer BOX A1R

(5'-CTACGGCAAGGCGACGTCGACG-3') [25]. PCR amplifications were carried out with a model PTC 100 cycler (MJ Research, Waltham, U.S.A.) in a 25 μl reaction mixture. The PCR cycling programs consisted of an initial denaturation at 93°C for 7 min, followed by 34 cycles of 92°C for 1 min, 51.5°C for 1 min, and 65°C for 8 min, and a final extension at 65°C for 16 min. After PCR amplification, 10 μl samples of the REP-PCR products were separated by electrophoresis on horizontal 1.5% agarose gels at 43 V for 7 h. After the agarose gels were stained with ethidium bromide (0.5 μg/ml), images of the gels were analyzed by using an image analyzer BIO-PROFIL V.97 (Vilber Lourmat, France). Computer-assisted analysis of genomic fingerprints was performed by using the NTSYS program (Version 1.8; Exeter Software, New York, U.S.A.). Cluster analysis of similarity matrices was performed by the unweighted pair group method using arithmetic averages [30].

RIS-LP Analysis

The 16S-23S rDNA spacer regions were amplified with primers S1391f (5'-TTGTACACACCGCCCGTC-3') and L208r (5'-GGTACTTAGATGTTTCAGTTC-3') (*E. coli* numbering) [11]. The resultant PCR products contain the complete RIS and parts of the flanking rDNAs (ca. 152 bp of 16S rDNA and 210 bp of 23S rDNA). PCR amplification was performed in 25-μl reaction mixtures containing 10×PCR buffer (200 mM Tris/HCl, 500 mM KCl, pH 8.4), 100 ng of template DNA, 25 pmol of each primer, 200 μM of each dNTP (Boehringer Mannheim), and 2 U of *Taq* polymerase. PCR cycles consisted of an initial denaturation at 95°C for 2 min, followed by 33 cycles of 94°C for 30 sec, 47°C for 30 sec, and 72°C for 2 min, and a final extension at 72°C for 5 min. After PCR amplification, 10 μl samples of the RIS-PCR products were separated by electrophoresis on horizontal 1.5% agarose gels.

Strain-Specific 16S rDNA PCR Assay

Strain-specific primers were designed for the most prominent antagonist, DF14, which exhibited antifungal activity against all of the three phytopathogenic fungi. The primers used for strain-specific PCR were designed by comparing the entire 16S rDNA sequence of strain DF14 with those of the other bacilli isolates. The groups of rDNA sequences were aligned using ClustalW, and hypervariable regions were screened as PCR primer candidates. Based on the 16S rDNA sequence variations, primers df14f (5'-GATCATGGCTCAGGACGAA-3') and df14r (5'-CCCT-ATTTGAAGGGCACTT-3') were selected for strain DF14-specific PCR assay. PCR amplifications were carried out in a thermocycler (MJ Research, Waterton, U.S.A.)

Table 1. Origin, identification data, and antagonistic properties of the *Bacillus* isolates.

Isolate	Soil sites	Nearest relative[a]	Similarity (%)	Antibiosis[b] against		
				Fusarium oxysporum	*Rhizoctonia solani*	*Pythium ultimum*
DF3	Gimhae, Gyoungnam	*Bacillus subtilis*	99	++	+	-
DF7	Cheongwon, Chungbuk	*Bacillus subtilis*	99	+	++	-
DF11	Gimhae, Gyoungnam	*Bacillus subtilis*	99	+	+	-
DF12	Seosan, Chungnam	*Bacillus* sp.	100	+	+	+
DF14	Gimhae, Gyoungnam	*Bacillus* sp.	99	++	++	++
DF15	Gimhae, Gyoungnam	*Bacillus* sp.	99	-	+	-
DF16	Hadong, Gyoungnam	*Bacillus* sp.	99	+	-	-
DF18	Gimhae, Gyoungnam	*Bacillus* sp.	99	+	-	-
DF19	Gimhae, Gyoungnam	*Bacillus* sp.	99	+++	++	-
DF20	Cheonan, Chungnam	*Bacillus pumilus*	99	++	-	+
DF21	Gimhae, Gyoungnam	*Bacillus* sp.	99	+++	++	-
DF27	Sangju, Gyoungbuk	*Bacillus* sp.	99	+	-	-
DF30	Gimhae, Gyoungnam	*Bacillus* sp.	99	+	-	+
DF36	Buan, Jeonbuk	*Bacillus* sp.	99	++	-	+
DF39	Buan, Jeonbuk	*Bacillus pumilus*	99	++	-	+
DF41	Milyang, Gyoungbuk	*Bacillus subtilis*	99	+	+	-
DF44	Hoengseong, Gangwon	*Bacillus* sp.	99	+	-	+
DF48	Hwasun, Jeonnam	*Bacillus* sp.	99	-	+	-
DF49	Yeonggwang, Jeonnam	*Bacillus* sp.	99	++	+	++
DF56	Gwangju	*Bacillus* sp.	99	+	+	++
DF60	Jangseong, Jeonnam	*Bacillus subtilis*	99	+	+	-
DF61	Gangjin, Jeonnam	*Bacillus subtilis*	99	-	-	+

