Identification of *Saccharomonospora* Strains by the Use of Genomic DNA Fragments and rRNA Gene Probes

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Restriction digestion fragments of DNAs extracted from 14 representative strains of *Saccharomonospora azurea*, “*Saccharomonospora caesia*,” *Saccharomonospora cyanea*, *Saccharomonospora glauca*, and *Saccharomonospora viridis* and six “*Saccharomonospora*”-like isolates were separated by electrophoresis, Southern blotted onto nylon membranes, and hybridized by using two rRNA gene probes cloned from *Streptomyces griseus* subsp. *griseus* KCTC 9080. The following four restriction endonucleases were used: *Bam*HI, *Sal*II, *Pvu*II, and *Xho*I. The resultant five ribotype patterns were considered species specific. The genomic diversity revealed by ribotyping indicates that this method can be used to both characterize and identify *saccharomonosporae*. All of the test strains contained DNA with three rRNA gene clusters.

The genus *Saccharomonospora* was proposed by Nonomura and Ohara (21) for aerobic, gram-positive, non-acid-fast, non-motile, monosporic actinomycetes which typically form non-fragmenting, branched substrate mycelia with walls that contain meso-diaminopimelic acid, arabinose, and galactose (wall chemotype IV sensu Lechevalier and Lechevalier [20]). Additional chemical markers include the presence of major amounts of iso and anteiso fatty acids, 2-hydroxy fatty acids, phosphatidylethanolamine, and tetrahydrogenated menaquinones with nine isoprene units (17). This genus forms a distinct clade within the evolutionary radiation encompassed by the family *Pseudonocardiaceae* (16).

There are currently four validly described *Saccharomonospora* species, namely, *Saccharomonospora azurea* Runmao 1987 (23), *Saccharomonospora cyanea* Runmao and Guizhen 1988 (24), *Saccharomonospora glauca* Greiner-Mai et al. 1988 (8), and *Saccharomonospora viridis* Nonomura and Ohara 1970 (21), the type species of the genus. A fifth taxon, “*Saccharomonospora caesia*,” was proposed by Greiner-Mai et al. (9) for strains previously classified as *Micropolyspora caesia* Kalakoutskii (12, 17); this name was not included on the Approved Lists of Bacterial Names (25) and has not been validly published. However, reliable procedures are still needed to distinguish validly published and putatively novel *Saccharomonospora* species. It is important to distinguish *Saccharomonospora* species as some strains are agents of hypersensitivity pneumonitis (18). *Saccharomonospora viridis* has been strongly implicated as a causal agent of farmer's lung disease (1, 7).

Improvements in the classification of the genus *Saccharomonospora* have made it possible to choose good representative strains for comparative taxonomic studies. Thus, in a comprehensive numerical taxonomic survey, Kim equated numerically circumscribed clusters with *Saccharomonospora azurea*, “*Saccharomonospora caesia*,” *Saccharomonospora cyanea*, *Saccharomonospora glauca* and *Saccharomonospora viridis* (15). It is also encouraging that representatives of validly described and putatively novel *Saccharomonospora* species can be

separated on the basis of 16S rRNA gene (rDNA) sequence data (16) although *Saccharomonospora azurea* K161^T (T = type strain) and “*Saccharomonospora caesia*” K163 have been shown to have identical 16S rRNA gene sequences.

Recent advances in nucleic acid fingerprinting techniques have been found to be effective as rapid and specific aids in the characterization and identification of bacterial species that are difficult to identify (27). In particular, species- and strain-specific ribosomal restriction endonuclease patterns can be visualized after hybridization of rRNA gene probes to genomic DNAs isolated from members of diverse bacterial genera (10). Species-specific differences in ribotype patterns have been used to identify several bacterial species for which conventional laboratory identification methods are inadequate. This method has been used in actinomycete systematics, notably with respect to the genera *Actinomyces* (2, 5), *Corynebacterium* (26), *Gordona* (22), *Mycobacterium* (4, 13), *Rhodococcus* (19), and *Streptomyces* (6).

The primary aim of this investigation was to determine the value of ribotyping in the classification and identification of *Saccharomonospora* species. To this end, two rDNA probes were used to determine rRNA operon-targeted polymorphisms in 20 judiciously chosen representatives of the genus *Saccharomonospora*. The resultant data were also used to determine the copy numbers of the rRNA gene clusters in the test strains.

MATERIALS AND METHODS

Organisms and culture conditions. The test strains (Table 1) were grown in shake flasks containing tryptone soy broth supplemented with glucose (0.75%, wt/vol) at 45°C for 48 h. The broth cultures were checked for purity before they were harvested by centrifugation.

