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Inter- and Intraspecific Genetic Analysis of the Genus *Saccharomonospora* with 16S to 23S Ribosomal DNA (rDNA) and 23S to 5S rDNA Internally Transcribed Spacer Sequences

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In order to clarify interspecific relationships and to investigate the intraspecific phylogenetic structure of the genus *Saccharomonospora*, 16S to 23S ribosomal DNA (16S-23S) and 23S to 5S ribosomal DNA (23S-5S) internally transcribed spacers (ITSs) were used for sequence analyses. The 16S-23S and 23S-5S ITSs from 22 *Saccharomonospora* strains were amplified by PCR and directly sequenced. The average levels of nucleotide similarity of the 16S-23S and 23S-5S ITSs for the four valid species were 87.6% ± 3.9% and 83% ± 2.2%, respectively. For the most part, intraspecific sequence differences were not found in the two ITSs; the only exception was *Saccharomonospora glauca* K194, which differed from other *S. glauca* strains by 1 bp in the 23S-5S ITS. The *Saccharomonospora viridis* strains had a smaller 16S-23S ITS region than the other strains, which may be useful for differentiating these organisms from other *Saccharomonospora* species. The characteristics of the two ITS regions make them more useful than 16S rRNA sequences as a tool for defining and identifying *Saccharomonospora* strains. However, *Saccharomonospora azurea* K161^T had two types of 23S-5S ITSs; *rrnB*, separated by *Xho*I digestion, had two additional nucleotides inserted between positions 52 and 55. Most of the 16S-23S and 23S-5S ITS sequences of *S. azurea* K161^T and strains of "*Saccharomonospora caesia*" were identical; the only exception was *rrnB* in *S. azurea* K161^T. The lengths and levels of sequence divergence of the two ITSs of *Saccharomonospora* sp. strain K180 were different from the lengths and levels of sequence divergence of the ITSs of other species. These findings suggest that a taxonomic revision of the genus *Saccharomonospora* is necessary. Two trees based on 16S-23S and 23S-5S ITS sequences revealed distinct interspecific relationships in the genus *Saccharomonospora*.

The genus *Saccharomonospora* was proposed by Nonomura and Ohara (20) for monosporic actinomycetes containing *meso*-diaminopimelic acid, arabinose, and galactose in the cell wall peptidoglycan (wall chemotype IV sensu Lechevalier and Lechevalier [18]). Representatives of this genus form nonfragmenting, branched mycelia and develop aerial hyphae that bear single spores. The additional physical and chemical markers that characterize the genus have been described elsewhere (12, 39).

The original type species of the genus is *Saccharomonospora viridis* (20). In addition to *S. viridis*, three *Saccharomonospora* species, *Saccharomonospora azurea* (24), *Saccharomonospora cyanea* (25), and *Saccharomonospora glauca* (7), have been validly described. Although "*Saccharomonospora caesia*" was proposed by Greiner-Mai et al. (8) as a fifth taxon for strains previously classified as *Micropolyspora caesia* (9, 13), this organism was not included on the Approved Lists of Bacterial Names (28) and has not been validly published on subsequent Approved Lists or Validation Lists. The results of recent numerical phenetic studies (11) and results obtained by nucleic acid techniques (12, 39–41) suggest that the strains of "*S. caesia*" are very closely related to *S. azurea* K161^T. It has been determined that strains of *S. viridis* cause farmer's lung disease (1, 6) and are significant agents of hypersensitivity pneumonitis (14).

Recently, a 16S ribosomal DNA (rDNA) sequence analysis showed that the genus *Saccharomonospora* is a distinct phyletic group within the evolutionary radiation of the family *Pseudonocardiaceae* (12). However, the 16S rDNA sequences of the four validly described *Saccharomonospora* species exhibited relatively high levels of nucleotide similarity (97.5% ± 1.0%), and the levels of homology between strains of the same species were high. The 16S rDNAs of most *Saccharomonospora* species were more than 98% similar; the only exception was *S. viridis* 16S rDNA. *S. azurea* K161^T and "*S. caesia*" strains had identical 16S rDNA sequences. Intraspecific sequence variations were not found in *S. viridis* and "*S. caesia*," and the 16S rDNA sequence of the type strain of *S. glauca* differed by only one nucleotide from the 16S rDNA sequences of other *S. glauca* strains. In addition, *Saccharomonospora* sp. strain K180 had a sequence that was different from the sequences of the four validly described *Saccharomonospora* species. Currently, a level of 16S rRNA sequence similarity of 97% is recognized as the threshold value for species definition in bacteriology (30). The high levels of nucleotide similarity obtained for the 16S rDNA sequences of *Saccharomonospora* species make defining species and consequently describing new species difficult. These values also make describing the phylogenetic relationships between closely related organisms difficult. Therefore, it is necessary to use additional variable genetic markers to clarify interspecific relationships and to investigate intraspecific phylogenetic relationships in the genus *Saccharomonospora*. These markers are also valuable in polyphasic taxonomic studies (36) for describing new species. For these reasons, 16S to

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TABLE 1. Strains used in this study and accession numbers of 16S-23S and 23S-5S ITS

Laboratory no.	Species	Source and/or other designation(s)[a]	16S-23S ITS accession no.	23S-5S ITS accession no.
K161^T	*Saccharomonospora azurea*	H. Runmao, NA-128 (= SIIA 86128)	U73397	U73398 (*rrnA* and *rrnC*), U73399 (*rrnB*)
K76^T	"*Saccharomonospora caesia*"	KCTC 9152	U73428	U73429
K163	"*Saccharomonospora caesia*"	DSM 43068	U73400	U73401
K182	"*Saccharomonospora caesia*"	E. Greiner-Mai, Kol8	U73410	U73411
K200	"*Saccharomonospora caesia*"	J. Lacey, A1932	U73422	U73423
SB-01	"*Saccharomonospora caesia*"	S.-B. Kim	U73430	U73431
SB-22	"*Saccharomonospora caesia*"	S.-B. Kim	U73432	U73433
SB-58	"*Saccharomonospora caesia*"	S.-B. Kim	U73440	U73441
K168^T	*Saccharomonospora cyanea*	H. Runmao, NA-134 (= SIIA 86134)	U73402	U73403
K169^T	*Saccharomonospora glauca*	DSM 43769	U73404	U73405
K179	*Saccharomonospora glauca*	A. J. McCarthy, BD-125	U73406	U73407
K194	*Saccharomonospora glauca*	J. Lacey, A66	U73416	U73417
K195	*Saccharomonospora glauca*	J. Lacey, A1450	U73418	U73419
K202	*Saccharomonospora glauca*	J. Ruan, 350	U73424	U73425
SB-37	*Saccharomonospora glauca*	S.-B. Kim	U73438	U73439
K73^T	*Saccharomonospora viridis*	NCIB 9602	U73426	U73427
K185	*Saccharomonospora viridis*	E. Greiner-Mai, Llv	U73412	U73413
K191	*Saccharomonospora viridis*	E. Greiner-Mai, R25	U73414	U73415
K197	*Saccharomonospora viridis*	J. Lacey, A1905	U73420	U73421
SB-31	*Saccharomonospora viridis*	S.-B. Kim	U73434	U73435
SB-33	*Saccharomonospora viridis*	S.-B. Kim	U73436	U73437
K180	*Saccharomonospora* sp.	A. J. McCarthy	U73408	U73409

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

23S rDNA (16S-23S) and 23S to 5S rDNA (23S-5S) internally transcribed spacer (ITS) regions were studied.