[a]The genus and/or species most similar to the strain is given based on similarities of 16S rDNA sequence.
[b]*In Vitro* Bioassay: -, no inhibition zone; +, <5 mm; ++, 5- 10 mm; +++, 10- 15 mm wide growth inhibition zone.

according to the following profile: initial denaturation at 94°C for 5 min; 30 cycles consisting of 94°C for 1 min, 64°C for 1 min, and 72°C for 1.5 min, and a final extension at 72°C for 10 min. After PCR amplification, 10 μl samples of the PCR products were separated by electrophoresis on horizontal 1.5% agarose gels.

RESULTS AND DISCUSSION

Isolation and Identification of *Bacilli* Antagonists

Using the heat treatment procedure for the selection of spore-forming bacteria, 712 colonies were subcultured on nutrient agar. In antifungal activity assays, 65 of these isolates were observed to produce any zone of inhibition against plant pathogenic fungi, *F. oxysporum*, *R. solani*, and/or *P. ultimum*. The analyses of the partial rDNA fragment sequences revealed that the 65 isolates were related to members of the genera *Bacillus*, *Paenibacillus*, and *Streptomyces* (M. J. Joe *et al.* 2002. *Abstr. Annu. Meet. Kor. Soc. Microbiol.*, Cheong-ju, Korea, B516). Of these isolates, 22 Gram-positive strains belonging to the genus *Bacillus* were selected and their complete rDNA sequences were further analyzed using the SIMILARITY-RANK program of the RDP (Table 1). Most of the bacilli isolates showed a high degree of similarities to the 16S rDNA sequences of *Bacillus* species in the GenBank database. The bacilli isolates consisted of 14 *Bacillus* species, 6 *Bacillus subtilis* strains, and 2 *Bacillus pumilus* strains. The antifungal properties of the 22 bacilli strains are also shown in Table 1. Strain DF14 exhibited the most prominent antagonistic activity against the three phytopathogenic fungi.

REP-PCR Analyses

A REP-PCR experiment was performed to type and differentiate each bacilli strain among the isolates. Figure 1 shows genomic DNA fingerprints for the bacilli isolates generated by using REP-PCR with the BOX A1R primer. Complex chromosomal DNA fingerprint patterns were obtained for all of the isolates studied and most of the isolates shared several common bands. Versalovic *et al.* [32] reported that most Gram-positive species, such as *Bacillus* and *Streptococcus* species, produced very limited DNA bands in REP-PCR, leading to limited applicability of this method for *Bacillus* species. Recently, Marten *et al.* [22] used the REP-PCR method to differentiate plant-associated *B. subtilis* isolates, but they obtained minimal DNA bands from only 50% of their isolates. In the present study, REP-PCR programs were optimized empirically. With the modified PCR programs, complex genomic DNA band patterns were obtained for all of the isolates, which made it possible to use the REP-PCR approach for the rapid and molecular differentiation of the Gram-positive *Bacillus* species. When each strain was subjected to REP-PCR in three replicates, all of them exhibited the same genomic DNA fingerprint patterns (data not shown), indicating that the REP-PCR method is highly reproducible, as reported previously in other studies [32, 33]. The REP-PCR analysis revealed that the 22 isolates produced 20 different DNA fingerprint patterns. Identical PCR DNA patterns were observed for strains DF20 and DF39, which were identified as *B. pumilus* in 16S rDNA sequence analysis. Strains DF19 and DF21, which were identified as *Bacillus* sp., also exhibited the same genomic DNA fingerprint patterns. Identical patterns indicate that the strains are very closely related or siblings. Since many of these strains were isolated from different soil locations, their detection frequencies reflect the extent of their dominance in the soils examined.

