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Restriction Fragment Length Polymorphism Analysis of PCR-Amplified 16S Ribosomal DNA for Rapid Identification of *Saccharomonospora* Strains

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Twenty-one strains of *Saccharomonospora azurea*, *Saccharomonospora cyanea*, *Saccharomonospora glauca*, *Saccharomonospora viridis*, and *"Saccharomonospora caesia"* were examined to evaluate the discriminatory value of 16S ribosomal DNA (rDNA) fingerprints. The 16S rDNAs were amplified by PCR by using oligonucleotide primers complementary to 16S rRNA genes. A restriction fragment length polymorphism (RFLP) analysis of the 16S rDNAs was performed with *Sma*I and *Mlu*I. The four validly described *Saccharomonospora* species could be differentiated on the basis of their characteristic 16S rDNA restriction patterns. The strains of *"S. caesia"* gave a restriction pattern identical to that of *S. azurea* K161^T (T = type strain). This result was anticipated from the previous report that *S. azurea* K161^T and the strains of *"S. caesia"* have identical 16S rRNA sequences. We found that purification of amplified 16S rDNA products following PCR was necessary for our RFLP analysis.

The genus *Saccharomonospora* was first proposed by Nonomura and Ohara (13) for monosporic actinomycetes containing *meso*-diaminopimelic acid, arabinose, and galactose in the peptidoglycan (wall chemotype IV sensu Lechevalier and Lechevalier (12)). This genus is a genus of gram-positive, aerobic, non-acid-fast actinomycetes with non-motile spores that typically form nonfragmenting, branched substrate mycelia (13). 16S rRNA sequence data have shown that the genus is a distinct homogeneous group within the evolutionary radiation encompassed by the family *Pseudonocardiaceae* (9).

Up to this point, the genus *Saccharomonospora* has been described as a genus with four valid species, namely, *Saccharomonospora azurea* (14), *Saccharomonospora cyanea* (15), *Saccharomonospora glauca* (4), and *Saccharomonospora viridis* (13). "*Saccharomonospora caesia*" was proposed as a fifth species by Greiner-Mai et al. (5) for strains previously classified as *Micropolyspora caesia* (7, 10). However, this species was not included on the Approved Lists of Bacterial Names (17) and has not been validly published on subsequent Approved Lists. It has been found recently that strains of "*S. caesia*" are very closely related to *S. azurea* K161^T (T = type strain) as determined by numerical phenetic studies (8) and data based on nucleic acid techniques (9, 21), and more investigations are needed to determine the detailed relationship between the two species. It has been determined that strains of *S. viridis* cause farmer's lung disease (1, 3) and are significant agents of hypersensitivity pneumonitis (11). Therefore, it is important to be able to distinguish rapidly among the existing taxa and between validly published and putatively novel *Saccharomonospora* species.

rRNAs, especially the 16S or 18S rRNA genes, have been widely studied both in prokaryotes and in eukaryotes (19, 20). Because the 16S rRNA gene contains highly conserved regions found in all prokaryotic organisms and diagnostic variable sequence regions that are unique to particular organisms, it has been used to determine phylogenetic relationships between bacterial species (19, 20) and has also been used in the classification and identification of *Saccharomonospora* species (9). However, since sequence analysis of 16S rRNA is somewhat expensive and time-consuming, there are difficulties in routine application of this technique to rapid identification of large numbers of strains.

In this study, PCR-amplified 16S ribosomal DNAs (rDNAs) of 21 strains of the genus *Saccharomonospora* were analyzed on the basis of their restriction fragment length polymorphisms (RFLPs). The usefulness of PCR-RFLP analysis for rapid identification of members of the genus *Saccharomonospora* was examined. The existence of differences between digests of purified and unpurified PCR products was also investigated.

MATERIALS AND METHODS

Bacterial strains. Table 1 summarizes the bacterial strains used in this study. The sources of the bacteria have been described previously (9, 21). All of the test strains were grown in shake flasks containing tryptone soy broth supplemented with glucose (0.75%, wt/vol) at 45°C for 48 h.

Isolation of DNA. The chromosomal DNAs were isolated by the method described previously (21).