With some exceptions (15, 33, 35), most rRNA gene loci of prokaryotes are arranged in the order 16S-23S-5S, and each rRNA gene is separated by intergenic spacer regions and flanking regions (29). Recently, PCR techniques and recognition of the existence of conserved regions in each rRNA gene have made it possible to amplify intergenic spacer regions. In particular, the sequence variability of the 16S-23S intergenic spacer region has been shown to be useful in typing, restriction fragment length polymorphism (RFLP), and sequence analyses performed to differentiate between bacterial species and between strains belonging to the same species (4, 17, 19).

In this study, two types of 23S-5S ITS region were found in *S. azurea* K161^T; that is, two additional nucleotide-inserted 23S-5S ITSs were shown to exist in a certain rRNA gene cluster. Strains belonging to the genus *Saccharomonospora* were shown to have three copies of the rRNA gene cluster on their genomes (39). It was important to investigate which of the three rRNA gene clusters of *S. azurea* K161^T have additional nucleotides in order to determine the evolutionary relationship between *S. azurea* K161^T and strains of "*S. caesia*." It was possible to separate the three rRNA gene clusters on an agarose gel by using previously described ribotype patterns (39).

The principal aims of this study were to make more distinct taxonomic differentiation between *Saccharomonospora* species possible and to examine intraspecific genetic relationships by performing a 16S-23S and 23S-5S ITS sequence analysis. Another aim was to determine the relationship between *S. azurea* K161^T and "*S. caesia*" strains and the taxonomic position of *Saccharomonospora* sp. strain K180 by performing ITS sequence analyses. An additional objective was to compare the evolutionary status of *S. azurea* K161^T and strains of "*S. caesia*" by using the heterogeneity found in the 23S-5S ITS regions of these organisms.

MATERIALS AND METHODS

Organisms and culture conditions. 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. The broth cultures were checked for purity before they were harvested by centrifugation. Table 1 shows the strain designations and the GenBank accession numbers for the 16S-23S and 23S-5S ITS sequences. The sources of strains have been described previously (12, 39).

Isolation of chromosomal DNAs. Chromosomal DNAs were isolated by a previously described method (39).

PCR amplification of 16S-23S and 23S-5S ITS regions. The PCR primers used for amplification of DNA fragments containing the 16S-23S intergenic spacer were selected from sequences corresponding to the conserved region of 16S rDNA and the 5′ region of 23S rDNA. The oligonucleotide primer annealing to 16S rDNA (primer 16SF) was designed by using a highly conserved region described previously (16), and its sequence was 5′-CAGCMGCCGCGGTA ATSC-3′ (positions 519 to 536 [*Escherichia coli* 16S rRNA numbering]). The oligonucleotide primer annealing at the region close to the 5′ end of 23S rDNA (primer 23SR) was designed by using 5′ sequences of the 23S rRNA genes of *Bacillus subtilis* (5), *Frankia* sp. (21), and some *Streptomyces* species (10, 22, 31), and its sequence was 5′-AGGCATCCACCGTGCGCCCT-3′ (positions 34 to 14 [*E. coli* 23S rRNA numbering]). The PCR product containing the 23S-5S intergenic spacer was amplified with primers corresponding to the conserved regions of 23S rDNA and 5S rDNA. The sequence of the oligonucleotide primer annealing to 23S rDNA (primer 23SF) was 5′-GCGAAATTCCTTGTCGGGTA-3′ (positions 1933 to 1952 [*E. coli* 23S rRNA numbering]), which was shown to be conserved from an alignment of the 23S rRNA sequences of *B. subtilis* (5), *Frankia* sp. (21), and some *Streptomyces* species (10, 22, 31). The sequence of the oligonucleotide primer annealing to 5S rDNA (primer 5SR) was 5′-TGTCCTA CTCTCCCAC-3′ (positions 109 to 94 [*E. coli* 5S rRNA numbering]), and this primer was selected from an alignment of 5S rRNA sequences of *Saccharomonospora* species obtained from the GenBank database. The 5′ ends of primers 16SF and 5SR were phosphorylated by using T4 polynucleotide kinase (New England Biolabs, Inc., Beverly, Mass.). Each PCR 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 performed for 35 cycles with a DNA thermal cycler (model 480; Perkin-Elmer Co.) under the following conditions; denaturation at 94°C for 1 min, primer annealing at 55°C for 1 min, and extension at 72°C for 2 min. The final cycle included an additional 10 min of extension at 72°C. Following the PCR, each reaction tube was frozen at −70°C in a deep freezer for 10 min, and then the mineral oil layer was removed with a pipette. The PCR mixture was transferred to a new tube. The

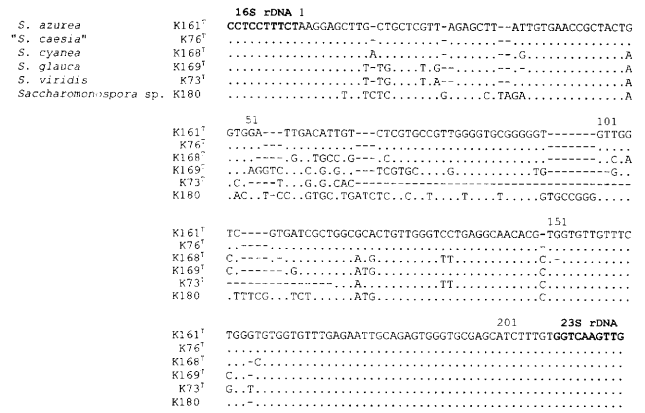

FIG. 1. Alignment of the 16S-23S ITS sequences of representative strains of the genus *Saccharomonospora*.

PCR product was precipitated with 9 μl of 3 M sodium acetate (pH 5.2) and 70 μl of isopropanol and resuspended in 10 μl of distilled water.