Comparison of Phylogenetic and Genetic Characteristics of the Bacilli Isolates

To determine the phylogenetic relationships among the 22 bacilli strains, a phylogenetic tree (unrooted) was constructed based on the complete 16S rDNA sequences (Fig. 2A). Six groups (groups A to F) were observed among the isolates.

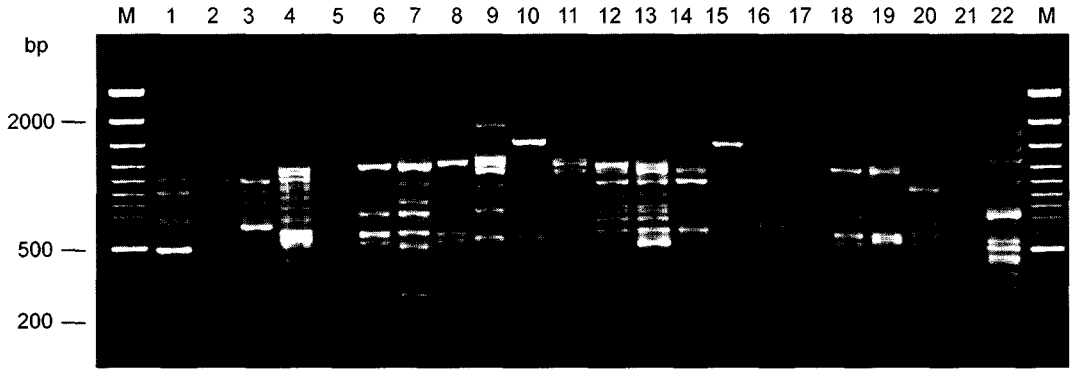
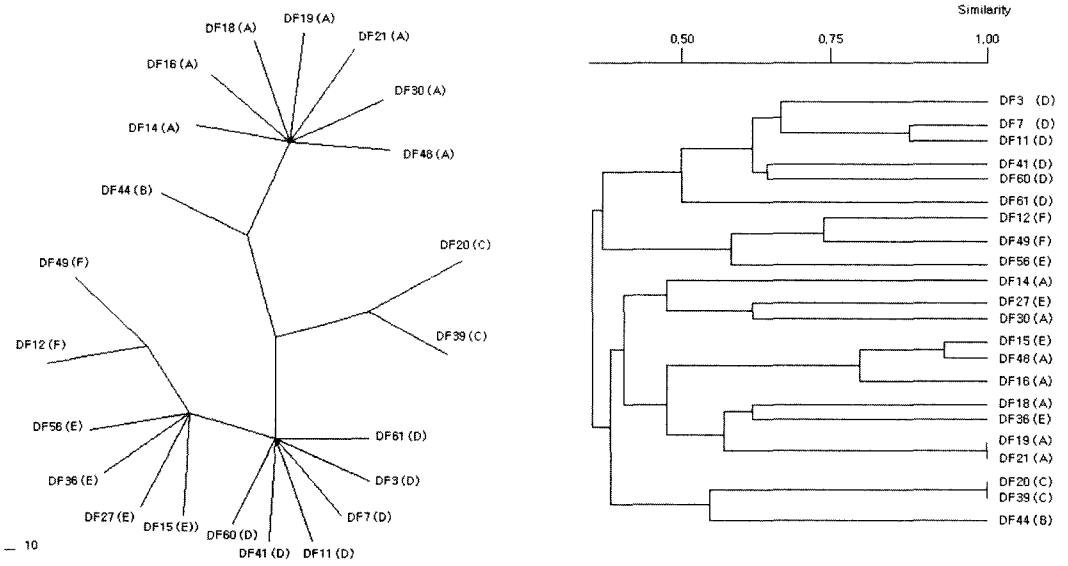


Fig. 1. REP-PCR DNA fingerprint patterns of the bacilli isolates.
Lanes: 1, DF3; 2, DF7; 3, DF11; 4, DF12; 5, DF14; 6, DF15; 7, DF16; 8, DF18; 9, DF19; 10, DF20; 11, DF21; 12, DF27; 13, DF30; 14, DF36; 15, DF39; 16, DF41; 17, DF44; 18, DF48; 19, DF49; 20, DF56; 21, DF60; 22, DF61; M, DNA size marker.



