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A Phylogenetic Analysis of the Genus *Saccharomonospora* Conducted with 16S rRNA Gene Sequences

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Nearly complete sequences of 16S rRNA genes of representative strains of the genus *Saccharomonospora* were determined following the isolation and cloning of the amplified genes. The sequences were aligned with those of representatives of the family *Pseudonocardiaceae*, and a phylogenetic tree was inferred by the neighbor-joining method. The genus *Saccharomonospora* formed a distinct clade within the evolutionary radiation encompassed by the family *Pseudonocardiaceae*. The average nucleotide similarity value found between the type strains of the four validly described *Saccharomonospora* species was 97.5% ± 1.0%. The most distant relationship was found between *Saccharomonospora azurea* and *Saccharomonospora viridis* K73 (96.3% similarity). In contrast, *Saccharomonospora azurea* K161 and "*Saccharomonospora caesia*" K163 had identical 16S rRNA gene sequences. The nucleotide sequence data suggest that the genus *Saccharomonospora* contains several new centers of variation.

The genus *Saccharomonospora* was proposed by Nonomura and Ohara (30) for monosporic actinomycetes that contained *meso*-diaminopimelic acid, arabinose, and galactose in the peptidoglycan (wall chemotype IV sensu Lechevalier Lechevalier [25]). Subsequently, additional chemical markers were found to characterize the taxon, notably the presence of major amounts of *iso*- and *anteiso*-fatty acids, 2-hydroxy fatty acids, phosphatidylethanolamine, and menaquinones that were tetrahydrogenated with nine isoprene units (21, 25).

There are currently four validly described *Saccharomonospora* species, namely, *Saccharomonospora azurea* (32), *Saccharomonospora cyanea* (33), *Saccharomonospora glauca* (17), and *Saccharomonospora viridis* (30), the type species of the genus. A fifth species, "*Saccharomonospora caesia*," was proposed by Greiner-Mai et al. (18) for organisms previously classified as *Micropolyspora caesia* (20, 22); this organism did not appear in the Approved Lists of Bacterial Names (35) and has not been added subsequently, and hence, it has not been validly published. It is important to distinguish between members of validly described and putatively new *Saccharomonospora* species, as some kinds of saccharomonosporae can cause hypersensitivity pneumonitis. *S. viridis* is strongly implicated as one the causal agents of farmer's lung disease (3, 16).

It is also important to determine the taxonomic structure of the genus *Saccharomonospora* in order to discover the relationships between the existing taxa and between them and prospective new species. It appears from preliminary 16S rRNA sequence studies that the genus *Saccharomonospora* forms a distinct phyletic line within the evolutionary radiation encompassed by the family *Pseudonocardiaceae* (5, 9, 10, 28, 36). To date, three *Saccharomonospora* strains have been the subject of 16S rRNA sequencing analyses, the type strain of *S. viridis*, the putative type strain of "*S. caesia*," and an isolate from stored grain, *Saccharomonospora* sp. strain A1206, that was tentatively considered, subject to additional sequencing studies, to be the nucleus of a new species (36). In the present investigation, the partial sequences of the 16S rRNA genes (16S rDNA) of an additional 13 representative *Saccharomonospora* strains were examined.

MATERIALS AND METHODS

Selective isolation. Mushroom compost samples collected from Somerset, United Kingdom, and a hay sample from Rothamsted Experimental Station, Harpenden, United Kingdom, were dried at room temperature for 2 or 3 days. The dried hay (5 g) and mushroom compost samples (2 g) were transferred to nylon mesh bags which were inflated with compressed air prior to sealing. The preparations were vigorously shaken for 5 min to produce aerial spore suspensions that were thoroughly mixed for 5 min by the electric fan in a sedimentation chamber. The bags were left to stand for 90 min, and the spore cloud was sampled with a six-stage viable-particle sampler (Andersen 2000 Inc., Atlanta, Ga.) (2).