Sequencing of the 16S-23S and 23S-5S ITS regions. The strand containing phosphorylated primer from the PCR product was selectively digested by using λ exonuclease (Novagen, Inc., Madison, Wis.). The single-stranded DNA template produced was used directly for sequencing. Sequencing was performed as described previously (12) by using α-^{35}S-labeled dATP and a DNA sequencing kit (U.S. Biochemicals, Cleveland, Ohio). The primer used to sequence the 16S-23S intergenic spacer was designed by using a conserved region described previously (16), and its sequence was 5′-GYACACACCGCCCGT-3′ (positions 1392 to 1406 [*E. coli* 16S rRNA numbering]). The primer used to sequence the 23S-5S intergenic spacer was 5SR.

Cloning and sequencing of the 23S-5S ITS-containing PCR product from *S. azurea* K161^T and "*S. caesia*" K76^T. Cloning and sequencing of the PCR product were carried out by using a previously described method (12). The 23S-5S intergenic spacer regions of *S. azurea* and "*S. caesia*" were sequenced by using five clones.

Southern hybridization with *S. azurea* K161^T genomic DNA fragments and 23S-5S ITS probe. Digestion of *S. azurea* K161^T chromosomal DNA, electrophoresis, Southern blotting, and hybridization were performed as described previously (39), except that we used the 23S-5S ITS-containing PCR product as the probe.

Recovery of DNA fragments containing three rRNA gene clusters from *S. azurea* K161^T. Chromosomal DNA was digested overnight with *Xho*I as recommended by the manufacturer (New England Biolabs, Inc.). Restriction fragments were separated by electrophoresis on a 0.7% (wt/vol) agarose gel in Tris-acetate buffer (0.04 M Tris-acetate, 0.001 M EDTA; pH 8.3). The positions of three gel fractions containing each rRNA gene cluster were determined from the previously determined *Xho*I ribotype pattern. DNA fragments in gel slices were recovered by electroelution (27) and were purified by phenol-chloroform extraction, chloroform-isoamyl alcohol extraction, and ethanol precipitation.

Sequencing of the 23S-5S ITS regions from three rRNA gene clusters of *S. azurea* K161^T. PCR amplification and sequencing of three 23S-5S spacer regions were performed by using the methods described above.

Data analysis. The sequences of the 16S-23S and 23S-5S ITSs were aligned by using CLUSTAL W software (34), and then the alignments were manually corrected. The overall similarity values and similarity values based on data that included nucleotide gaps were manually calculated from the alignments. Trees were constructed by the neighbor-joining method (26) from a distance matrix calculated with the CLUSTAL W software. The topologies of the trees were evaluated by performing a bootstrap analysis (100 replications) of the sequence data with CLUSTAL W software.

Nucleotide sequence accession numbers. 16S-23S and 23S-5S ITS sequences have been deposited in the GenBank data library under the accession numbers shown in Table 1.

RESULTS

Sequence analysis of 16S-23S ITS regions. The 16S-23S intergenic spacer regions of *S. azurea* K161^T, "*S. caesia*" strains, *S. cyanea* K168^T, *S. glauca* strains, *S. viridis* strains, and *Saccharomonospora* sp. strain K180 (Fig. 1) were 185, 185, 183, 182, 144, and 205 bp long, respectively (Table 2). The 5′ and 3′ ends of the 16S-23S ITS regions were deduced from the 3′ ends of the 16S rDNAs and the 5′ ends of the 23S rDNAs of *B. subtilis* (5), *Frankia* sp. (21), and some *Streptomyces* species (2, 10, 22, 31, 37) described previously. The data show that *S. viridis* strains have the shortest 16S-23S ITS regions. In contrast, *Saccharomonospora* sp. strain K180 has the longest 16S-23S ITS region. It is interesting that intraspecific sequence variation was not found in any *Saccharomonospora* strains except single strains of *S. azurea* and *S. cyanea* and *Saccharomonospora* sp. strain K180. In addition, *S. azurea* K161^T and the seven strains of "*S. caesia*" had identical 16S-23S ITS sequences.

The average level of nucleotide similarity for the 16S-23S ITS regions of the four validly described *Saccharomonospora* species was 87.6% ± 3.9%. The most distant relationship was the relationship between *S. glauca* and *S. cyanea*, for which a level of similarity of 83.7% was obtained (Table 3). The average level of nucleotide similarity including gap sites for the four validly described *Saccharomonospora* species was 77.1% ± 10.6%. The most distant relationship was the relationship between *S. viridis* and *Saccharomonospora* sp. strain K180, for which a level of similarity of 59.2% was obtained (Table 3). When *Saccharomonospora* sp. strain K180 was compared with the four validly described *Saccharomonospora* species, the average level of nucleotide similarity and the average level of similarity when gaps were included were 84.5% ± 0.9% and 67.5% ± 8.3%, respectively (Table 3).

The tree constructed by using 16S-23S ITS sequences shows the inter- and intraspecific genealogical relationships of the *Saccharomonospora* species (Fig. 2). The four validly described *Saccharomonospora* species and *Saccharomonospora* sp. strain K180 formed distinct genetic lineages on the tree based on 16S-23S ITS sequences. The branching pattern makes it possible to exactly classify or identify *Saccharomonospora* strains. While *Saccharomonospora* sp. strain K180 was phylogenetically related to *S. cyanea* K168^T on the basis of 16S rDNA sequence analysis data (12), the 16S-23S ITS sequence-based tree showed that *Saccharomonospora* sp. strain K180 forms a distinct genetic lineage that is separated from the four valid species and "*S. caesia*" strains.

Sequence analysis of 23S-5S ITS regions. The 23S-5S intergenic spacer regions of *S. azurea* K161^T, "*S. caesia*" strains, *S. cyanea* K168^T, *S. glauca* strains, *S. viridis* strains, and *Saccharomonospora* sp. strain K180 were 73 (or 75), 73, 75, 69, 74, and 60 bp long, respectively (Table 2). The 5′ end of the 23S-5S ITS was deduced from the 3′ ends of the 23S rDNAs of *B. subtilis* (5), *Frankia* sp. (21), and some *Streptomyces* sp. (10, 22, 23) described previously. The 3′ end of the 23S-5S ITS was determined from the 5′ ends of the 5S rRNA sequences of *Saccharomonospora* strains obtained from a nucleotide sequence database. *Saccharomonospora* sp. strain K180 had a shorter 23S-5S ITS region than the other *Saccharomonospora* strains

TABLE 2. Sizes of 16S-23S and 23S-5S ITSs of *Saccharomonospora* strains

Strain(s)	Size of 16S-23S ITS (bp)	Size of 23S-5S ITS (bp)
S. azurea K161^T	185	73 (or 75)
"*S. caesia*" K76^T, K163, K182, K200, SB-01, SB-22, and SB-58	185	73
S. cyanea K168^T	183	75
S. glauca K169^T, K179, K194, K195, K202, and SB-37	182	69
S. viridis K73^T, K185, K191, K197, SB-31, and SB-33	144	74
Saccharomonospora sp. strain K180	205	60