PCR amplification of 16S rDNA. The oligonucleotide primers used for amplification of 16S rDNA were synthesized by KOREA BIOTECH., Inc., Taejeon, Republic of Korea. Primers annealing at the 5′ and 3′ ends of the 16S rRNA genes were 5′-GAGTT TGATC CTGGC TCAG-3′ (positions 9 to 27 [*Escherichia coli* 16S rRNA numbering]) and 5′-AGAAA GGAGG TGATC CAGCC -3′ (positions 1542 to 1525 [*E. coli* 16S rRNA numbering]), respectively. PCR amplification was performed in a final reaction volume of 100 μl, and the reaction mixture contained each primer at a concentration of 0.5 μM, each deoxynucleoside triphosphate at a concentration of 200 μM, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 0.01% (wt/vol) gelatin, and 2.5 U of *Taq* DNA polymerase (Perkin-Elmer Co., Norwalk, Conn.). Each reaction mixture was overlaid with mineral oil, and the PCR was run for 35 cycles with a DNA thermal cycler (model 480; Perkin-Elmer Co.). The following thermal profile was used for the PCR: denaturation at 94°C for 1 min, primer annealing at 60°C for 1 min, and extension at 72°C for 2 min. The final cycle included extension for 10 min at 72°C to ensure full extension of the products. On completion of the reaction, each reaction tube was frozen at −70°C in a deep freezer for 10 min, and then the

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TABLE 1. Strains used in this study

Laboratory no.	Species	Source and/or other designation(s)[a]
K161^T	*S. azurea*	H. Runmao, NA-128^T (= SIIA 86128^T)
K76^T	*"S. caesia"*	KCTC 9152^T
K163	*"S. caesia"*	DSM 43068
K182	*"S. caesia"*	E. Greiner-Mai, Ko18
K200	*"S. caesia"*	J. Lacey, A1932
SB-01, SB-22, SB-58	*"S. caesia"*	S.-B. Kim
K168^T	*S. cyanea*	H. Runmao, NA-134^T (= SIIA 86134^T)
K169^T	*S. glauca*	DSM 43769^T
K179	*S. glauca*	A. J. McCarthy, BD-125
K194	*S. glauca*	J. Lacey, A66
K195	*S. glauca*	J. Lacey, A1450
K202	*S. glauca*	J. Ruan, 350
SB-37	*S. glauca*	S.-B. Kim
K73^T	*S. viridis*	NCIB 9602^T
K185	*S. viridis*	E. Greiner-Mai, L1v
K191	*S. viridis*	E. Greiner-Mai, R25
K197	*S. viridis*	J. Lacey, A1905
SB-31, SB-33	*S. viridis*	S.-B. Kim

[a] Abbreviations: SIIA, Sichuan Industrial Institute of Antibiotics, Chengdu, Sichuan, People's Republic of China; KCTC, Korean Collection for Type Cultures, Taejeon, Republic of Korea; DSM, Deutsche Sammlung von Mikroorganismen, Braunschweig, Germany; NCIB, National Collection of Industrial and Marine Bacteria, Ltd., Aberdeen, United Kingdom.

mineral oil layer was removed with a pipette tip. The PCR products were analyzed by electrophoresis of a 5-μl aliquot through a 1.0% (wt/vol) agarose gel, stained with ethidium bromide, and visualized by UV transillumination.

Purification of 16S rDNA PCR products. Purification with a GENECLEAN II kit (Bio 101, La Jolla, Calif.) and a Sephaglas BandPrep kit (Pharmacia Biotech., Uppsala, Sweden) was carried out by following the manufacturers' instructions. The PCR products were precipitated with 9 μl of 3 M sodium acetate (pH 5.2) and 60 μl of isopropanol and resuspended in 50 μl of distilled water. Extraction of the PCR products with chloroform was carried out as described previously (16).

Enzymatic digestion of amplified DNA and electrophoresis. Purified DNA (400 ng) and 5 μl of chloroform-extracted and unpurified DNA were digested for 2 h in 10-μl volumes with restriction endonucleases according to the manufacturer's instructions. The following two restriction enzymes were used: *Sma*I and *Mlu*I (New England Biolabs, Inc., Beverly, Mass.). Double digestion with *Sma*I and *Mlu*I was performed as follows. DNA was first digested for 1 h at 25°C with *Sma*I. Following addition of the buffer recommended by the manufacturer, *Mlu*I was added and the reaction mixture was incubated for 1 h at 37°C. The resulting DNA fragments were electrophoresed in 1.5% (wt/vol) agarose gels in Tris-acetate buffer (0.04 M Tris-acetate, 0.001 M EDTA; pH 8.3) at 100 V for 30 min. The gels were stained with ethidium bromide, and DNA fragments were visualized by UV transillumination and photographed.