The inflated bags were attached to the inlet cone of the Andersen sampler, and air containing the spores was drawn through the system for 20 s with a vacuum pump. The spore clouds were impacted onto open petri dishes that contained R8 agar (1), half-strength tryptic soy agar (Difco), and half-strength tryptic soy agar supplemented with adenine (0.4%, wt/vol) and streptomycin (16 μg/ml); all three media also contained cycloheximide at 50 μg/ml. The agar plates were prepared in duplicate; one set was incubated at 30°C and the other was incubated at 50°C for 2 weeks, when the number of *Saccharomonospora*-like colonies was counted. Saccharomonosporae were readily recognized on isolation plates, as they produced a characteristic grayish-green aerial spore mass. The aerial hyphae were seen to carry single spores when examined with an Optiphot binocular light microscope fitted with a long distance objective (X400 magnification; Nikon Kogaru K.K., Tokyo, Japan).

Sixty-seven colonies were taken from the isolation plates with sterile toothpicks and inoculated onto R8 agar (1) plates that were incubated at either 30°C for 7 days or 50°C for 3 days (depending on the temperature at which the original isolation plates were incubated). Suspensions of spores and hyphal fragments were prepared in sterile glycerol (20%, vol/vol; Analar; BDH) and kept at −20°C (37). The tests strains were also maintained on R8 agar (1) plates.

Organisms and culture conditions. Representatives of *Saccharomonospora* species were examined together with five randomly chosen strains (Table 1) isolated from the hay and mushroom compost samples on R8 agar plates incubated at 50°C. Biomass of the test strains was prepared in shake flasks of tryptic soy broth, supplemented with glucose (0.75%, wt/vol), at 45°C for 48 h. At maximum growth, the broth cultures were checked for purity, harvested by centrifugation, and washed twice in 10 ml of saline-EDTA (0.15 M NaCl, 0.1 M EDTA; pH 8.0). Competent cells of *Escherichia coli* DH5αF′ (Promega Co.,

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TABLE 1. Strains used in the 16S rDNA sequence analyses

Laboratory no.[a]	Species	Strain designation and/or source[b]	Nucleotide sequence accession no.	Source of data[c]
K209^T	*Actinokineospora riparia*	IFO 14541^T	X76953	RDP
K210^T	*Amycolatopsis azurea*	NRRL 11412^T	X53199	RDP
K156^T	*Amycolatopsis fastidiosa*	ATCC 31181^T	X53200	RDP
K98^T	*Amycolatopsis mediterranei*	ATCC 13685^T	X76957	RDP
239^T	*Amycolatopsis methanolica*	NRRL 11412^T	X54274	RDP
K99^T	*Amycolatopsis orientalis*	ATCC 19795^T	X76958	RDP
K159^T	*Kibdelosporangium aridum*	ATCC 39323^T	X53191	RDP
NA	*Mycobacterium bovis*	Strain BCG	M20940	RDP
K211^T	*Pseudonocardia alni*	V. N. Akimov; VKM AC901^T	X76954	RDP
K402^T	*Pseudonocardia autotrophica*	ATCC 19727^T	X54288	RDP
P435^T	*Pseudonocardia compacta*	DSM 43592^T	X55609	RDP
K141^T	*Pseudonocardia halophobica*	DSM 43089^T	Z14111	RDP
K428^T	*Pseudonocardia hydrocarbonoxydans*	DSM 43281^T	X76955	RDP
K212^T	*Pseudonocardia nitrificans*	DSM 46012^T	X55609	RDP
K213^T	*Pseudonocardia petroleophila*	DSM 43193^T	X55608	RDP
A195^T	*Pseudonocardia saturnea*	DSM 43195^T	X76956	RDP
K160^T	*Pseudonocardia thermophila*	DSM 19285^T	X53195	RDP
K161^T	*Saccharomonospora azurea*	H. Runmao; NA128^T	Z38017	This study
K76^T	"*Saccharomonospora caesia*"	INMI 19125^T	X76960	RDP
K163	"*Saccharomonospora caesia*"	DSM 43068	Z38019	This study
K168^T	*Saccharomonospora cyanea*	H. Runmao; NA134^T	Z38018	This study
K169^T	*Saccharomonospora glauca*	DSM 43769^T	Z38003	This study
K202	*Saccharomonospora* sp.	J.-S. Ruan; 350; soil	Z38004	This study
K73^T	*Saccharomonospora viridis*	NCIB 9602^T	Z38007	This study
K191	*Saccharomonospora viridis*	E. Greiner-Mai; R25	Z38021	This study
K180	*Saccharomonospora* sp.	A. J. McCarthy; municipal refuge compost, Germany	Z38020	This study
SB-01	*Saccharomonospora* sp.	S.-B. Kim; mushroom compost, phase I	Z38022	This study
SB-22	*Saccharomonospora* sp.	S.-B. Kim; cooked-out mushroom compost	Z38023	This study
SB-33	*Saccharomonospora* sp.	S.-B. Kim; hay[d]	Z38005	This study
SB-37	*Saccharomonospora* sp.	S.-B. Kim; hay[d]	Z38006	This study
SB-58	*Saccharomonospora* sp.	S.-B. Kim; mushroom compost, end of phase II	Z38024	This study
NA	*Saccharomonospora* sp.	J. Lacey; A1206	X76961	RDP
D432	*Saccharopolyspora erythraea*	NRRL 2338	X53198	RDP
A85^T	*Saccharopolyspora gregorii*	NCIB 12823^T	X76962	RDP
K16^T	*Saccharopolyspora hirsuta*	ATCC 27875^T	X53196	RDP
A54^T	*Saccharopolyspora hordei*	NCIB 12824^T	X53197	RDP
F1^T	*Saccharopolyspora rectivirgula*	ATCC 33515^T	X53194	RDP
K204^T	*Saccharothrix autraliensis*	ATCC 31947^T	X54193	RDP
K207^T	*Saccharothrix mutabilis*	DSM 43853^T	X76966	RDP