Extraction of DNA. Chromosomal DNA was extracted and purified by using a previously described procedure (11). Wet biomass (ca. 3 g) was suspended in 10 ml of TE buffer (10 mM Tris-HCl, 1 mM EDTA; pH 8.0) supplemented with lysozyme (10 mg/ml) and incubated for 15 min at 37°C before 1 ml of 20% (wt/vol) sodium dodecyl sulfate (SDS), 10 ml of Tris-HCl (pH 8.0)-saturated phenol, and 1.5 ml of 5 M NaCl were added. The resulting preparation was centrifuged at 5,000 × g for 10 min, the supernatant was extracted twice with an equal volume of chloroform, and the crude DNA precipitate was recovered with an equal volume of isopropanol. The chromosomal DNA was resuspended in 10 ml of TE buffer and incubated first with RNase A (20 µg/ml; Sigma) for 60 min at 50°C and then with proteinase K (100 µg/ml; Sigma) for 60 min at 37°C.

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TABLE 1. Designations and sources of test strains

Laboratory no. ^a	Taxon	Other designation(s) and/or source ^b
K161 ^T	<i>Saccharomonospora azurea</i>	H. Runmao NA-128 (SIIA 86128); Sichuan Industrial Institute of Antibiotics, Chengdu, Sichuan, People's Republic of China
K163	" <i>Saccharomonospora caesia</i> "	DSM 43068, CCM 3262, R. Hütter ETH 28747, Corbaz 168; moldy hay
K182	" <i>Saccharomonospora caesia</i> "	E. Greiner-Mai Ko18; Institut für Mikrobiologie, Technische Hochschule, Darmstadt, Germany
K200	" <i>Saccharomonospora caesia</i> "	J. Lacey A1932, Plant Pathology Department, Rothamsted Experimental Station, Harpenden, United Kingdom
K168 ^T	<i>Saccharomonospora cyanea</i>	H. Runmao NA-134 (SIIA 86134); soil, Guangyuan City, People's Republic of China
K169 ^T	<i>Saccharomonospora glauca</i>	DSM 43769
K179	<i>Saccharomonospora glauca</i>	A. J. McCarthy BD-125; hay, Department of Genetics and Microbiology, University of Liverpool, Liverpool, and Rothamsted Experimental Station, Harpenden, United Kingdom
K194	<i>Saccharomonospora glauca</i>	J. Lacey A66; hay, Rothamsted Experimental Station, Harpenden, United Kingdom
K195	<i>Saccharomonospora glauca</i>	J. Lacey A1450; air sample, cotton mill, Cramlington, United Kingdom
K202	<i>Saccharomonospora glauca</i>	J. Ruan 350; Institute of Microbiology, Beijing, People's Republic of China
K73 ^T	<i>Saccharomonospora viridis</i>	NCIB 9602, E. Küster P101; peat, Ireland
K185, K191	<i>Saccharomonospora viridis</i>	E. Greiner-Mai L1v and R25
K197	<i>Saccharomonospora viridis</i>	J. Lacey A1905; mushroom compost, Avon, United Kingdom
SB-01, SB-22, SB-58	" <i>Saccharomonospora</i> "-like isolates	S.-B. Kim, mushroom compost, Somerset, United Kingdom
SB-31, SB-33, SB-37	" <i>Saccharomonospora</i> "-like isolates	S.-B. Kim, hay, Rothamsted Experimental Station, Harpenden, United Kingdom

^a The 16S rRNA sequences of strains K161^T, K163, K168^T, K169^T, K202, K73, K191, SB-01, SB-22, SB-58, SB-33, and SB-37 were determined by Kim et al. (16).

^b Abbreviations: CCM, Czechoslovak Collection of Microorganisms, Masaryk University, Brno, Czech Republic; DSM, Deutsche Sammlung von Mikroorganismen, Braunschweig, Germany; NCIB, National Collection of Industrial and Marine Bacteria, Ltd., Aberdeen, United Kingdom.

Following this the DNA was extracted once with phenol and twice with chloroform. It was then precipitated with isopropanol, spooled out with a glass rod, dried, and resuspended in TE buffer. The concentration and quality of each DNA preparation were determined spectrophotometrically and by agarose gel electrophoresis.

Preparation and labelling of probes. The PCR was used to amplify an 899-bp region close to the 5' end of the 16S rDNA (positions 4 to 902 [*Streptomyces griseus* subsp. *griseus* KCTC 9080 16S rDNA numbering]) and a 660-bp region close to the 3' end of the 23S rDNA (positions 2154 to 2813 [*Streptomyces griseus* subsp. *griseus* KCTC 9080 23S rDNA numbering]) from the *rne* gene cluster of *Streptomyces griseus* subsp. *griseus* KCTC 9080. The probes were labelled by using a nonradioactive ECL direct nucleic acid labelling and detection system (Amersham International plc, Buckinghamshire, United Kingdom) and the manufacturer's instructions.