(A) 16S rDNA gene sequences (B) REP-PCR genomic fingerprints

Fig. 2. Phylogenetic and genetic relatedness of the bacilli isolates based on 16S rDNA sequences (A) and REP-PCR genomic fingerprints (B).

Group designations (Groups A to F) based on 16S rDNA sequences are shown in parentheses. Group branch points have bootstrap values greater than 82%. The scale bar represents a 10% estimated difference in nucleotide sequences.

Strains identified as *Bacillus* species were distributed into groups A, B, E, and F. Group C contained strains of *B. pumilus*, and Group D contained only strains of *B. subtilis*. Although some of the *Bacillus* species belonging to the same 16S rDNA group were distributed throughout the dendrogram generated by cluster analysis of REP-PCR genomic fingerprints (Fig. 2B), there were certain trends: all of the isolates belonging to *B. subtilis* (Group D) were in the same branch of the genomic DNA fingerprint dendrogram, and strains of Group C (*B. pumilus*) and strains of Group F (*Bacillus* species) were very closely related in the genomic DNA fingerprint patterns, respectively. The results indicate that the bacilli isolates of this study were genetically diverse, but their genetic properties were more or less related to their phylogenetic diversity.

rDNA-RFLP Analysis

Nearly full-length 16S rDNAs of the 22 isolates were PCR amplified with universal primers 27f and 1492r [18]. The amplified PCR products were digested with enzymes *Cfo*I and *Msp*I, and the resulting restriction fragments were separated on 1.5% agarose gels (Fig. 3). The *Cfo*I digestion yielded 3-4 DNA bands and revealed three DNA fingerprint patterns among the isolates (Fig. 3A). Strains DF44 and DF61 showed unique RFLP patterns, respectively. The other 20 isolates displayed the same DNA fingerprint pattern, forming a major separate group. The *Msp*I digestion also revealed three genotypes among the 22 isolates (Fig. 3B). Strains DF44 and DF61 exhibited different DNA fingerprint patterns, respectively, and the other 20 strains showed the same DNA fingerprint pattern, belonging to a separate group. RFLP analysis of PCR-amplified 16S rDNA has been useful in identifying and grouping closely related members of various genera, such as *Lactobacillus* and *Bradyrhizobium* species [5, 33]. In the present study, 20 strains out of the 22 isolates were grouped into a major group with the rDNA-RFLP analysis when digested with either *Cfo*I or *Msp*I. Moreover, the bacilli isolates belonging to different species, such as *B. subtilis*, *B. pumilus*, and *Bacillus* sp., exhibited the same DNA fingerprint patterns in rDNA-RFLP analysis. The results indicate that the rDNA-RFLP analysis has less discriminatory power than the REP-PCR method in identifying and differentiating each bacilli isolate at the strain level.

RIS-LP Analysis

The 16S-23S rDNA spacer regions of the isolates were amplified with primers S1391f and L208r [11] to investigate whether their spacer-length variations could be distinguished for typing each strain among the isolates. Electrophoretic separation of the total RIS amplification products revealed that the 22 isolates produced 1-4 major DNA bands and displayed four different RIS-LP patterns (Fig. 4). Eleven strains and seven strains of the isolates exhibited the same band patterns, respectively, forming