[a] NA, not assigned; ^{T}, type strain.
[b] Abbreviations: ATCC, American Type Culture Collection, Rockville, Md.; DSM, Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany; IFO, Institute for Fermentation, Higashiyodogawa, Osaka, Japan; INMI, Institute of Microbiology, Moscow, Russia; NCIB, National Collection of Industrial Bacteria, Aberdeen, Scotland; NRRL, Northern Regional Research Laboratory, Agricultural Research Service, U.S. Department of Agriculture, Peoria, Ill. Sources of cultures: V. N. Akimov, Department of Type Cultures, Institute of Microbiology, Moscow, Russia; E. Greiner-Mai, Institut für Mikrobiologie, Technische Hochschule Darmstadt, Darmstadt, Germany; J. Lacey, Plant Pathology Department, Rothamsted Experimental Station, Harpenden, Hertfordshire, United Kingdom; A. J. McCarthy, Department of Genetics and Microbiology, University of Liverpool, Liverpool, United Kingdom; J.-S. Ruan, Institute of Microbiology, Academia Sinica, Zhong Guan Cun, Beijing People's Republic of China; H. Runmao, New Antibiotic Research Department, Sichuan Industrial Institute of Antibiotics, Chengdu, Sichuan, People's Republic of China.
[c] RDP, Ribosomal Database Project release 4 (24).
[d] Isolated on R8 agar (1) at 50°C.

Madison, Wis.) were used for transformation with the recombinant plasmid pBluescript II SK(+) (Stratagene, La Jolla, Calif.).

DNA extraction. Chromosomal DNA was extracted and purified by a modification of the method of Marmur (26). Wet biomass (ca. 5 g) was suspended in 10 ml of TE buffer (10 mM Tris-HCl, 1 mM EDTA; pH 8.0) with lysozyme (10 mg/ml) for 15 min at 37°C prior to the addition of 1 ml of 20% (wt/vol) sodium dodecyl sulfate, 10 ml of TE-saturated phenol, and 1.5 ml of 5 M NaCl. This preparation was centrifuged at 10,000 $\times$ *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 30 min at 37°C and then with proteinase K (100 μg/ml; Sigma) at 50°C for a further 30 min. Following this, the DNA was extracted once again with phenol and twice with chloroform. It was then precipitated with isopropanol, dried, and resuspended in TE buffer.