Southern blotting and hybridization. Aliquots (2 µg) of the chromosomal DNAs of the test strains were digested with 4 U of *Bam*HI, *Sal*I, *Pvu*II, and *Xho*I overnight at 37°C as recommended by the manufacturer (Boehringer Mannheim GmbH Biochemica, Mannheim, Germany). Chromosomal DNA from *Saccharomonospora glauca* K169^T was also digested with *Bgl*II by the same procedure. Restriction nuclease fragments were then separated by electrophoresis in a 0.7% (wt/vol) agarose gel in Tris-acetate buffer (0.04 M Tris-acetate, 0.001 M EDTA). The separated DNA fragments were denatured in the gel, transferred to a nylon

membrane (Hybond N+; Amersham International plc), fixed with UV light for 3 min, and then hybridized in tubes in an oven (model HB-OV-BL/Bs; Hybaid, Ltd., Middlesex, United Kingdom). Prehybridization was performed in a hybridization solution (ECL direct nucleic acid labelling and detection system; Amersham International plc) for 1 h. One of the two labelled probes was added to the hybridization solution, and the preparation was incubated for 8 h at 42°C. Following hybridization, the membrane was washed once with a primary working solution (0.1× SSC, 0.4% [wt/vol] SDS) for 10 min at 55°C and once with a secondary working solution (2× SSC) for 5 min at room temperature (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate). The hybrids on the membrane were detected by using detection reagents (ECL direct nucleic acid labelling and detection system; Amersham International plc). Hybridization with the second probe was carried out with the same preparation by using the same conditions once the probe hybridized on the membrane in the first experiment had been eliminated by stringent agitation with reprobing solution (0.2 N NaOH, 0.1% [wt/vol] SDS) for 30 min at 65°C.

RESULTS AND DISCUSSION

DNA samples were checked for the absence of extensive shearing and degradation prior to digestion with the restriction

FIG. 1. Ribotype patterns generated from DNA digests of representative *Saccharomonospora* strains hybridized with the 5' end probe of 16S rDNA from *Streptomyces griseus* subsp. *griseus* KCTC 9080. The strains used were *Saccharomonospora azurea* K161^T (A), "*Saccharomonospora caesia*" K163 (B), *Saccharomonospora cyanea* K168^T (C), *Saccharomonospora glauca* K169^T (D), and *Saccharomonospora viridis* K73^T (E). Lanes 1, molecular weight markers (restriction fragments of bacteriophage λ DNA digested with *Hind*III); lanes 2, *Bam*HI digests; lanes 3, *Sal*I digests; lanes 4, *Xho*I digests; lanes 5, *Pvu*II digests.

FIG. 2. Ribotype patterns generated from DNA digests of representative *Saccharomonospora* strains hybridized with the 3' end probe of 23S rDNA from *Streptomyces griseus* subsp. *griseus* KCTC 9080. The strains used were *Saccharomonospora azurea* K161^T (A), "*Saccharomonospora caesia*" K163 (B), *Saccharomonospora cyanea* K168^T (C), *Saccharomonospora glauca* K169^T (D), and *Saccharomonospora viridis* K73^T (E). Lanes 1, molecular weight markers (restriction fragments of bacteriophage λ DNA digested with *Hind*III); lanes 2, *Bam*HI digests; lanes 3, *Sal*I digests; lanes 4, *Xho*I digests; lanes 5, *Pvu*II digests.

enzymes and after electrophoresis of the genomic digests. The DNA preparations had high molecular weights and were free from the products of extensive shearing. The absence of high-molecular-weight DNA in the agarose gel electrophoretic patterns of the genomic digests showed that the DNA samples were completely digested. The restriction fragment length polymorphisms of the ribosomal genes of the representative strains of *Saccharomonospora azurea*, "*Saccharomonospora caesia*," *Saccharomonospora cyanea*, *Saccharomonospora glauca*, and *Saccharomonospora viridis* allow these taxa to be distinguished (Fig. 1 and 2). The additional "*Saccharomonospora caesia*," *Saccharomonospora glauca*, and *Saccharomonospora viridis* strains produced ribotype patterns identical to those of the corresponding representative strains. Interestingly, isolates SB-01, SB-22, and SB-58 produced ribotype patterns identical to the pattern produced by the representative strain of "*Saccharomonospora caesia*," SB-37 produced a pattern identical to the pattern produced by the representative strain of *Saccharomonospora glauca*, and SB-31 and SB-33 produced patterns identical to the pattern produced by the representative strain of *Saccharomonospora viridis*.