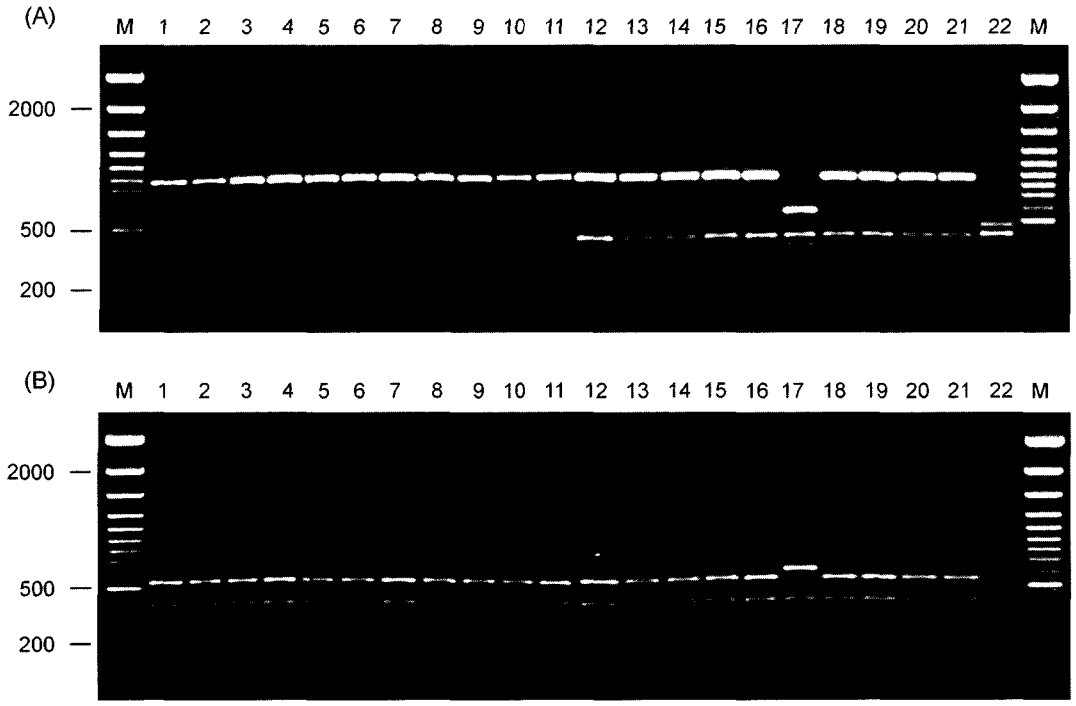


Fig. 3. Restriction patterns with *Cfo*I (A) and *Msp*I (B) of PCR-amplified product of 16S rDNA of the bacilli isolates.
Lanes: 1, DF3; 2, DF7; 3, DF11; 4, DF12; 5, DF14; 6, DF15; 7, DF16; 8, DF18; 9, DF19; 10, DF20; 11, DF21; 12, DF27; 13, DF30; 14, DF36; 15, DF39; 16, DF41; 17, DF44; 18, DF48; 19, DF49; 20, DF56; 21, DF60; 22, DF61; M, DNA size marker.

two major groups in RIS-LP analysis. Interestingly, the isolates (except DF61) identified as *B. subtilis* in 16S rDNA sequence analysis exhibited the same RIS-LP pattern, and so did the isolates of *B. pumilus*. On the other hand, the isolates identified as *Bacillus* sp. in 16S rDNA sequencing exhibited three different RIS-LP DNA patterns. It is of note that identical REP-PCR patterns corresponded to identical RIS-LP patterns, whereas many of the isolates belonging to the same RIS-LP pattern exhibited different REP-PCR patterns. Analysis of PCR-amplified fragments of the 16S-23S rRNA intergenic spacer regions has been useful in differentiating and typing closely related members of various genera, such as Gram-negative *Pseudomonas* species [17] and Gram-positive *Clostridium* species [4, 10]. The results of this study on the bacilli isolates indicate that RIS-LP analysis could not differentiate them properly at the strain level. Most of the bacilli isolates examined produced only three to four major bands, possibly leading to limited spacer-length variations between strains. The RIS-LP approach was apparently less