PCR amplification of 16S rDNA. 16S rDNAs were amplified with two primers, namely, 5'-TCACG GAGAG TTTGA TCCTG-3' (in *E. coli* numbering, positions 2 to 21) and 5'-AGAAA GGAGG TGATC-3' (in *E. coli* numbering, positions 1544 to 1530). The PCR mixtures (100 μl) were prepared with 2.5 μl of each of the primers (20 μM), 16 μl of deoxynucleoside triphosphate mix (0.5 mM; dATP, dCTP, dGTP, and dTTP), 1 μl of *Taq* DNA polymerase (5 U: AmpliTaq; Perkin-Elmer), and 10 μl of 10× AmpliTaq buffer (Perkin-Elmer). Chromosomal DNA (ca. 200 ng) was added to this solution, which had been made up to 100 μl with distilled water. The thermal cycler (Perkin-Elmer model 480) used for the thermal amplification was programmed as follows: 35 cycles of

TABLE 2. Numbers of *Saccharomonospora* colonies isolated on R8 agar plates incubated for 14 days

Sample	Temp (°C)	CFU g^{-1}
Hay	30	3.1×10^3
	50	2.7×10^3
Mushroom compost		
Phase I	50	8.30×10^2
Phase II	50	5.70×10^2
End of phase II	50	2.30×10^2
Cooked-out phase	50	4.90×10^2