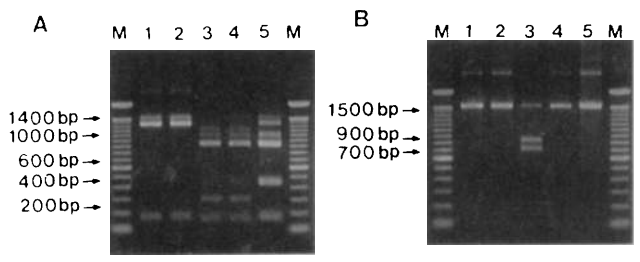

FIG. 1. Restriction patterns of 16S rDNAs purified with a GENECLEAN II kit. (A) 16S rDNAs digested with *Sma*I. (B) 16S rDNAs digested with *Mlu*I. Lanes M, 100-bp DNA ladder (Bethesda Research Laboratories); lane 1, *S. azurea* K161^T; lane 2, *"S. caesia"* K163; lane 3, *S. cyanea* K168^T; lane 4, *S. glauca* K169^T; lane 5, *S. viridis* K73^T.

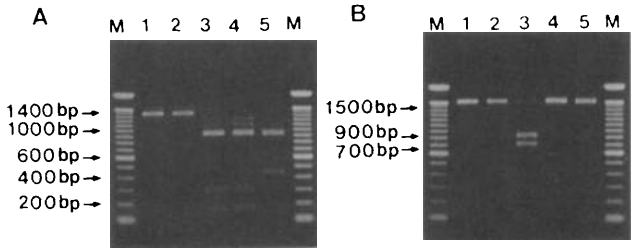

FIG. 2. Restriction patterns of 16S rDNAs purified with a Sephaglas BandPrep kit. (A) 16S rDNAs digested with *Sma*I. (B) 16S rDNAs digested with *Mlu*I. Lanes M, 100-bp DNA ladder (Bethesda Research Laboratories); lane 1, *S. azurea* K161^T; lane 2, *"S. caesia"* K163; lane 3, *S. cyanea* K168^T; lane 4, *S. glauca* K169^T; lane 5, *S. viridis* K73^T.

RESULTS AND DISCUSSION

Saccharomonospora species have been phylogenetically analyzed by using 16S rRNA gene sequences (9). However, many strains cannot be rapidly characterized by 16S rRNA sequence analysis. This problem required the development of simple methods for identification of *Saccharomonospora* species.

Our previous studies showed that each *Saccharomonospora* species could be differentiated by using genomic DNA restriction fragments and rRNA gene probes. The resulting ribotype patterns are species specific (21). This method is appropriate and more rapid than 16S rRNA sequencing for identification of *Saccharomonospora* species but is slightly laborious. In the present study PCR-based RFLP analysis of 16S rDNAs was performed without probing rRNA genes.

Approximately 1,500-bp 16S rDNAs of test strains were amplified by using the universal primers described previously (18) (data not shown). Representative strains of the four validly described *Saccharomonospora* species (*S. azurea* K161^T, *S. cyanea* K168^T, *S. glauca* K169^T, and *S. viridis* K73^T) and *"S. caesia"* K163 were first used in an RFLP analysis of 16S rDNAs. The RFLPs of 16S rDNAs were determined by using two restriction enzymes, *Sma*I and *Mlu*I. The characteristic restriction profiles generated by digestion with *Sma*I, *Mlu*I, and *Sma*I plus *Mlu*I allowed representative strains of the four validly described *Saccharomonospora* species to be differentiated (Fig. 1 to 6).

S. azurea K161^T and *"S. caesia"* strains produced the same *Sma*I, *Mlu*I, and *Sma*I plus *Mlu*I restriction patterns (Fig. 1 to 7). *"S. caesia"* K76^T (data not shown) gave RFLP patterns identical to those of other *"S. caesia"* strains (Fig. 1 to 7). Since it has been reported that *S. azurea* K161^T and strains of *"S. caesia"* have identical 16S rRNA sequences (9) and that the two species are closely related (8, 21), it was anticipated that

