Streptomyces bungoensis sp. nov.

TORU EGUCHI,¹ NOBUO TAKADA,² SHOJI NAKAMURA,¹ TOSHIYUKI TANAKA,¹
TAKETOSHI MAKINO,¹ AND YASUJI OSHIMA^{2*}

Research and Development Division, Sunstar Inc., 3-1 Asahi-Machi, Takatsuki-shi, Osaka 569,¹ and
Department of Biotechnology, Faculty of Engineering, Osaka University,
2-1 Yamadaoka, Suita-shi, Osaka 565,² Japan

Two actinomycete strains, MS16-10G and MS16-10W, isolated from soil and producing an antibiotic, were examined and assigned to the genus *Streptomyces*, because they have a gray aerial mass, spiral spore chains, and a spiny spore surface; form a melanoid pigment on tyrosine agar, on peptone-yeast extract-iron agar, and in tryptone-yeast extract broth; and have cell wall chemotype I. The DNA base compositions and DNA-DNA reassociation of the isolates were compared with those of four strains of authentic species of the genus *Streptomyces*: two synonymous strains of *Streptomyces cyaneus* (*Streptomyces afghaniensis* and *Streptomyces echinatus*) and one strain each of *Streptomyces chromofuscus* and *Streptomyces antibioticus* showing similar morphological characteristics. The results indicated that the two isolates differed from the four authentic species and represent a single new species for which the name *Streptomyces bungoensis* is proposed. The type strain is MS16-10G, which has been deposited in the collection of the Fermentation Research Institute, Agency of Industrial Sciences and Technology, Tsukuba, Japan, with accession no. FERM 8432^T.

During screening for microorganisms producing new antibiotics against anaerobic bacteria in the human oral cavity, we isolated two distinct actinomycete strains from soil samples collected in the Yabakei area of Ohita Prefecture, Japan. This report deals with the taxonomical characterization of the two isolates by conventional taxonomical procedures, by determination of their DNA base composition, and by DNA-DNA reassociation experiments with four strains of related authentic species of the genus *Streptomyces*. Results showed that these two isolates should be classified as a single new species of the genus *Streptomyces*. We propose the name *S. bungoensis* for the new species.

MATERIALS AND METHODS

Microorganisms. Two actinomycete strains, MS16-10G and MS16-10W, were isolated from soil samples by standard plating techniques. Four strains of authentic species of the genus *Streptomyces* were used as reference strains in DNA-DNA reassociation experiments. Three strains, *S. cyaneus* (formerly named *S. afghaniensis*) IFO 12831 (= ISP 5528), *S. cyaneus* (syn. *S. echinatus*) IFO 12763 (= ISP 5013), and *S. antibioticus* (syn. *S. lucensis*) IFO 13056 (= ISP 5317), were obtained from the Institute for Fermentation, Osaka (IFO), Japan. The other strain, *S. chromofuscus* JCM 4354 (= ISP 5273), was obtained from the Japan Collection of Microorganisms (JCM), Institute of Physical and Chemical Research, Wako, Saitama, Japan.

Methods for taxonomical characterization. A medium containing 10 g of yeast extract and 10 g of glucose per liter and adjusted to pH 7.2 (yeast extract-glucose medium) was used for general cultivation of actinomycete strains. The media used for morphological characterization were nutrient agar (Difco Laboratories, Detroit, Mich.), Czapek's solution agar (5), sucrose-nitrate agar (9), glucose-asparagine agar (14), tryptone-yeast extract broth (ISP medium 1) (9), yeast extract-malt extract agar (ISP medium 2) (9), oatmeal agar (ISP medium 3) (9), inorganic salts-starch agar (ISP medium 4) (9), glycerol-asparagine agar (ISP medium 5) (9), peptone

yeast extract-iron agar (ISP medium 6) (9), and tyrosine agar (ISP medium 7) (9). The utilizations of various carbon compounds were examined on ISP medium 9 (9). For preparation of DNA, Trypticase soy broth (BBL Microbiology Systems, Cockeysville, Md.) was used for cultivation of cells. These media (100 ml) were inoculated with 1-ml volumes of cell suspensions prepared from cells cultivated in 100 ml of yeast extract-glucose medium by shaking for 48 h at 27°C, harvested, washed, and suspended in 10 ml of sterile water.

Morphological characteristics of cells in various media were recorded after incubation for 28 days at 27°C by the recommended method (9). Spore morphologies of the organisms grown on yeast extract-malt extract agar at 27°C for 10 days were examined with a microscope. For scanning electron micrography of spores and sporophores, 7-day-old slide cultures were prepared in yeast extract-malt extract agar by incubation at 27°C. The slide cultures were then exposed to 2% Os₂O₄ vapor in situ at room temperature for 2 h, washed in 70% acetone, and dehydrated in an increasing acetone series. After critical point drying, the slides were mounted on specimen stubs with silver paste, sputter coated with gold-palladium, and observed with a Hitachi S-430 scanning electron microscope.