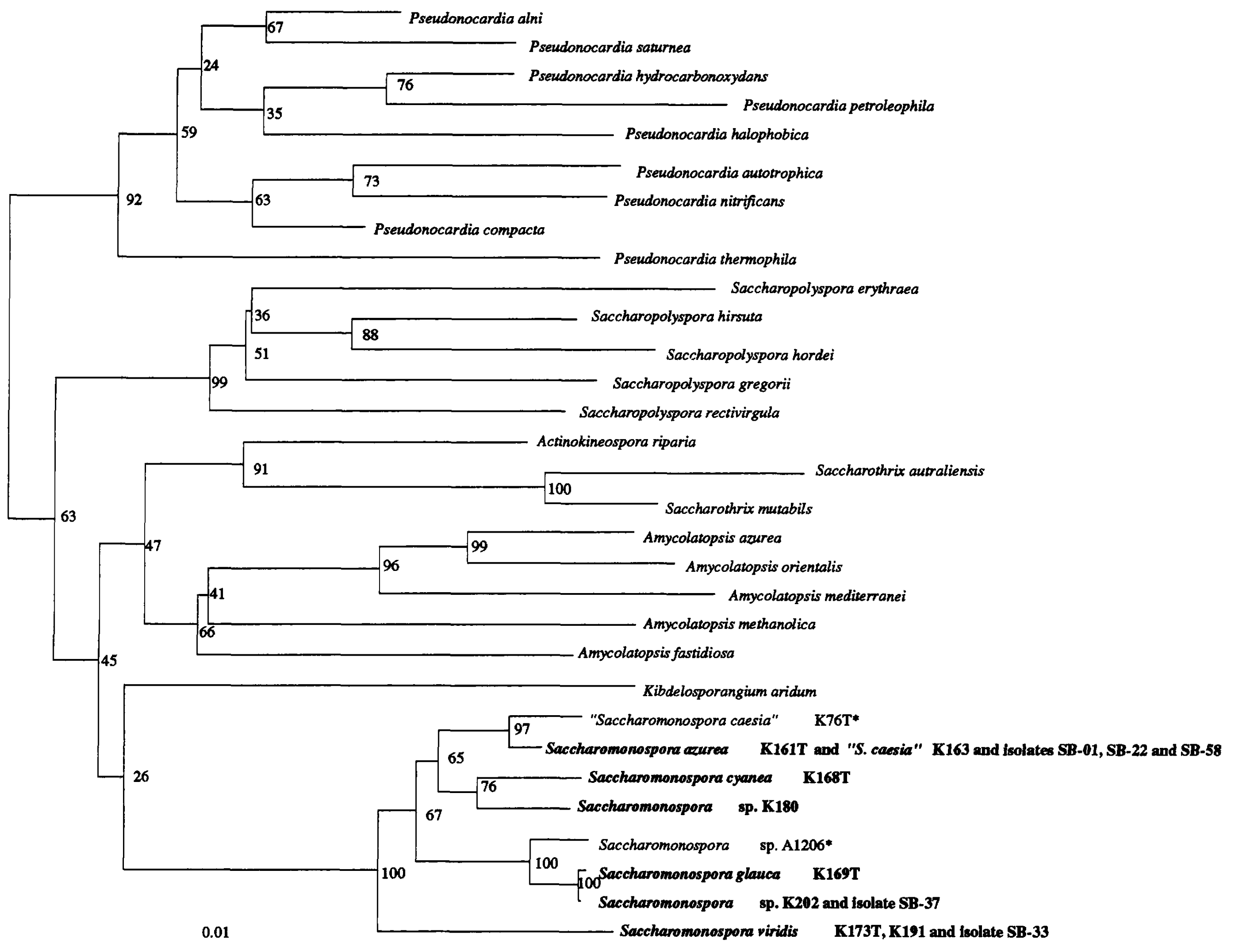

FIG. 1. Phylogenetic tree showing the relationships between different *Saccharomonospora* species and between them and representatives of genera classified in the family *Pseudonocardiaceae*. The tree was generated by the neighbor-joining (34) method. The numbers at the nodes indicate the levels of bootstrap (11) support based on data for 1,000 replicates; the scale bar indicates 0.01 substitution per nucleotide position. *, 16S rRNA data from T. M. Embley.

denaturation at 95°C for 1 min, primer annealing at 50°C for 1 min, and primer extension at 72°C for 2 min.

Cloning of amplified 16S rDNA. Amplified 16S rDNA was recovered from 1% agarose gels in Tris-acetate buffer (0.04 M Tris-acetate, 0.001 M EDTA) and purified by using a Gene Clean II Kit (Bio 101, La Jolla, Calif.). The DNA was dried and dissolved in 10 μl of distilled water. Each preparation of PCR fragments (9 μl) was mixed with 2 μl of pBluescript T vector digested with *Eco*RV, 1.5 μl of 10 mM ATP, 1 μl of T4 DNA ligase, and 1.5 μl of 10× ligase buffer (30 mM Tris-HCl [pH 7.8], 100 mM $MgCl_2$, 100 mM dithiothreitol, and 5 mM ATP) and incubated at 16°C for 16 h. White colonies were selected after transformation to DH5αF′.

Sequencing of double-stranded 16S rDNA. At least three colonies from the same ligation reaction were selected to confirm the final sequence data and were cultured, and plasmid DNA was isolated. Purified plasmids were sequenced with DNA Sequencing Kits (Biochemical Corp.), according to the manufacturer's protocol, using the primers T3 (5′-ATTAACCCTCACTAAAG-3′) and T7 (5′-

TABLE 3. Matrix of similarity values (lower part) and the number of differences (from 1,488 to 1,490 sequences; upper part) of the 16S rDNA sequences of the 13 *Saccharomonospora* strains

Strain identity	K161^{T}	K168^{T}	K169^{T}	K202	K73^{T}	K180
S. azurea K161^{Ta}		24/1,489	29/1,489	28/1,489	55/1,490	18/1,489
S. cyanea K168^{T}	98.4		29/1,489	28/1,489	52/1,488	20/1,489
S. glauca K169^{T}	98.1	98.1		1/1,490	45/1,488	34/1,489
Saccharomonospora sp. strain K202[b]	98.1	98.1	99.9		46/1,488	33/1,489
S. viridis K73^{Tc}	96.3	96.5	97.0	96.9		50/1,488
Saccharomonospora sp. K180	98.8	98.7	97.7	97.8	96.6	