TABLE 3. Levels of nucleotide similarity and levels of nucleotide similarity with gaps included for 16S-23S ITS sequences of *Saccharomonospora* strains

Strain	% Similarity to[a]:					
	S. azurea K161^T	"*S. caesia*" K76^T	*S. cyanea* K168^T	*S. glauca* K169^T	*S. viridis* K73^T	*Saccharomonospora* sp. strain K180
S. azurea K161^T		100 (185/185)	87.6 (163/186)	81.0 (153/189)	67.2 (125/186)	75.7 (156/206)
"*S. caesia*" K76^{Tb}	100 (185/185)		87.6 (163/186)	81.0 (153/189)	67.2 (125/186)	75.7 (156/206)
S. cyanea K168^T	89.6 (163/182)	89.6 (163/182)		79.7 (149/187)	66.5 (123/185)	74.6 (153/205)
S. glauca K169^{Tc}	86.0 (153/178)	86.0 (163/182)	83.7 (149/178)		70.7 (130/184)	75.6 (155/205)
S. viridis K73^{Td}	87.4 (125/143)	87.4 (125/143)	86.6 (123/142)	91.5 (130/142)		59.2 (122/206)
Saccharomonospora sp. strain K180	84.8 (156/184)	84.8 (156/184)	83.6 (153/183)	85.1 (155/182)	85.3 (122/143)	

[a] The values on the lower left are levels of nucleotide similarity, and the values on the upper right are levels of nucleotide similarity with gaps included. The values in parentheses are number of nucleotides that are identical/number of nucleotides examined.
[b] Identical results were obtained for "*S. caesia*" K76^T, K163, K182, K200, SB-01, SB-22, and SB-58.
[c] Identical results were obtained for *S. glauca* K169^T, K179, K194, K195, K202, and SB-37.
[d] Identical results were obtained for *S. viridis* K73^T, K185, K191, K197, SB-31, and SB-33.

(Table 2), although it had a longer 16S-23S ITS. Intraspecific size and sequence variations of the 23S-5S ITS regions were not observed in "*S. caesia*" and *S. viridis*. Similarly, for the most part *S. glauca* strains exhibited no intraspecific variation in the 23S-5S ITS; the only exception was *S. glauca* K194, which differed from other *S. glauca* strains by 1 bp (Fig. 3).

Conversely, heterogeneity in the 23S-5S ITS region within an organism was observed in *S. azurea* K161^T; that is, *S. azurea* K161^T was found to have two types of 23S-5S ITS region when we performed a sequence analysis of five clones containing the 23S-5S ITS region (Fig. 4). Three *rrn* loci were separated by *Xho*I digestion (Fig. 5), and *rrnB* was found to have a 23S-5S ITS with two additional nucleotides between positions 52 and 55 (Fig. 3 and 4). The 23S-5S ITS sequences of *rrnA* and *rrnC* from *S. azurea* K161^T were identical to the 23S-5S ITS sequences of the seven strains of "*S. caesia*" examined (Fig. 3).

The average level of nucleotide similarity for the four validly described *Saccharomonospora* species was 83% ± 2.2%, and the most distant relationship was the relationship between *S. azurea* K161^T and *S. cyanea* K168^T, which exhibited a level of similarity of 80.8% (Table 4). The average level of nucleotide similarity when gap sites were included for the four validly described *Saccharomonospora* species was 80% ± 4.0%, and the most distant relationship was the relationship between *S. glauca* K194 and *S. cyanea* K168^T, which exhibited a level of similarity of 76% (Table 4). *Saccharomonospora* sp. strain K180 exhibited an average level of similarity of 90% ± 1.7% and a level of similarity with gaps included of 75.2% ± 3.2% when it was compared with the four validly described *Saccharomonospora* species (Table 4).

Figure 6 is a tree which shows the inter- and intraspecific genetic relationships of the members of the genus *Saccharomonospora* as determined by using 23S-5S ITS sequences. Although the positions of the species that formed a branching lineage on the 23S-5S ITS-derived tree were different from the positions on the tree based on 16S-23S ITS sequences, it is still true that the four validly described *Saccharomonospora* species formed a distinct genetic lineage which was consistent with the 16S-23S ITS tree. *Saccharomonospora* sp. strain K180 was found to be more closely related to other species in this analysis than when two other species were compared to each other. This could be explained by the results of an analysis of the alignment of the 23S-5S ITS sequences (Fig. 3). *Saccharomonospora* sp. strain K180 has a shorter 23S-5S ITS region than other *Saccharomonospora* species, and deletions of nucleotides are concentrated in the variable region between positions 43 and 63 (Fig. 3). Since nucleotide gaps were not included in the distance calculation, *Saccharomonospora* sp. strain K180 was found to have a close relationship with other species. *S. glauca* K194 is the only strain which exhibited intraspecific sequence divergence, but this strain does not confuse the species boundary between *S. glauca* and other *Saccharomonospora* species.

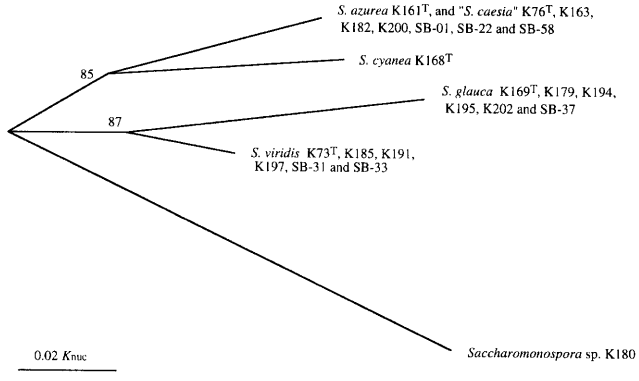

FIG. 2. Unrooted tree showing the genetic relationships of *Saccharomonospora* strains based on 16S-23S ITS sequences. Scale bar = 2 inferred nucleotide substitutions per 100 nucleotides.

DISCUSSION

The PCR was used to amplify the DNA fragment containing the intergenic spacer between the 16S rDNA and the 23S rDNA (16S-23S ITS) and the DNA fragment containing the intergenic spacer between the 23S rDNA and the 5S rDNA

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                                    23S rDNA 1
S. azurea   K161T rrnA & B  GCTTGACCTACAAAGATGCTACGCACCCACTCTGCAACTCTGAAACACC
S. azurea   K161T rrnC      ....................................................
"S. caesia"          K76T   ....................................................
S. cyanea            K168T  .....C..-.....C...................................
S. glauca            K169T  ........-.......C.................G.................
S. glauca            K194   ........-.......C.................G.................
S. viridis           K73T   ........-......G...................................
Saccharomonospora sp. K180  ........-.....C...................................