The same ribotype patterns were produced by the *Saccharomonospora azurea*, "*Saccharomonospora caesia*," and *Saccharomonospora viridis* strains irrespective of which of the probes was used, indicating that the four restriction enzymes

did not digest either the structural genes or the interspacer regions of the rRNA operons (Fig. 1 and 2). In contrast, different ribotype patterns were produced when the *Xho*I digests of the *Saccharomonospora glauca* strains were treated with the two rDNA probes. It seems that these strains have an *Xho*I site(s) in either the structural genes or the interspacer regions of the rRNA operons. Similarly, *Saccharomonospora cyanea* K168^T appears to have a *Sal*I site(s) in one of the two regions of the rRNA operons. Similar results have been obtained with 16S and 23S rRNA probes (3).

Saccharomonospora azurea K161^T and the "*Saccharomonospora caesia*" strains produced identical ribotype patterns when the products of the *Bam*HI, *Sal*I, and *Pvu*II digests were treated with the rDNA probes. However, in corresponding studies performed with *Xho*I digests, *Saccharomonospora azurea* K161^T could be differentiated from the "*Saccharomonospora caesia*" strains (Fig. 1 and 2). Previously, members of these taxa were found to be closely related in numerical phenetic (15) and 16S rRNA sequencing studies (16). DNA-DNA relatedness experiments are needed to determine the exact taxonomic relationship between *Saccharomonospora azurea* and "*Saccharomonospora caesia*" before a new taxon can be validly described. It is also interesting that the *Saccharomonospora azurea*, "*Saccharomonospora caesia*," *Saccharomonospora cyanea*, *Saccharomonospora glauca*, and *Saccharomonospora viridis* strains produced identical ribotype patterns when the products of the *Bam*HI, *Sal*I, and *Pvu*II digests were treated with the rDNA probes.

FIG. 3. Ribotype analysis of *Xho*I-digested DNAs from representative *Saccharomonospora* strains. (A) Hybridization with the 5' end probe of 16S rDNA from *Streptomyces griseus* subsp. *griseus* KCTC 9080. (B) Hybridization with the 3' end probe of 23S rDNA from *Streptomyces griseus* subsp. *griseus* KCTC 9080. Lanes 1 and 7, molecular weight markers (restriction fragments of bacteriophage λ DNA digested with *Hind*III); lanes 2, *Saccharomonospora viridis* K73^T; lanes 3, "*Saccharomonospora caesia*" K163; lanes 4, *Saccharomonospora azurea* K161^T; lanes 5, *Saccharomonospora cyanea* K168^T; lanes 6, *Saccharomonospora glauca* K169^T.

FIG. 4. Ribotype analysis of *Bgl*II-digested DNA from *Saccharomonospora glauca* K169^T. (A) Hybridization with the 5' end probe of 16S rDNA from *Streptomyces griseus* subsp. *griseus* KCTC 9080. (B) Hybridization with the 3' end probe of 23S rDNA from *Streptomyces griseus* subsp. *griseus* KCTC 9080. Lanes 1, molecular weight markers (restriction fragments of bacteriophage λ DNA digested with *Hind*III); lanes 2, *Saccharomonospora glauca* K169^T.

spora viridis strains produced distinct ribotype patterns when *Xho*I digests were treated with the rRNA probes (Fig. 3).

The number of bands and their relative intensities indicate that the chromosomes of the *Saccharomonospora azurea*, "*Saccharomonospora caesia*," *Saccharomonospora cyanea*, and *Saccharomonospora viridis* strains contain three rRNA gene clusters. In contrast, the *Saccharomonospora glauca* strains gave ambiguous results when the *Bam*HI and *Pvu*II digests were probed (Fig. 1 and 2). However, we found that *Saccharomonospora glauca* K169^T also contains three rRNA gene clusters when *Bgl*II digests were treated with the two rDNA probes (Fig. 4). There is evidence that the copy number of rRNA genes may provide taxonomically useful information (2, 4).

We concluded that the ribotyping method in which four restriction endonucleases are used provides a useful and reliable way to distinguish *Saccharomonospora azurea*, "*Saccharomonospora caesia*," *Saccharomonospora cyanea*, *Saccharomonospora glauca*, and *Saccharomonospora viridis* strains. The five ribotype patterns which we obtained can be considered species specific as members of these taxa formed distinct taxospecies in the comprehensive numerical taxonomic study of Kim et al. (14). In theory, members of these taxa can be distinguished merely by probing *Xho*I digests with the rDNA probes. However, in practice, the remaining restriction enzymes should be used in order to help ensure the detection of members of putatively novel *Saccharomonospora* species.

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