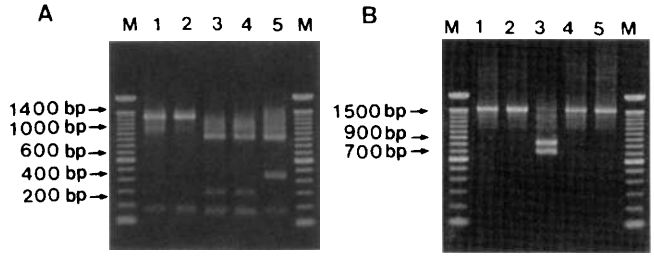

FIG. 3. Restriction patterns of 16S rDNAs precipitated with isopropanol. (A) 16S rDNAs digested with *Sma*I. (B) 16S rDNAs digested with *Mlu*I. Lanes M, 100-bp DNA ladder (Bethesda Research Laboratories); lane 1, *S. azurea* K161^T; lane 2, *"S. caesia"* K163; lane 3, *S. cyanea* K168^T; lane 4, *S. glauca* K169^T; lane 5, *S. viridis* K73^T.

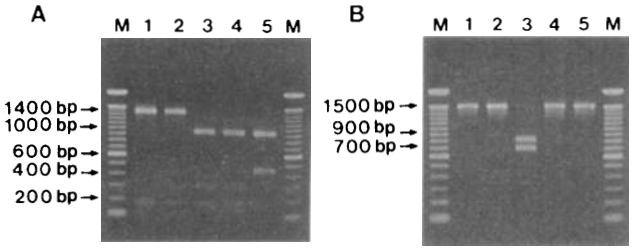

FIG. 4. Restriction patterns of 16S rDNAs extracted with chloroform. (A) 16S rDNAs digested with *Sma*I. (B) 16S rDNAs digested with *Mlu*I. Lanes M, 100-bp DNA ladder (Bethesda Research Laboratories); lane 1, *S. azurea* K161^T; lane 2, "*S. caesia*" K163; lane 3, *S. cyanea* K168^T; lane 4, *S. glauca* K169^T; lane 5, *S. viridis* K73^T.

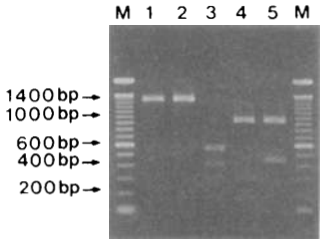

FIG. 6. Restriction patterns of 16S rDNAs without any treatment after double digestion with *Sma*I plus *Mlu*I. Lanes M, 100-bp DNA ladder (Bethesda Research Laboratories); lane 1, *S. azurea* K161^T; lane 2, "*S. caesia*" K163; lane 3, *S. cyanea* K168^T; lane 4, *S. glauca* K169^T; lane 5, *S. viridis* K73^T.

these organisms would have the same 16S rDNA RFLP patterns. However, DNA-DNA relateness experiments or other taxonomic studies are needed to determine the exact taxonomic relationship between the two species. *S. cyanea* K168^T and *S. glauca* K169^T gave the same 16S rDNA RFLP patterns after *Sma*I digestion (Fig. 1A, 2A, 3A, 4A, and 5A). However, digestion of the 16S rDNAs with *Mlu*I allowed the two species to be distinguished (Fig. 1B, 2B, 3B, 4B, and 5B). A *Mlu*I restriction site was found only in the 16S rDNA of *S. cyanea* K168^T (Fig. 1B, 2B, 3B, 4B, and 5B). *S. viridis* K73^T was distinguished from other *Saccharomonospora* species only by its *Sma*I restriction profile (Fig. 1A, 2A, 3A, 4A, and 5A).

The additional 10 test strains of *S. glauca* and *S. viridis* gave RFLP patterns identical to those of the corresponding representative strains after *Sma*I and *Mlu*I digestion (Fig. 7). These observations were in good agreement with the results of a 16S rRNA sequence analysis (9) and ribotyping (21); that is, intraspecific differences were not observed in the *S. glauca*, *S. viridis*, and "*S. caesia*" strains when the two analyses were performed (the only exception was *S. glauca* K169^T, which differed at one nucleotide position from other *S. glauca* strains). Since other strains of *S. cyanea* were not available, RFLP patterns of additional *S. cyanea* strains were not investigated. Five test strains identified as *S. glauca* by their *Sma*I restriction patterns did not produce a restriction pattern when they were treated with *Mlu*I (Fig. 7B). Thus, we concluded that strains of *S. cyanea* were not present in the additional test strains.