                                          51                          5S rDNA
            K161T rrnA & B  ACACCCCGGAAAC--GATCCTG--GGTTGTTTCACAGTGTTTCGGTGG
            K161T rrnC      .............AT.......--.........................
                     K76T   .............--.......--.........................
                     K168T  ..C..GG.A.C..--ACC.GGCGT.A.......C...A...........
                     K169T  ..C.TGGT.TGC.--AG.------.............A...........
                     K194   ....TGGT.TGC.--AG.------.............A...........
                     K73T   .......CAC...--ACCGGGC-T.............A...........
                     K180   ...---------.--AC.------.............AC...A...C..
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FIG. 3. Alignment of the 23S-5S ITS sequences of representative strains of the genus *Saccharomonospora*.

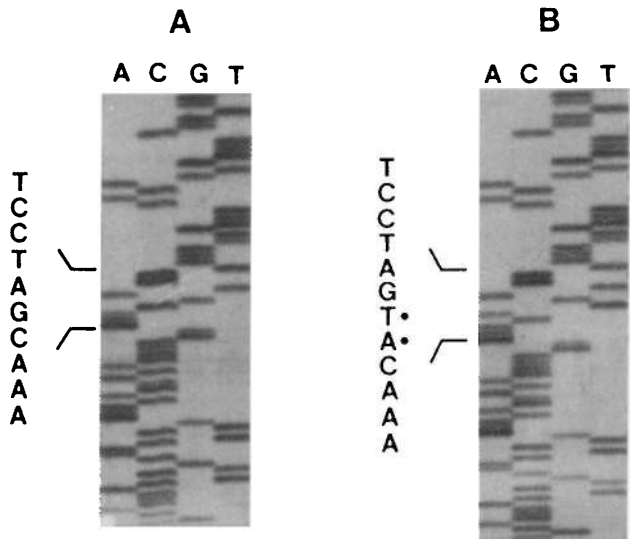

FIG. 4. Nucleotide sequences of 3′ regions from two types of 23S-5S ITSs found in *S. azurea* K161^T. (A) *rrnA* and *rrnC*. (B) *rrnB*. The dots indicate two inserted nucleotides found between positions 52 and 55 of *rrnB*.

(23S-5S ITS) from 22 *Saccharomonospora* strains. The primers used for amplification of the two ITSs were designed by using conserved regions of three rDNAs. The forward primers annealing to 16S and 23S rDNAs were selected from a conserved region far from the 3′ end of each rDNA. Although the conserved sequences are in regions close to 3′ ends of the 16S and 23S rDNAs, the forward primers designed by using these regions also produced nonspecific secondary PCR products. These secondary PCR products were thought to affect direct sequencing of the two ITSs.

All of the strains yielded one PCR product for each 16S-23S ITS and 23S-5S ITS as determined by agarose gel electrophoresis of PCR amplicons (data not shown). However, in a subsequent sequence analysis, one strain, *S. azurea* K161^T was found to have two types of 23S-5S ITS (Fig. 4). In a previous study, it was shown that *Saccharomonospora* strains have three rRNA gene clusters on their genomes (39). The presence of single PCR amplicons on the agarose gel suggests that 16S-23S ITSs and 23S-5S ITSs from the three *rrn* loci of each strain are identical or very similar. The presence of one PCR product of the 16S-23S ITS has also been described for some *Bifidobacterium* species (17). However, electrophoresis of the 16S-23S ITS PCR products of *Staphylococcus aureus* strains revealed 6 to 11 bands, some of which may have been secondary PCR products (4).

Most *Saccharomonospora* strains were found to have a 16S-23S ITS and a 23S-5S ITS which were similar in size, as determined by agarose gel electrophoresis; the only exceptions were *S. viridis* strains, which had 16S-23S ITS PCR amplicons that were smaller than those of other species (data not shown). Strains of *S. viridis* have been implicated as causal agents of farmer's lung disease (1, 6). It is, therefore, important to distinguish *S. viridis* from other *Saccharomonospora* species. The smaller 16S-23S ITS found in *S. viridis* strains is a trait that is useful for differentiating these organisms from other *Saccharomonospora* strains. Although *Saccharomonospora* sp. strain K180 was determined to have a slightly longer 16S-23S ITS and a shorter 23S-5S ITS than other *Saccharomonospora* strains by sequence analysis, agarose gel electrophoresis did not result in resolution of these products from the two ITS-containing PCR products of species other than *S. viridis*.

The nucleotide sequences of the 16S-23S ITS and 23S-5S ITS regions were determined by directly sequencing nonphosphorylated strands of PCR-amplified fragments whose 5′-phosphorylated strands were selectively digested by λ exonuclease. The length of the 16S-23S ITS ranged from 144 to 205 bp and varied only a small amount in *Saccharomonospora* strains other than *S. viridis* strains and *Saccharomonospora* sp. strain K180 (Table 2). The 16S-23S ITSs of *Saccharomonospora* species are smaller than those of other microorganisms. The 16S-23S ITS of *E. coli*, including tRNA (3), is 400 bp long; the 16S-23S ITSs of some *Bifidobacterium* species, not including tRNA (17), are 274 to 552 bp long; and the 16S-23S ITSs of some *Streptomyces* species, not including tRNA (2, 10, 22, 31), are 277 to 304 bp long. This means that despite the small size of the 16S-23S ITS of *Saccharomonospora* species, it is sufficient for processing 16S rRNA and 23S rRNA from the primary rRNA transcript. *S. viridis* had a smaller 16S-23S ITS region (144 bp) than other species, as determined by agarose gel electrophoresis of the PCR amplicon. *S. viridis* strains could be differentiated from their positions on the phylogenetic tree based on 16S rRNA sequences (12).

It is thought that due to their small size, tRNA-like structures do not exist in the 16S-23S ITS regions of *Saccharomonospora* species, although secondary-structure models were not constructed by using 16S-23S ITS sequences. The results of comparisons with previously published tRNA sequences (38) also suggested that tRNA sequences are not found in the 16S-23S ITS regions of *Saccharomonospora* strains. This is consistent with previous results which showed that no tRNA gene occurred in the 16S-23S ITS sequences of *Frankia* sp. (21), some *Streptomyces* species (2, 10, 22, 31), and some *Bifidobacterium* species (17).