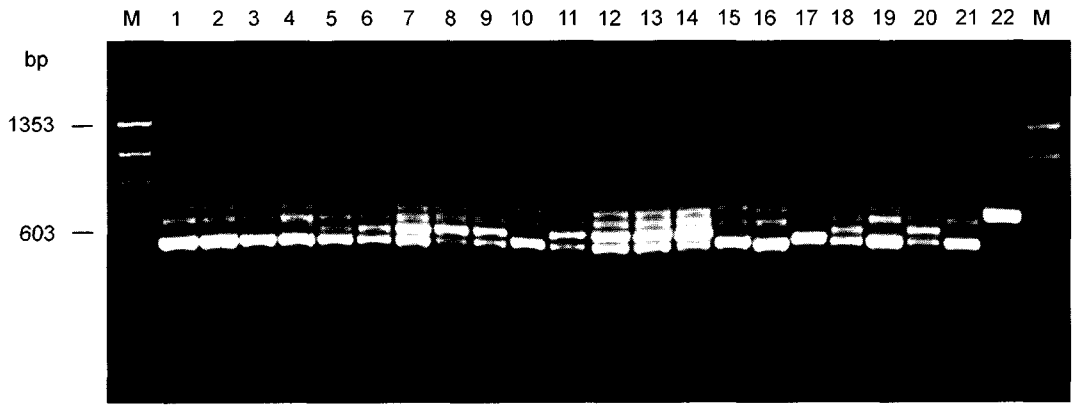


Fig. 4. RIS-LP banding patterns of the bacilli isolates.
Lanes: 1, DF3; 2, DF7; 3, DF11; 4, DF12; 5, DF14; 6, DF15; 7, DF16; 8, DF18; 9, DF19; 10, DF20; 11, DF21; 12, DF27; 13, DF30; 14, DF36; 15, DF39; 16, DF41; 17, DF44; 18, DF48; 19, DF49; 20, DF56; 21, DF60; 22, DF61; M, DNA size marker.

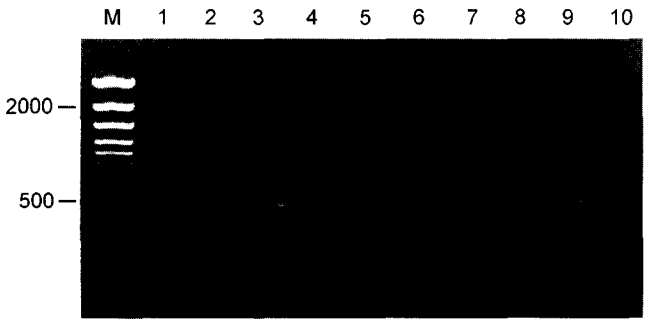


Fig. 5. Amplified DNA bands obtained from the representative isolates and soils with strain DF14-specific primers designed based on 16S rDNA sequences.
Lanes: 1, DF3; 2, DF11; 3, DF14; 4, DF20; 5, DF30; 6, DF36; 7, DF44; 8, DF60; 9, soil DNA inoculated with DF14 at 10^4 cells/g soil; 10, control soil DNA not inoculated with DF14; M, DNA size marker.

powerful than the REP-PCR method in differentiating and typing the bacilli isolates.

Strain-Specific rDNA-PCR Assays

The 16S rDNA sequences used to identify the bacilli isolates were used to design strain-specific PCR primers for differentiation and detection of a specific strain among the isolates. Strain DF14 was selected as a target strain, because this strain exhibited potent antagonistic activity against all of the three phytopathogenic fungi (Table 1). This strain is being applied to top soil of a vegetable garden as a biological control agent in a mesocosm experiment, which makes it necessary to develop a rapid and reliable method of detecting this strain in soil. Strain DF14-specific primers, df14f and df14r, were designed based on sequence comparisons to maximize specificity for the strain. The result of strain-specific rDNA PCR performed with bacterial DNA and total soil DNA is shown in Figure 5. The expected DNA bands of 464 bp in size were observed only from the target strain, DF14, and the total soil DNA inoculated with this strain. The result indicated that the PCR assay with the primer pair df14f/df14r was very specific for strain DF14, and thus it could be used to track and detect the target strain in environmental samples.

In conclusion, the results indicate that the REP-PCR genomic DNA fingerprinting method is more applicable to Gram-positive *Bacillus* species than rDNA-RFLP and RIS-LP assays for typing and differentiating them at the strain level. The rapidity and the relative ease of the REP-PCR assay allow this technique to be employed in the routine environmental microbiology laboratory. The REP-PCR assay, when combined with the strain-specific rDNA PCR method, could be used for tracking and detecting a specific target *Bacillus* species during production and storage of biocontrol products, and in inoculated soils with and without cultivation.

Acknowledgment

This study was supported by Technology Development Program for Agriculture and Forestry, Ministry of Agriculture and Forestry, Republic of Korea.