[a] Identical results were obtained for *S. azurea* K161^{T}, "*S. caesia*" K163, and *Saccharomonospora* strains SB-01, SB-22, and SB-58.
[b] Identical results were obtained for *Saccharomonospora* strains K202 and SB-37.
[c] Identical results were obtained for *S. viridis* K73^{T} and K192 and *Saccharomonospora* strain SB-33.

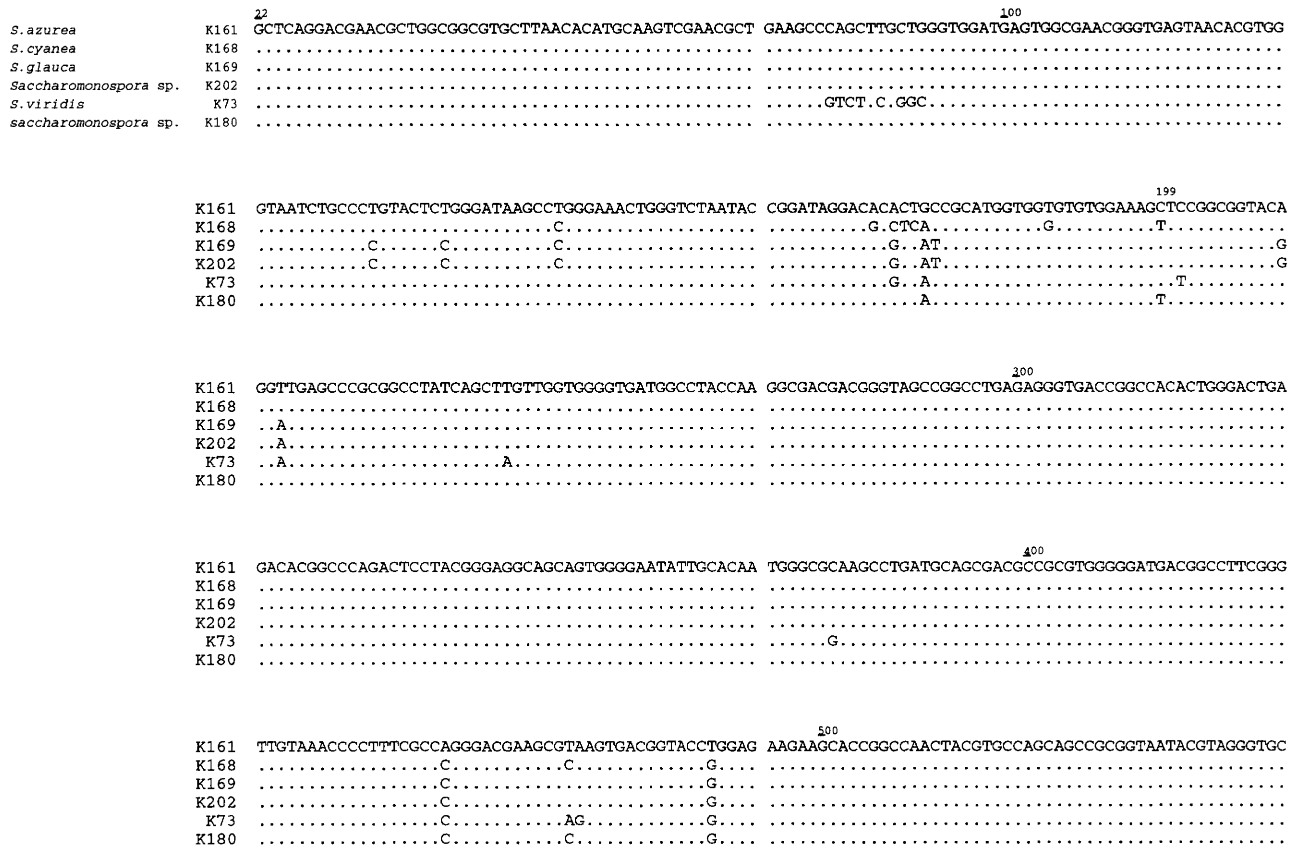

FIG. 2. Aligned 16S rDNA sequences including signatures distinguishing between representatives of the genus *Saccharomonospora*. The numbering system is that of *E. coli* (6).