"*S. caesia*," *S. glauca*, and *S. viridis* did not exhibit intraspecific length polymorphisms and sequence divergence in their 16S-23S ITSs (Table 3). The 16S-23S ITS is known to have a high rate of evolution, resulting from multiple substitutions at a nucleotide position and a high frequency of insertion and deletion (17). These properties of the 16S-23S ITS have made it a useful molecule for investigating intraspecific relationships,

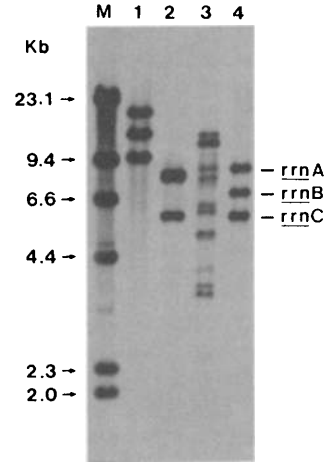

FIG. 5. RFLP patterns generated from DNA digests of *S. azurea* K161^T hybridized with a 23S-5S ITS-containing DNA fragment probe. Lane M, molecular weight markers (restriction fragments of bacteriophage λ DNA digested with *Hin*dIII); lane 1, *Bam*HI digest; lane 2, *Sal*I digest; lane 3, *Pvu*II digest; lane 4, *Xho*I digest.

TABLE 4. Levels of nucleotide similarity and levels of nucleotide similarity with gaps included for 23S-5S ITS sequences of *Saccharomonospora* strains

Strain or sequence(s)	% Similarity to[a]:							
	S. azurea K161^T *rrnA* and *rrnC*	*S. azurea* K161^T *rrnB*	*"S. caesia"* K76^T	*S. cyanea* K168^T	*S. glauca* K169^T	*S. glauca* K194	*S. viridis* K73^T	*Saccharomonospora* sp. strain K180
S. azurea K161^T *rrnA* and *rrnB*		97.3 (73/75)	100 (73/73)	78.7 (59/75)	76.7 (56/73)	78.1 (57/73)	82.4 (61/74)	75.3 (55/73)
S. azurea K161^T *rrnC*	100 (73/73)		97.3 (73/75)	76.6 (59/77)	74.7 (56/75)	76.0 (59/75)	80.3 (61/76)	73.3 (55/75)
"S. caesia" K76^{Tb}	100 (73/73)	100 (73/73)		78.7 (59/75)	76.7 (56/73)	78.1 (57/73)	82.4 (61/74)	75.3 (55/73)
S. cyanea K168^T	80.8 (59/73)	80.8 (59/73)	80.8 (59/73)		77.3 (58/75)	76.0 (57/75)	84.0 (63/75)	72.0 (54/75)
S. glauca K169^{Tc}	81.2 (56/69)	81.2 (56/69)	81.2 (56/69)	84.1 (58/69)		98.6 (68/69)	78.4 (58/74)	76.8 (53/69)
S. glauca K194	82.6 (57/69)	82.6 (57/69)	82.6 (57/69)	82.6 (57/69)	98.6 (68/69)		77.0 (57/74)	78.3 (54/69)
S. viridis K73^{Td}	83.6 (61/73)	83.6 (61/73)	83.6 (61/73)	85.1 (63/74)	84.1 (58/69)	82.6 (57/69)		74.3 (55/74)
Saccharomonospora sp. strain K180	91.7 (55/60)	91.7 (55/60)	91.7 (55/60)	90.0 (54/60)	88.3 (53/60)	90.0 (54/60)	91.7 (55/60)	

[a] The values on the lower left are levels of nucleotide similarity, and the values on the upper right are levels of nucleotide similarity with gaps included. The values in parentheses are number of nucleotides that are identical/number of nucleotides examined.
[b] Identical results were obtained for *"S. caesia"* K76^T, K163, K182, K200, SB-01, SB-22, and SB-58.
[c] Identical results were obtained for *S. glauca* K169^T, K179, K195, K202, and SB-37.
[d] Identical results were obtained for *S. viridis* K73^T, K185, K191, K197, SB-31, and SB-33.

as well as interspecific relationships. PCR-RFLP analysis of the 16S-23S intergenic spacer was used to characterize natural populations of *Nitrobacter* species (19). Length polymorphisms and sequence divergence of 16S-23S ITS for strains belonging to the same species, as well as for strains belonging to different species, have been reported for some *Bifidobacterium* species (17). The polymorphisms in the spacer region between 16S and 23S rDNAs were also used to type *Staphylococcus aureus* (4). Therefore, it is interesting that intraspecific sequence divergence was not found in the 16S-23S ITSs of *Saccharomonospora* species. The intraspecific homogeneity of the genus *Saccharomonospora* was illustrated by the 16S rRNA sequences in which only one strain of *S. glauca* exhibited intraspecific sequence divergence at only one nucleotide (12). Such intraspecific homogeneity of the 16S-23S ITS sequences, together with the 16S rRNA sequences, provides information about the evolutionary status of the genus *Saccharomonospora*.

The 16S-23S ITSs of *S. viridis* and *Saccharomonospora* sp. strain K180 exhibited high levels of nucleotide similarity with other species despite extensive deletions and insertions, respectively (Table 3). These findings resulted from the fact that the deletions and insertions were concentrated in the variable region and the resulting nucleotide gaps were not included when levels of nucleotide similarity were calculated. Thus, nucleotide similarity values in which gap sites were included were calculated separately to clarify the relationships among *Saccharomonospora* species. *S. viridis* strains and *Saccharomonospora* sp. strain K180 exhibited levels of nucleotide similarity when gaps were included of 59.2 to 70.7 and 59.2 to 75.7%, respectively, with other *Saccharomonospora* species (Table 3).

S. azurea K161^T and the seven strains of *"S. caesia"* had identical 16S-23S ITS sequences. Two species have also been reported to have identical 16S rRNA sequences (12). According to Leblond-Bourget et al. (17), the 16S-23S ITS has an evolutionary rate that is 10 times greater than that of the 16S rRNA gene itself. Nevertheless, the fact that *S. azurea* K161^T and *"S. caesia"* have identical 16S-23S ITS sequences and identical 16S rDNAs suggests that taxonomic revision of these two taxa is necessary.