In this study, it was found that purification of PCR products was not necessary for a satisfactory RFLP analysis. Digestion of 16S rDNA PCR products purified with commercial purification kits, such as GENECLEAN II and Sephaglas BandPrep kits, was no better than digestion of unpurified PCR products (Fig. 1 and 2). The restriction patterns of the 16S rDNAs precipitated with isopropanol allowed each species to be differentiated, albeit with difficulty since the patterns were not distinct (Fig. 3). The 16S rDNA products extracted with chloroform and directly used without any treatment following the PCR gave good restriction results (Fig. 4 and 5). Therefore, the restriction of DNA was not adversely affected by the small amount of mineral oil still present. The elimination of the purification step is important in terms of saving experimental time and effort in RFLP analyses of unknown strains.

Rapid identification of *Saccharomonospora* species is now possible by PCR amplification of 16S rDNA followed by digestion(s) with *Sma*I and *Mlu*I. This represents an alternative to methods based on phenotypic characterization and existing molecular approaches. PCR-RFLP analysis of 16S rDNAs is simple, reproducible, and species specific. The method was found to be more rapid and less laborious than ribotyping, as it eliminates the requirement for blotting and probe hybridization procedures.

It has been reported recently that 16S rDNAs can be directly amplified by using crude cell lysates from small quantities of bacterial cells (2, 6). In our study, 16S rDNAs of *Saccharomonospora* species were also successfully amplified without preliminary DNA extraction and purification from cells by the

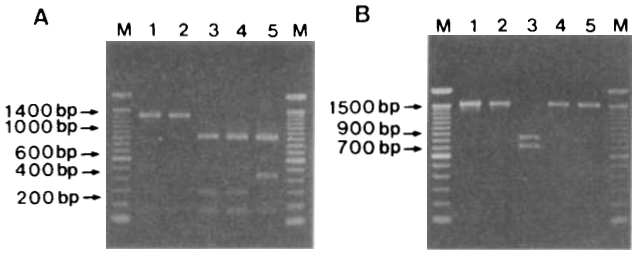

FIG. 5. Restriction patterns of 16S rDNAs without any treatment. (A) 16S rDNAs digested with *Sma*I. (B) 16S rDNAs digested with *Mlu*I. Lanes M, 100-bp DNA ladder (Bethesda Research Laboratories); lane 1, *S. azurea* K161^T; lane 2, "*S. caesia*" K163; lane 3, *S. cyanea* K168^T; lane 4, *S. glauca* K169^T; lane 5, *S. viridis* K73^T.

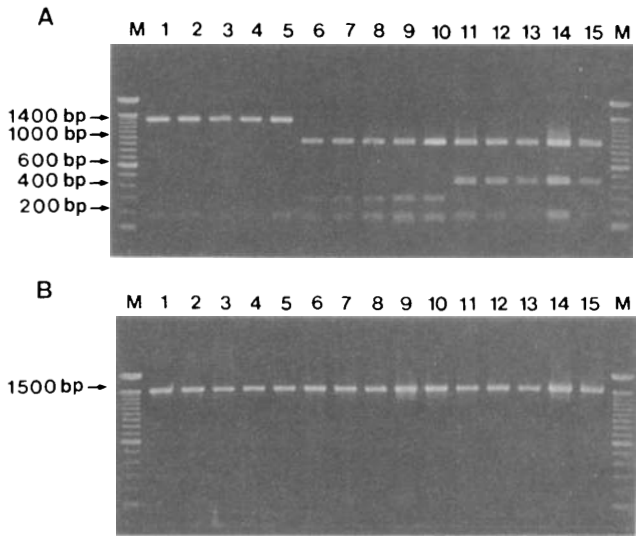

FIG. 7. Restriction patterns of 16S rDNAs from 15 test strains. (A) 16S rDNAs digested with *Sma*I. (B) 16S rDNAs digested with *Mlu*I. Lanes M, 100-bp DNA ladder (Bethesda Research Laboratories); lane 1, K182; lane 2, K200; lane 3, SB-01; lane 4, SB-22; lane 5, SB-58; lane 6, K179; lane 7, K194; lane 8, K195; lane 9, K202; lane 10, SB-37; lane 11, K185; lane 12, K191; lane 13, K197; lane 14, SB-31; lane 15, SB-33.

method described previously (data not shown) (6). Our results demonstrate that a more rapid method for bacterial characterization based on 16S rDNA RFLP analysis may be possible.

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