REFERENCES

1. Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman. 1990. Basic local alignment tool. *J. Mol. Biol.* **215:** 403-410.
2. Bohlool, B. B. and E. L. Schmidt. 1980. The immunofluorescence approach in microbial ecology. *Adv. Microb. Ecol.* **4:** 203-242.
3. Bouzar, H., J. B. Jones, R. E. Stall, F. J. Louws, M. Schneider, J. L. W. Rademaker, F. J. de Bruijn, and L. E. Jackson. 1999. Multiphasic analysis of xanthomonads causing bacterial spot disease on tomato and pepper in the Caribbean and Central America: Evidence for common lineages within and between countries. *Phytopathology* **89:** 328-335.
4. Cartwright, C. P., F. Stock, S. E. Beekmann, E. C. Williams, and V. J. Gill. 1995. PCR amplification of rRNA intergenic spacer regions as a method for epidemiologic typing of *Clostridium difficile*. *J. Clin. Microbiol.* **33:** 184-187.
5. Chang, C. E., S. I. Pavlova, L. Tao, E. K. Kim, S. C. Kim, H. S. Yun, and J. S. So. 2002. Molecular identification of vaginal *Lactobacillus* spp. isolated from Korean women. *J. Microbiol. Biotechnol.* **12(2):** 312-317.
6. Crawford, D. L., J. M. Lynch, J. M. Whipps, and M. A. Ousley. 1993. Isolation and characterization of actinomycete antagonists of a fungal root pathogen. *Appl. Environ. Microbiol.* **59:** 3899-3905.
7. de Bruijn, F. J. 1992. Use of repetitive (repetitive extragenic palindromic and enterobacterial repetitive intergeneric consensus) sequences and the polymerase chain reaction to fingerprint the genomes of *Rhizobium meliloti* isolates and other soil bacteria. *Appl. Environ. Microbiol.* **58:** 2180-2187.
8. Dombek, P., L. K. Johnson, S. T. Zimmerley, and M. J. Sadowsky. 2000. Use of repetitive DNA sequences and the PCR to differentiate *Escherichia coli* isolates from human and animal sources. *Appl. Environ. Microbiol.* **66:** 2572-2577.
9. Emmert, E. A. B. and J. Handelsman. 1999. Biocontrol of plant disease: A (Gram-) positive perspective. *FEMS Microbiol. Lett.* **171:** 1-9.
10. Gurtler, V. 1993. Typing of *Clostridium difficile* strains by PCR-amplification of variable length 16S-23S rDNA spacer regions. *J. Gen. Microbiol.* **139:** 3089-3097.
11. Gurtler, V. and V. A. Stanisich. 1996. New approaches to typing and identification of bacteria using the 16S-23S rDNA spacer region. *Microbiology* **142:** 3-16.
12. Handelsman, J. and E. V. Stabb. 1996. Biocontrol of soil-borne plant pathogens. *Plant Cell* **8:** 1855-1869.
13. Howell, C. R. and R. D. Stipanovic. 1979. Control of *Rhizoctonia solani* on cotton seedlings with *Pseudomonas*

