Chemical And Molecular Classification of *Saccharomonospora* Strains

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The results of our experiments showed that the 5'-terminal sequences of 23S rRNAs can be used to distinguish different genera of actinomycetes, including the genera *Streptomyces*, *Micromonospora*, *Amycolatopsis*, and *Saccharomonospora*. There are small differences (<1%) among the sequences of some strains belonging to the genera *Streptomyces* (two strains) and *Saccharomonospora* (seven strains). On the basis of the results of morphological and biochemical analyses, strain 9022 belongs in the genus *Saccharomonospora*; however, there are distinct differences in the cell wall compositions and the 5' termini of the 23S rRNA sequences of this strain and members of the genus *Saccharomonospora*. Hence, strain 9022 cannot be classified in the genus *Saccharomonospora*.

The genus *Saccharomonospora* was established by Nonomura and Ohara in 1971 (10, 12) for organisms that produce single spores predominantly on aerial hyphae. The spores are nonmotile and heat sensitive, and formation of spores occurs at the tips of simple, unbranched sporophores of variable length. On solid medium, branched vegetative mycelia protrude from leathery colonies that are usually covered with aerial hyphae bearing spores. Members of the genus *Saccharomonospora* have type IV cell walls (meso-diaminopimelic acid and sugar pattern A [i.e., arabinose and galactose are present]). The cell walls do not contain mycolic acids. The phospholipid type is type II, and the predominant menaquinone is tetrahydrogenated with nine isoprene units [MK-9(H₄)]. The G+C content of the DNA is 67 to 74 mol% (as determined by the thermal denaturation method).

During the course of screening for novel actinomycetes and antibiotics, seven strains were isolated from soil samples obtained in Yunnan and Fujian Provinces of the People's Republic of China. On the basis of their morphological characteristics, these strains belong to the genus *Saccharomonospora*. To further determine the taxonomic positions of these organisms, they were examined by using chemical and molecular techniques. In this paper, we describe the cultural and chemical characteristics and the 23S rRNA sequences of these *Saccharomonospora* strains.

MATERIALS AND METHODS

Strain isolation and cultivation. Seven strains (strains 10, 13, 23, 350, N, 9022, and 4153) were isolated from soil samples collected in Yunnan and Fujian Provinces of the People's Republic of China. These strains were cultivated on glycerol-asparagine agar, which contained 10 g of glycerol, 1 g of asparagine, 1 g of K₂HPO₄, 1 ml of a trace salt solution (0.1 g of FeSO₄ · 7H₂O per liter, 0.1 g of MnCl₂ · 4H₂O per liter, 0.1 g of ZnSO₄ · 7H₂O per liter), 1,000 ml of water, and 20 g of Difco agar. The pH was adjusted to 7.2 before sterilization.

Morphological and physiological properties were determined by using the standard procedures described by Ruan (16) and Shirling and Gottlieb (17). Cell wall composition was determined by the methods of Hasegawa et al. (5), Becker et al. (2), and Lechevalier and Lechevalier (9). Mycolic acids

were analyzed by the procedures described by Minnikin et al. (11). Phospholipids were analyzed by techniques described by Lechevalier and Lechevalier (9). Menaquinones were analyzed by the method of Kroppenstedt (8) and Collins (3).

The strains used for comparison in this study included *Saccharomonospora viridis* JCM 3306^T (T = type strain), *Amycolatopsis azurea* C43, *Streptomyces griseus* C51, "*Saccharomonospora yunnanensis*" 4650 (6), and the seven isolates mentioned above.

RNA sequencing and analysis. RNA sequences were determined directly from total RNA extracted by the LiCl method (1). Primer P457, which was used for direct rRNA sequencing, was a 20-nucleotide sequence complementary to a conserved motif at the 5' end of eubacterial 23S rRNA (positions 457 to 477 in *Escherichia coli* 23S rRNA; 3'-TCATGGCACTCCCT TACCAC-5'). Rapid rRNA sequencing was carried out as described by Qu et al. (13). The sequences were aligned to derive similarity values. Evolutionary distance (*K*_{nuc}) values were calculated by the Kimura method (7). Phylogenetic trees were constructed by using the unweighted pair group with mathematical average method (7) and the method of Fitch and Margoliash (4).

Nucleotide sequence accession numbers. The 23S rRNA nucleotide sequences of strains C51, C43, JCM 3306^T, 9022, and 4153 have been deposited in the EMBL database under accession numbers Z26646, X75549, X75548, Z26915, and Z26916, respectively.