AATACGACTCACTATAG-3′) of pBluescript II, universal primers (23), and synthesized primers. The samples were electrophoresed in a 6% (wt/vol) polyacrylamide–8.3 M urea gel.

Data analysis. The 16S rDNA sequences of the test strains were aligned manually against corresponding sequences of representative *Pseudonocardiaceae* strains obtained from the Ribosomal Database Project release 4 (24) with the AL16S program (7). Pairwise similarity values were calculated and converted to distances with the algorithm of Jukes and Cantor (19) in the DNADIST program (in PHYLIP, version 3.5c [12]). A phylogenetic tree was generated by the neighbor-joining method (34); *Mycobacterium bovis* BCG was used as the out-group strain. In order to determine the stability of the resultant phylogenetic tree, the sequence data were resampled 1,000 times for bootstrap analysis (11) using the SEQBOOT program (PHYLIP, version 3.5c).

Nucleotide sequence accession numbers. The 16S rDNA sequences determined in this study have been deposited in the EMBL database under the accession numbers listed in Table 1.

RESULTS AND DISCUSSION

Selective isolation of *Saccharomonospora* strains. Saccharomonosporae were isolated from the hay and mushroom compost samples only on the R8 agar plates. The target strains were readily recognized, given their capacity to form the green aerial spore mass characteristic of *Saccharomonospora* strains. The highest number of saccharomonosporae was isolated from the hay sample (Table 2).

16S rDNA sequence analysis. It is evident from the phylogenetic tree (Fig. 1) derived from the nucleotide similarity values (Table 3) that the genus *Saccharomonospora* forms a distinct phyletic line within the evolutionary radiation encompassed by the family *Pseudonocardiaceae* (36). These data lend weight to the results of earlier studies which showed the genus *Saccharomonospora* to be a homogeneous taxon on the basis of chemical, morphological, and molecular systematic evidence (5, 10, 17, 18, 31, 36). The close relationships found between the genus *Saccharomonospora* and the genera *Actinokineospora*, *Amycolatopsis*, *Kibdelosporangium*, and *Saccharothrix* need to be interpreted with care, given the low bootstrap values shown in Fig. 1.

The average nucleotide similarity values found between the type strains of the four validly described *Saccharomonospora* species was 97.5% ± 1.0%. The most distant relationship was found between "*S. caesia*" K163 and *S. viridis* K73^T (96.3% similarity). In contrast, *S. azurea* K161^T and "*S. caesia*" K163 had identical 16S rDNA sequences. Additional taxonomic studies are needed to determine the fine relationship between these taxa, as it is well known that sequence identity may not be sufficient to ensure species identity (13, 14). This is especially so since *S. azurea* K161^T and "*S. caesia*" K76^T are also closely related (Fig. 1). It is interesting that all three strains isolated from the mushroom compost were identical in nucleotide sequences both to one another and to the type strain of *S. azurea*. Similarly, strain SB-33 from hay had a nucleotide sequence identical to that of the type strain of *S. viridis*. The second hay isolate, strain SB-37, had only one nucleotide difference from the sequence shown by the type strain of *S. glauca*.

