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 *Pseudonocardiaaceae* (9).

Up to this point, the genus *Saccharomonospora* has been described as a genus with four valid species, namely, *Saccharomonospora azurea* (14), *Saccharomonospora cyanaea* (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 *Mircopolyspora 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 that strains of "S. caesia" are very closely related to *S. azurea* K161T (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.

16S 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%, w/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 rDNA genes were 5'-GAGTGT TGATC CTGGC TCAG-3' (positions 9 to 27 [Escherichia coli 16S rRNA numbering]) and 5'-AGAAA GGAGG TGATC CAGCC-3' (positions 1542 to 1555 [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 MgCl2, 0.01% (w/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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The PCR products were precipitated with 9 volumes 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: Smal and MluI (New England Biolabs, Inc., Beverly, Mass.). Double digestion with Smal and MluI was performed as follows. DNA was first digested for 1 h at 25°C with Smal. Following addition of the buffer recommended by the manufacturer, MluI 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.

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, Smal and MluI. The characteristic restriction profiles generated by digestion with Smal, MluI, and Smal plus MluI 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 Smal, MluI, and Smal plus MluI 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

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**TABLE 1. Strains used in this study**

<table>
<thead>
<tr>
<th>Laboratory no.</th>
<th>Species</th>
<th>Source and/or other designation(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>K161^T</td>
<td>S. azurea</td>
<td>H. Runmoo, NA-128^T ( = SIA 86128^T)</td>
</tr>
<tr>
<td>K76^T</td>
<td>“S. caesia”</td>
<td>KCTC 9152^T</td>
</tr>
<tr>
<td>K163</td>
<td>“S. caesia”</td>
<td>DSM 43068</td>
</tr>
<tr>
<td>K182</td>
<td>“S. caesia”</td>
<td>E. Greiner-Mai, Ko18</td>
</tr>
<tr>
<td>K200</td>
<td>“S. caesia”</td>
<td>J. Lacey, A1932</td>
</tr>
<tr>
<td>SB-01, SB-22, SB-58</td>
<td>“S. caesia”</td>
<td>S.-B. Kim</td>
</tr>
<tr>
<td>K161^T</td>
<td>S. cyanura</td>
<td>H. Runmoo, NA-134^T ( = SIA 86134^T)</td>
</tr>
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<td>S. glauca</td>
<td>DSM 43769^T</td>
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</tr>
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<td>S. glauca</td>
<td>J. Lacey, A66</td>
</tr>
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<td>S. glauca</td>
<td>J. Lacey, A1450</td>
</tr>
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<td>S. glauca</td>
<td>J. Ruan, 350</td>
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<td>SB-37</td>
<td>S. glauca</td>
<td>S.-B. Kim</td>
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<td>K173^T</td>
<td>S. viridis</td>
<td>NCIB 9602^T</td>
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<td>S. viridis</td>
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<td>S. viridis</td>
<td>E. Greiner-Mai, R25</td>
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<td>K197</td>
<td>S. viridis</td>
<td>J. Lacey, A1905</td>
</tr>
<tr>
<td>SB-31, SB-33</td>
<td>S. viridis</td>
<td>S.-B. Kim</td>
</tr>
</tbody>
</table>

*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.*

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**FIG. 1. Restriction patterns of 16S rDNAs purified with a GENECLEAN II kit.** (A) 16S rDNAs digested with Smal. (B) 16S rDNAs digested with MluI. Lanes M, 100-bp DNA ladder (Bethesda Research Laboratories); lane 1, S. azurea K161^T; lane 2, “S. caesia” K163; lane 3, S. cyanura 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 relatedness experiments or other taxonomic studies are needed to determine the exact taxonomic relationship between the two species. S. cyanea K168T and S. glauca K169T gave the same 16S rDNA RFLP patterns after SmaI digestion (Fig. 1A, 2A, 3A, 4A, and 5A). However, digestion of the 16S rDNAs with MluI allowed the two species to be distinguished (Fig. 1B, 2B, 3B, 4B, and 5B). A MluI restriction site was found only in the 16S rDNA of S. cyanea K168T (Fig. 1B, 2B, 3B, 4B, and 5B). S. viridis K73T was distinguished from other Saccharomonospora species only by its SmaI 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 SmaI and MluI 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 K169T, 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 SmaI restriction patterns did not produce a restriction pattern when they were treated with MluI (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 SmaI and MluI. 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
method described previously (data not shown) (6). Our results
demonstrate that a more rapid method for bacterial character-
ization based on 16s rDNA RFLP analysis may be possible.

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