TABLE 1. Cultural characteristics of *Saccharomonospora viridis* JCM 3306^T and other strains

Group	Strain	Aerial mycelium	Substrate mycelium	Soluble pigment
A	JCM 3306 ^T	White to green	Brown to green	No
	4650	Green	Brown to green	No
	10	White to green	Green	No
	13	White to green	Green	No
	23	Green	Brown to green	No
	N	White	Green	No
B	350	Greyish green	Bluish green	Bluish green
C	9022	Yellow	Brown	No
D	4153	None or white (trace)	Green to brown	No

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C51	CGTAGAGGGT	AAGACCCCGG	TA* GCCTAAA	CATTGACCGT	TCG* * * TTTG	50
S.a	-----	-----	----- G---	--- CAG- G- C	-----	
3306 ^T	- G- - T- - -	G- - GT- - -	-- C- TT- - -	- TGCATGTCC	- T- A- - - - -	
10	- G- - T- - -	G- - GT- - -	-- C- TT- - -	- TGCATGTCC	- T- A- - - - -	
13	- G- - T- - -	G- - GT- - -	-- C- TT- - -	- TGCATGTCC	- T- A- - - - -	
23	- G- - T- - -	G- - GT- - -	-- C- TT- - -	- TGCATGTCC	- T- A- - - - -	
N	- G- - T- - -	G- - GT- - -	-- C- TT- - -	- TGCATGTCC	- T- A- - - - -	
350	- G- - T- - -	G- - GT- - -	-- C- TT- - -	- TGCATGTCC	- T- A- - - - -	
4650	- G- - T- - -	G- - GT- - -	-- C- TT- - -	- TGCATGTCC	- T- A- - - - -	
C43	- CG- - T- - -	T- - GT- - - C	-- C- GA- - -	- CGTTTT- G	ATTTTG- G- C	
4153	----- C- - -	T- T- GT- - -	----- GTG- - -	GTGGCCTG- C	- TC- - - - G- -	
9022	#####	#####	#####	- C- - CC- AAG	-----	
C51	AGAGACACCC	AAGTAGCACG	GGGCCCGAGA	AATCCCGTGT	TAATCTGGCG	100
S.a	-----	-----	-----	-----	-----	
3306 ^T	TG- TGT- - -	C- - - - - GC	- A- - TT- T- G	--- T- GC- TG	----- *	
10	TTG- TGT- - -	C- - - - - GC	- A- - TT- T- G	--- T- GC- TG	----- *	
13	TTG- TGT- - -	C- - - - - GC	- A- - TT- T- G	--- T- GC- TG	----- *	
23	TTG- TGT- - -	C- - - - - GC	- A- - TT- T- G	--- T- GC- TG	----- *	
N	TTG- TGT- - -	C- - - - - GC	- A- - TT- T- G	--- T- GC- TG	----- *	
350	TTG- TGT- - -	C- - - - - GC	- A- - TT- T- G	--- T- GC- TG	----- *	
4650	TTG- TGT- - -	C- - - - - GC	- A- - TT- T- G	--- T- GC- TG	----- *	
C43	T- G- TTT- - -	C- - - - - GC	- A- - TT- T- G	--- TTGC- - G	----- *	
4153	G- - CTC- - -	----- GGC	- A- - CTT- - -	--- TGCGCG	----- C- A	
9022	- G- GXT- - -	-----	----- XX- -	----- T- - -	----- C- A	
C51	GGACCACCCG	CTAAGCCTAA	ATATTCCTG	GTGACCGATA	GCGGA	150
S.a	-----	-----	-----	-----	-----	
3306 ^T	-----	G- - - - -	--- C- T- - -	-- T- - - C- -	-----	
10	-----	G- - - - -	--- C- T- - -	-- T- - - C- -	-----	
13	-----	G- - - - -	--- C- T- - -	-- T- - - C- -	-----	
23	-----	G- - - - -	--- C- T- - -	-- T- - - C- -	-----	
N	-----	G- - - - -	--- C- T- - -	-- T- - - C- -	-----	
350	-----	G- - - - -	--- C- T- - -	-- T- - - C- -	-----	
4650	-----	G- - - - -	--- C- T- - -	-- T- - - C- -	-----	
C43	-----	GG- - - - -	--- C- T- - -	AA- - - - C- -	-----	
4153	----- T- -	GG- - - - -	--- C- T- - -	-- T- - - C- -	-----	
9022	----- T- -	G- - - - -	--- C- - - - -	A- - - - - C- -	-----	

FIG. 1. Sequence alignment of the 5'-terminal regions of 23S rRNAs from actinomycetes corresponding to positions 401 to 542 in *Streptomyces ambofaciens*. Only the nucleotides that differ from the nucleotides in the strain C51 sequence are shown. The dashes indicate that the nucleotides were identical to the strain C51 nucleotides; the asterisks indicate deletions; and X and the number signs indicate undetermined nucleotides. S. a, *Streptomyces ambofaciens*; C51, *Streptomyces griseus* C51; C43, *Amiclatopsis azurea* C43; 3306^T, *Saccharomonospora viridis* JCM 3306^T; 9022, 4153, 10, 13, 23, N, and 350, unidentified strains; 4650, "*Saccharomonospora yunnanensis*" 4650.

FIG. 2. Phylogenetic tree based on partial 23S rRNA sequences. The tree was constructed by using the unweighted pair group with mathematical average method and the K_{nuc} values in Table 3. The distance between any two species was proportional to the sum of the branch lengths on the x axis (K_{nuc} values), while the distances on the y axis were arbitrary. 1, JCM 3306^T; 2, C43; 3, 4153; 4, 9022; 5, *Streptomyces ambofaciens*; 6, C51.