fluorescens and with an antibiotic produced by the bacterium. *Phytopathology* **69:** 480-482.
14. Hugenholtz, P., C. Pitulle, K. L. Hershberger, and N. R. Pace. 1998. Novel division level bacterial diversity in a Yellowstone hot spring. *J. Bacteriol.* **180:** 366-376.
15. Judd, A. K., M. Schneider, M. J. Sadowsky, and F. J. de Bruijn. 1993. Use of repetitive sequences and the polymerase chain reaction technique to classify genetically related *Bradyrhizobium japonicum* serocluster 123 strains. *Appl. Environ. Microbiol.* **59:** 1702-1708.
16. Katz, E. and A. L. Demain. 1977. The peptide antibiotics of *Bacillus*: Chemistry, biogenesis, and possible role. *Bacteriol. Rev.* **41:** 449-474.
17. Kostman, J. R., T. D. Edlind, J. J. LiPuma, and T. L. Stull. 1992. Molecular epidemiology of *Pseudomonas cepacia* determined by PCR ribotyping. *J. Clin. Microbiol.* **30:** 2084-2087.
18. Lane, D. J. 1991. 16S/23S rRNA sequencing, pp. 115-148. *In* E. Stackebrandt and M. Goodfellow (eds.), *Nucleic Acid Techniques in Bacterial Systematics*. John Wiley and Sons, Chichester, England.
19. Lim, H. S., J. M. Lee, and S. D. Kim. 2002. 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(2):** 249-257.
20. Maidak, B. L., J. R. Cole, T. G. Lilburn, C. T. Parker Jr., P. R. Saxman, J. M. Stredwick, G. M. Garrity, B. Li, G. J. Olsen, S. Pramanik, T. M. Schmidt, and J. M. Tiedje. 2000. The RDP (Ribosomal Database Project) continues. *Nucleic Acids Res.* **28(1):** 173-174.
21. Mari, M., M. Guizzardi, and G. C. Pratella. 1996. Biological control of gray mold in pears by antagonistic bacteria. *Biol. Control* **7:** 30-37.
22. Marten, P., K. Smalla, and G. Berg. 2000. Genotypic and phenotypic differentiation of an antifungal biocontrol strain belonging to *Bacillus subtilis*. *J. Appl. Microbiol.* **89:** 463-471.
23. Michereff, S. J., N. S. S. Silveira, A. Reis, and R. L. R. Mariano. 1994. Epiphytic bacteria antagonistic to *Curvularia* leaf spot of yam. *Microb. Ecol.* **28:** 101-110.
24. Nielsen, M. N., J. Sorensen, J. Fels, and H. C. Pedersen. 1998. Secondary metabolite- and endochitinase-dependent antagonism toward plant-pathogenic microfungi of *Pseudomonas fluorescens* isolates from sugar beet rhizosphere. *Appl. Environ. Microbiol.* **64:** 3563-3569.
25. Oda, Y., W. Wanders, L. A. Huisman, W. G. Meijer, J. C. Gottschal, and L. J. Forney. 2002. Genotypic and phenotypic diversity within species of purple nonsulfur bacteria isolated from aquatic sediments. *Appl. Environ. Microbiol.* **68:** 3467-3477.
26. Sadowsky, M. J., L. L. Kinkel, J. H. Bowers, and J. L. Schottel. 1996. Use of repetitive intergenic DNA sequences to classify pathogenic and disease-suppressive *Streptomyces* strains. *Appl. Environ. Microbiol.* **62:** 3489-3493.
27. Shanahan, P., D. J. O'Sullivan, P. Simpson, J. D. Glennon, and F. O'Gara. 1992. Isolation of 2,4-diacetylphloroglucinol from a fluorescent pseudomonad and investigation of physiological parameters influencing its production. *Appl. Environ. Microbiol.* **58:** 353-358.
28. Shoji, J. 1978. Recent chemical studies on peptide antibiotics from the genus *Bacillus*. *Adv. Appl. Microbiol.* **24:** 187-214.
29. Smibert, R. M. and N. R. Krieg. 1981. General characterization, pp. 409-443. *In* P. Gerhardt, R. G. E. Murray, R. N. Costilow, E. W. Nester, W. A. Wood, N. R. Krieg, and G. B. Phillips (eds.), *Manual of Methods for General Bacteriology*. American Society for Microbiology, Washington DC, U.S.A.
30. Sneath, P. H. A. and R. R. Sokal. 1973. *Numerical Taxonomy*. Freeman, San Francisco, California, U.S.A.
31. Swofford, D. L. 2002. PAUP: phylogenetic analysis using parsimony, version 4.0. Computer program distributed by Sinauer Associates, Sunderland, Massachusetts, U.S.A.
32. Versalovic, J., M. Schneider, F. J. de Bruijn, and J. R. Lupski. 1994. Genomic fingerprinting of bacteria using repetitive sequence-based polymerase chain reaction. *Methods Mol. Cell Biol.* **5:** 25-40.
33. Vinuesa, P., J. L. W. Rademaker, F. J. de Bruijn, and D. Werner. 1998. Genotypic characterization of *Bradyrhizobium* strains nodulating endemic woody legumes of the Canary islands by PCR-restriction fragment length polymorphism analysis of genes encoding 16S rRNA (16S rDNA) and 16S-23S rDNA intergenic spacers, repetitive extragenic palindromic PCR genomic fingerprinting, and partial 16S rDNA sequencing. *Appl. Environ. Microbiol.* **64:** 2096-2104.
34. Weller, D. M. 1988. Biological control of soilborne plant pathogens in the rhizosphere with bacteria. *Annu. Rev. Phytopathol.* **26:** 379-407.