The 23S-5S ITS sequences of some other microorganisms have been determined previously. The 23S-5S ITS sequence of *E. coli* is 92 bp long (3), the 23S-5S ITS sequence of *B. subtilis* is 55 bp long (5), the 23S-5S ITS sequence of *Frankia* sp. is 68 bp long (21), and the 23S-5S ITS sequences of some *Streptomyces* species are 72 to 109 bp long (10, 22, 23). The 23S-5S ITSs of *Saccharomonospora* species ranged from 60 to 75 bp long (Table 2). *Saccharomonospora* sp. strain K180 had a smaller 23S-5S ITS, which distinguished this organism from other *Saccharomonospora* species (Table 2). However, since nucleotide deletions are found in the variable region between positions 43 and 63, as shown in the alignment of 23S-5S ITS sequences, *Saccharomonospora* sp. strain K180 exhibits relatively high levels of nucleotide similarity (88.3 to 91.7%) to other *Saccharomonospora* species (Table 4). Although the deletions make it possible to differentiate *Saccharomonospora* sp. strain K180 from other species, they do not affect levels of similarity to other species. However, it was necessary to calculate levels of nucleotide similarity in which the nucleotide gaps were taken into consideration. *Saccharomonospora* sp. strain K180 exhibited levels of nucleotide similarity of 72 to 78.3% when nucleotide gaps were included in the calculations (Table 4). The characteristics of the 23S-5S ITS, together with the characteristics of the 16S-23S ITS, provide evidence that *Saccharomonospora* sp. strain K180 may be considered a member of a new species. The strains of *"S. caesia"* and *S. viridis* exhibited no intraspecific divergence in their 23S-5S ITS sequences (Table 4). These results are consistent with the 16S-

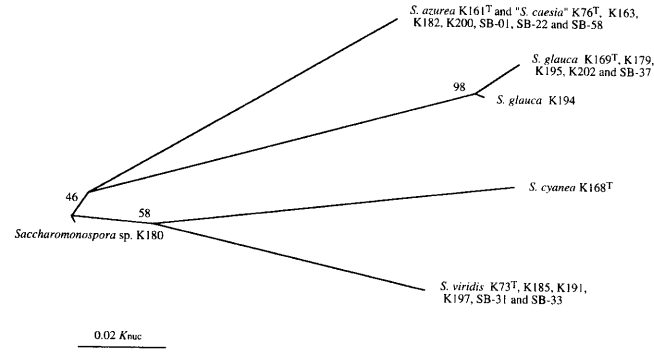

FIG. 6. Unrooted tree showing the genetic relationships of *Saccharomonospora* strains based on 23S-5S ITS sequences. Scale bar = 2 inferred nucleotide substitutions per 100 nucleotides.

23S ITS sequencing results. The 23S-5S ITS sequence of *S. glauca* K194 differed at only one nucleotide from the 23S-5S ITS sequences of the other five *S. glauca* strains (Fig. 3). This organism is the only *Saccharomonospora* strain that exhibited intraspecific divergence in this study.

S. azurea K161^T was found to have two types of 23S-5S ITS. When the PCR product containing the 16S-23S ITS of *S. azurea* K161^T was directly sequenced, a complex band pattern occurred above position 53 of the 23S-5S ITS sequence. We confirmed by using the results of other taxonomic studies that this phenomenon was not due to contamination (12, 39). It was thought that this observation was the result of heterogeneity of the 23S-5S ITS within *S. azurea* K161^T itself. When several clones containing the 23S-5S ITS region were sequenced, results which demonstrated the heterogeneity of the 23S-5S ITS within *S. azurea* K161^T were obtained; that is, 23S-5S ITS sequences with two nucleotides inserted between positions 52 and 55 were also found (Fig. 4). However, when several clones containing the 23S-5S ITS of "*S. caesia*" K76^T were sequenced, only one type of 23S-5S ITS sequence was found (data not shown).

All *Saccharomonospora* strains, including *S. azurea* K161^T, were shown to have three rRNA gene clusters on their genomes in our previous ribotyping study (39). It was unclear which one of the three *rrn* loci of *S. azurea* K161^T had the two nucleotides inserted into the 23S-5S ITS. This is an important consideration for elucidation of the evolutionary relationship between *S. azurea* K161^T and "*S. caesia*" strains. To answer this question, it was necessary to obtain restricted DNA fragments containing each rRNA gene cluster. Previously determined *Bam*HI and *Xho*I ribotype patterns (39) showed the positions of three DNA fragments on an agarose gel on which DNA fragments digested by corresponding restriction nucleases were separated. When the PCR product containing the 23S-5S ITS of *S. azurea* K161^T was used as the probe to investigate whether each rRNA gene cluster contained the 23S-5S ITS region, results identical to all of the previous ribotype patterns except the *Pvu*II pattern were obtained (Fig. 5). This indicated that each rRNA gene cluster separated by *Bam*HI and *Xho*I digestion on the agarose gel contained the 23S-5S ITS together with 5S rDNA since restriction sites for the four restriction nucleases used were not found in the 23S-5S ITS or 5S rRNA sequences of *S. azurea* K161^T. Three rRNA gene clusters separated by *Xho*I digestion were designated *rrnA*, *rrnB*, and *rrnC* (Fig. 5). The sequences of the 23S-5S ITSs obtained by using the three rRNA gene clusters showed that the 23S-5S ITS from *rrnB* had two additional nucleotides inserted between positions 52 and 55 (Fig. 4). The two inserted nucleotides were found only in *rrnB* of *S. azurea* K161^T and not in any of the other *Saccharomonospora* strains used in this study.

The trees constructed by using 16S-23S ITS sequences and 23S-5S ITS sequences are shown in Fig. 2 and 6, respectively. The two trees show the distinct interspecific relationships of members of the genus *Saccharomonospora*, but it was difficult to determine the relationships between *Saccharomonospora* species because of high levels of ITS sequence variation and length polymorphism. Deciding the exact positions of *S. viridis* and *Saccharomonospora* sp. strain K180 was particularly difficult, since high levels of insertion and deletion were found in these two species. The topologies of the two trees based on 16S-23S and 23S-5S ITS sequences differed from each other in certain respects and are not consistent with the topology of the 16S rRNA-based tree (12). Nevertheless, the two ITS sequence-derived trees provide reliable information for inferring phylogenetic relationships between *Saccharomonospora* species.

Bacillus subtilis	U G U U C U U U G A A A A C U A G A U A A
Streptomyces ambofaciens	C G U U G U U U G A G A A C U G C A C A G
Streptomyces coelicolr	C G U U G U U U G A G A A C U G C A C A G
Streptomyces griseus subsp. *griseus*	C G U U G C U U G A G A A C U G C A C A G
Saccharomonospora species	U G G U G U U U G A G A A U U G C A G A G

FIG. 7. Similarities between the processing sites found upstream from 23S rDNA in some gram-positive bacteria. The boldface letters indicate highly conserved nucleotides.