FIG. 3. Unrooted phylogenetic tree. The tree was constructed by using the methods of Fitch and Margoliash (4) and the K_{nuc} values in Table 3. 1, JCM 3306^T; 2, C43; 3, 4153; 4, 9022; 5, *Streptomyces ambofaciens*; 6, C51.

TABLE 2. Chemotaxonomic characteristics^a

Strain	Cell wall type		Menaquinone(s)
	Amino acids	Sugars	
JCM 3306 ^T	IV	Galactose, glucose, arabinose, ribose	MK-9(H ₄)
4650	IV	Galactose, glucose, arabinose, ribose	MK-9(H ₄)
10	IV	Galactose, glucose, arabinose, ribose	MK-9(H ₄)
13	IV	Galactose, glucose, arabinose, ribose	MK-9(H ₄)
23	IV	Galactose, glucose, arabinose, ribose	MK-9(H ₄)
350	IV	Galactose, glucose, arabinose, ribose	MK-9(H ₄)
N	IV	Galactose, glucose, arabinose, ribose	MK-9(H ₄)
9022	II (glycine variable)	Galactose, glucose, mannose, madurose, xylose, ribose	MK-9(H ₄ , H ₆)
4153	II	Galactose, glucose, arabinose, xylose, ribose	MK-9(H ₄), MK-10(H ₆)

^a In addition, the phospholipid type of all strains is type PII, and all strains lack mycolic acids.

RESULTS AND DISCUSSION

The morphological characteristics of *Saccharomonospora viridis* JCM 3306^T and strains 4650, 350, 10, 13, 23, N, and 9022 were determined on Bennett's agar. These organisms produced single spores on aerial hyphae at the tips of simple unbranched sporophores of variable length. As determined with an electron microscope, the spores appeared to be ovoid (0.9 to 1.1 by 1.3 to 1.5 μ m) and to have smooth surfaces.

On the basis of the cultural characteristics observed on glucose-asparagine and oatmeal media, the strains were divided into four groups (Table 1).

The results of the chemical tests are shown in Table 2. The chemical and morphological properties showed that strains 4650, 10, 13, 23, 350, and N belong to the genus *Saccharomonospora*. On the other hand, strain 4153 did not produce any aerial mycelium and had a type II cell wall (containing xylose) and thus should be placed in the genus *Micromonospora*. Strain 9022 was similar to the type strain of *Saccharomonospora viridis* in morphology and phospholipid and menaquinone contents. However, xylose and madurose were found in whole cells of strain 9022, while arabinose was not.

TABLE 3. Pairwise comparison of rRNA sequences

Taxon	Levels of similarity ^a					
	C51	<i>Streptomyces ambofaciens</i>	JCM 3306 ^T	C43	4153	9022
C51		1/130	33/130	37/130	38/130	12/102
<i>Streptomyces ambofaciens</i>	0.012		34/130	37/130	37/130	12/102
JCM 3306 ^T	0.310	0.322		18/130	32/130	23/102
C43	0.358	0.358	0.154		35/130	24/102
4153	0.370	0.358	0.300	0.344		23/102
9022	0.129	0.129	0.269	0.283	0.269	

^a The values on the upper right are the numbers of sequence differences/numbers of sequence positions examined; the values on the lower left are the K_{nuc} values as determined by the formula of Kimura.

Hence, the classification of this strain was not resolved by the morphological and chemical data.

In order to identify variations in the nucleotide sequences of the actinomycetes used in this study, particularly the *Saccharomonospora* strains and strain 9022, and to confirm the taxonomic positions of these organisms, a direct RNA sequencing analysis was carried out by studying a 5'-terminal region of 23S rRNA which had been calibrated previously for phylogenetic analysis (14, 15) of lower eukaryotes. The results of the sequence analysis are shown in Fig. 1 and Table 3.

It was evident that there were small differences (<1%) among the sequences of strains in the same genus (for example, *Streptomyces ambofaciens* and *Streptomyces griseus*). In the genus *Saccharomonospora*, no differences were found in the 140 nucleotide sequences determined for all seven strains. However, significant differences were observed among the genera of actinomycetes. This suggests that the partial sequences of the 5' termini of 23S rRNAs could be used to effectively distinguish the different genera of actinomycetes and to determine their affinity relationships. Phylogenetic trees based on the rRNA sequences were constructed (Fig. 2 and 3). It is noteworthy that *Saccharomonospora viridis* JCM 3306^T and *Amycolatopsis* sp. strain C43, have the same cell wall type (type IV) and exhibited relatively high levels of homology in their rRNA sequences. Notwithstanding the consistent cell wall composition characteristic, the significant level of sequence difference between strain 9022 and members of the genus *Saccharomonospora* suggests that strain 9022 belongs to a genus other than *Saccharomonospora* despite the similarity in the morphology of the taxa. The precise taxonomic position of strain 9022 will require further study.

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