The present study provides further evidence that the genus *Saccharomonospora* encompasses undescribed species (15, 27, 36). The separation of *Saccharomonospora* sp. strain A1206 from the other test strains (Fig. 1) confirms and extends the earlier work of Warwick et al. (36). *Saccharomonospora* sp. strain K180 also forms the nucleus of a distinct center of variation. However, additional comparative taxonomic studies

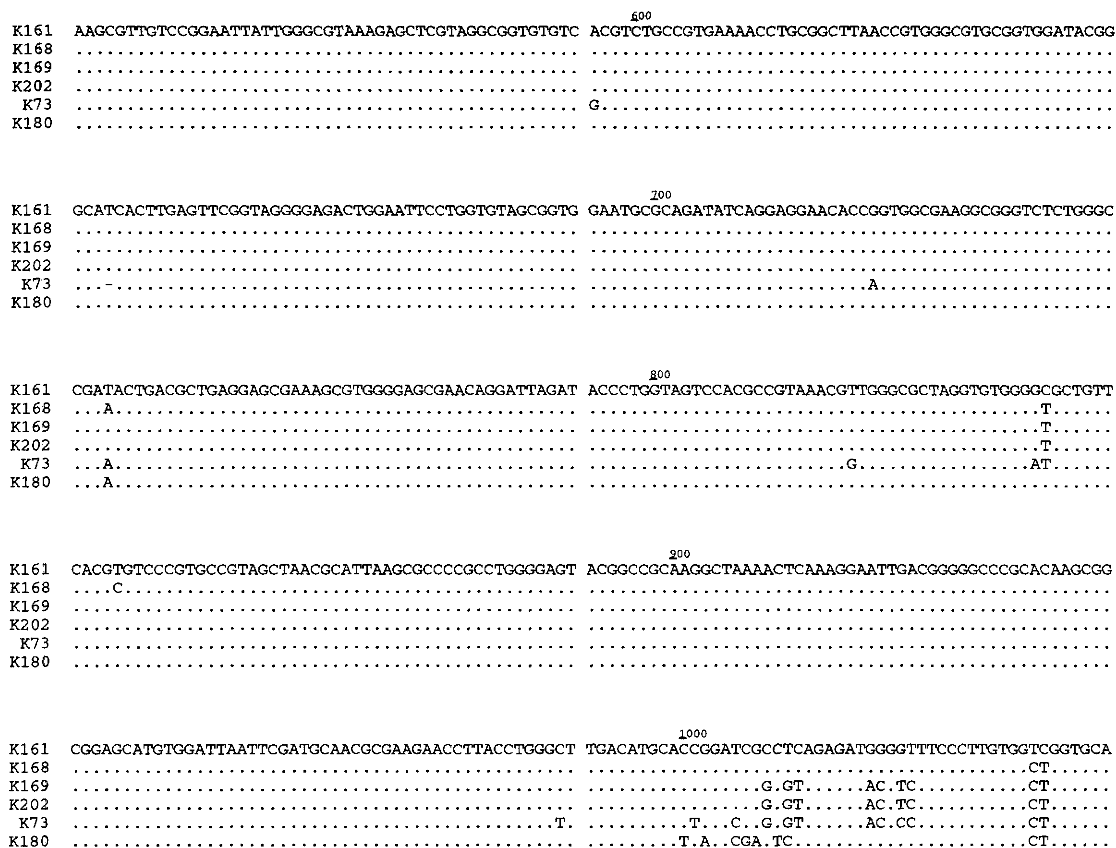

FIG. 2—*Continued.*

are needed to confirm the taxonomic status of these strains, as it is now generally accepted that the most stable and useful classifications are ones that are based on a combination of genotypic and phenotypic data (29).

The separation of validly described and putatively novel *Saccharomonospora* species on the basis of 16S rDNA sequence data not only is important in its own right but also opens up the prospect of developing PCR or oligonucleotide probes for identifying unknown saccharomonosporae from environmental samples and clinical material (4, 8). 16S rDNA nucleotide signatures that distinguish representatives of the test strains are shown in Fig. 2.

The present investigation provides additional evidence of the value of 16S rDNA sequence studies in underpinning the integrity of actinomycete genera and in clarifying relationships within such taxa. This approach together with the application of chemical, morphological, and other molecular systematic techniques can be confidently expected to fuel further advances in actinomycete systematics.

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