The great variability in ITS sequences and the length polymorphisms of ITSs due to deletion and insertion also make it difficult to elucidate phylogenetic relationships between high-ranked taxa and between distantly related organisms. This reduces the value of ITS sequences for database purposes and thus the value of ITS sequences for identification or phylogenetic analysis of an unknown organism. The fact that high levels of 16S-23S ITS sequence variation are not appropriate for inferring phylogenetic relationships between distantly related organisms has been described previously in a 16S-23S ITS sequence analysis of *Bifidobacterium* species (17). Nevertheless, such data are very useful in deciding the taxonomic status of *Saccharomonospora* species based on 16S-23S and 23S-5S ITS sequences. This fact may be very useful for identification or phylogenetic analysis of an unknown organism. The 16S-23S and 23S-5S ITSs of *Saccharomonospora* strains are smaller than the 16S rRNA gene; therefore, it is relatively easy to sequence them. In addition, for the most part the two ITSs of *Saccharomonospora* strains do not exhibit intraspecific sequence divergence; the only exception is the *S. glauca* K194 ITSs. Such intraspecific homogeneity, which has not been found in other taxa, helps clarify species definitions in the genus *Saccharomonospora*. The levels of interspecific divergence are also sufficient to make the relationships between *Saccharomonospora* species clear. The two trees based on ITS sequences allowed each species to be validly confirmed.

The two ITS regions, together with the upstream region from the 16S rRNA gene and the downstream region from the 5S rRNA gene, provide the sites for processing the primary rRNA transcripts to mature rRNAs (29). Therefore, despite their high evolutionary rate, ITS sequences are thought to have highly conserved regions together with variable regions. The 23S-5S ITSs of some *Streptomyces* species exhibit levels of sequence similarity of less than 50% with each other, but the levels of sequence similarity for the first 23 bp are more than 95% (10), suggesting that this region could be a putative processing site. Alignment of the 16S-23S ITS sequences and 23S-5S ITS sequences revealed the existence of highly conserved regions in *Saccharomonospora* strains. In the alignment of the 16S-23S ITS sequences, the region between positions 126 and 207 corresponding to the 3′ end of the 16S-23S ITS was shown to be highly conserved. The region between positions 126 and 146 is thought to provide a putative processing site downstream from the 16S rRNA. However, the nucleotide sequence upstream from the 16S rRNA gene is required to construct the secondary structure of a putative signal for processing of the 16S rRNA. The region between positions 168 and 188 of the 16S-23S ITS sequences is thought to provide a putative processing site for 23S rRNA by forming a helical structure with the region between positions 20 and 40 of the 23S-5S ITS sequences. The sequence for processing the signal upstream from the 23S rRNA gene of *Saccharomonospora* species was compared with the sequences of some *Streptomyces* species and *B. subtilis* (22), and no differences between the

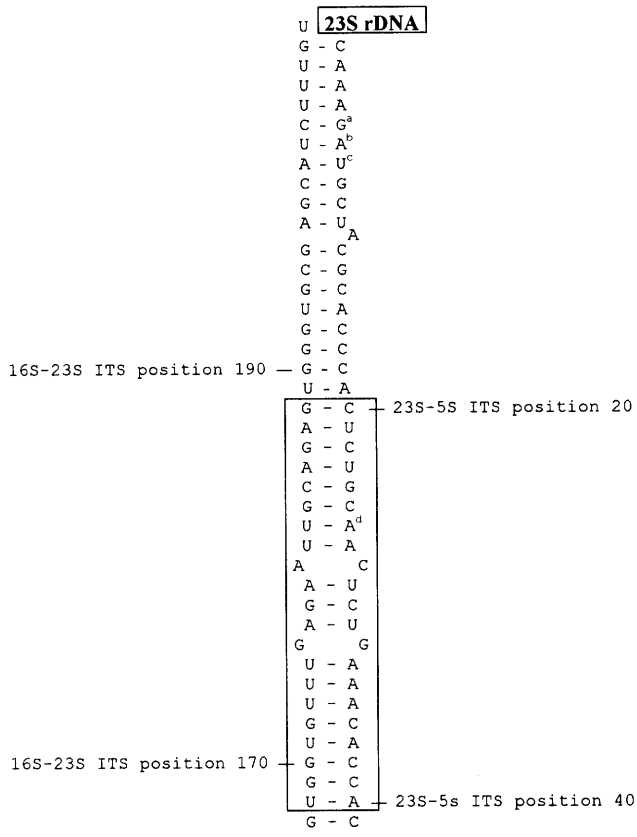

FIG. 8. Putative secondary structure formed by the 3′ region of the 16S-23S ITS and the 5′ region of the 23S-5S ITS. The superscript a indicates a nucleotide that is C in *S. cyanea* K168^T and *Saccharomonospora* sp. strain K180; the superscript b indicates a nucleotide that is G in *S. viridis* strains; the superscript c indicates a nucleotide that is C in *S. glauca* strains; and the superscript d indicates a nucleotide that is G in *S. glauca* strains.

highly conserved nucleotides described previously were found (Fig. 7). In the alignment of the 23S-5S ITS sequences, the region between positions 1 and 41 is highly conserved and exhibits an average level of nucleotide similarity of 96.4% (Fig. 3). This region forms a helical structure for processing of 23S rRNA with the region corresponding to the 3′ end (positions 167 to 207) of the 16S-23S ITS (Fig. 8). The putative secondary structure containing the conserved 3′ region of the 16S-23S ITS and the conserved 5′ region of the 23S-5S ITS is similar to secondary structures found in some *Streptomyces* species (10, 22).

Although PCR-RFLP analysis of 16S rDNA (40) and the multiplex PCR technique (41) are rapid methods for identifying *Saccharomonospora* strains, it is difficult to predict the results which will be obtained for a new species when the two techniques are used, and so they are not appropriate for classification of *Saccharomonospora* strains. However, the 16S-23S and 23S-5S ITS sequences can be used effectively for classification and identification of *Saccharomonospora* strains. The intraspecific homogeneity and interspecific divergence found in the 16S-23S ITSs and 23S-5S ITSs of members of the genus *Saccharomonospora* make these characteristics more useful than 16S rDNA sequences for species definition. A previous 16S rDNA sequence analysis revealed levels of nucleotide similarity of more than 98% for all *Saccharomonospora* species except *S. viridis* (12). This finding is not consistent with the similarity value for 16S rRNA sequences currently used for species definition in bacteriology (30). However, *Saccharomonospora* species were validly defined on the basis of the results of sequence analyses of the two ITSs. Therefore, in this study we showed that a level of 16S rRNA nucleotide similarity of more than 97% is not appropriate for species definition in all prokaryotes.

On the basis of the results of our studies of 16S-23S and 23S-5S ITSs, we make two propositions. The first is that *S. azurea* K161^T and "*S. caesia*" should be combined in one species on the basis of the identical 16S-23S ITS sequences and 23S-5S ITS sequences of all of the loci except the *rrnB* locus of *S. azurea* K161^T. Second, *Saccharomonospora* sp. strain K180 should be described as a member of a new species since this strain has 16S-23S and 23S-5S ITSs that are distinct from those of the other *Saccharomonospora* species, as described above.

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