Gelatin liquefaction, starch hydrolysis, and milk reaction were examined as described previously (9). Production of melanoid pigment was examined on tyrosine agar, on peptone-yeast extract-iron agar, and in tryptone-yeast extract broth. Sodium chloride tolerance was tested at concentrations of 1 to 15% in yeast extract-malt extract agar. Susceptibilities to streptomycin were determined by placing a sterilized disk (8 mm in diameter) of filter paper saturated with the antibiotic solution (100 µg/ml) on an agar plate that had been seeded with a cell suspension of the test organisms and then incubated at 27°C for 7 days. The chemotypes of cell walls were determined as described previously (1), with whole-cell hydrolysates prepared from washed cells grown for 48 h in yeast extract-glucose medium at 27°C.

For preparation of DNA, cells were shaken in Trypticase soy broth supplemented with 0.5% glycine for 48 h at 27°C, harvested, and washed with sterile water as described by

* Corresponding author.

TABLE 1. Cultural characteristics of strains MS16-10G and MS16-10W^a

Medium	Amount of growth	Color of:		Production of soluble pigment
		Aerial mycelium	Reverse side of colony	
Tryptone-yeast extract broth (ISP medium 1)	ND ^b			Yes (brownish black)
Yeast extract-malt extract agar (ISP medium 2)	Abundant	Gray	Reddish brown	Yes (brownish black)
Oatmeal agar (ISP medium 3)	Abundant	Gray	Gray	No
Inorganic salts-starch agar (ISP medium 4)	Abundant	Gray	Gray	No
Glycerol-asparagine agar (ISP medium 5)	Abundant	Grayish brown	Brownish yellow	No
Peptone-yeast extract-iron agar (ISP medium 6)	Abundant	White	Colorless	Yes (brownish black)
Tyrosine agar (ISP medium 7)	Abundant	Gray	Yellow	Yes (pale brown)
Glucose-asparagine agar	Abundant	Grayish brown	Brownish yellow	No
Sucrose-nitrate agar	Moderate	Gray	Colorless	No
Nutrient agar	Moderate	White	Colorless	Yes (reddish brown)
Czapek's solution agar	Moderate	White	Colorless	No

^a Color determinations were made after incubation for 28 days at 27°C by the method recommended by Shirling and Gottlieb (9).

^b ND, not determined.

Marmur (4). Base compositions of DNA were determined by high-performance liquid chromatography as described by Katayama-Fujimura et al. (3). DNA-DNA reassociation experiments were performed by the S1 nuclease procedure (2) with reference DNA labeled with ³²P by nick translation (8).

RESULTS AND DISCUSSION

Cultural and morphological characteristics of the two isolates. Both new isolates grew well on various ISP agar media but grew only moderately on sucrose-nitrate agar, nutrient agar, and Czapek's solution agar (Table 1). The aerial mass of strain MS16-10G was gray on yeast extract-malt extract agar after incubation at 27°C for 7 days, whereas that of strain MS16-10W grown on the same medium at 27°C was white after 10 days but became gray after 14 days. The mycelia of both strains excreted a brownish black melanoid pigment in tryptone-yeast extract broth and on peptone-yeast extract-iron agar and a pale brown melanoid pigment on tyrosine agar (Table 1). Both isolates produced a reddish brown soluble pigment during cultivation on nutrient agar in spite of their moderate cell growth on this medium and a brownish black pigment on yeast extract-malt extract agar. The reverse side of the colonies was reddish brown on yeast extract-malt extract agar, brownish yellow on glucose-asparagine agar and glycerol-asparagine agar, yellow on tyrosine agar, and gray on oatmeal agar and inorganic salts-starch agar. The substrate mycelia were colorless.

The aerial mycelia of these strains were moderately short with simple branches and formed spiral chains of spores on all of these agar media (Fig. 1). Each spore chain consisted of 20 or more ellipsoidal spores of 0.5 to 0.6 µm by 0.6 to 0.7 µm. The surface of the spores was spiny. No coccoid and/or bacillary fragments produced by division of substrate mycelia were observed in cultures of either isolate. No fragmentation of substrate mycelia was observed, and no sclerotia, sporangia, or flagellated spores were formed.

The isolates liquefied gelatin but did not hydrolyze starch. They induced peptonization but no coagulation of milk.

Analysis of cell hydrolysates of these strains revealed that their cell walls contained LL-diaminopimelic acid and glycine but no detectable arabinose, galactose, or meso-diaminopimelic acid. Thus, these strains have chemotype I cell walls according to the classification of Becker et al. (1). These observations indicate that strains MS16-10G and MS16-10W both belong to the genus *Streptomyces*.

We then compared the taxonomic characteristics of the

FIG. 1. Scanning electron micrographs of aerial mycelia of strains MS16-10G (A) and MS16-10W (B) grown on yeast extract-malt extract agar at 27°C for 7 days. Bars = 1 µm.

TABLE 2. Comparison of morphological characteristics of strains MS16-10G and MS16-10W with those in standard descriptions of 13 species of the genus *Streptomyces*^a

Strain or <i>Streptomyces</i> species described in:		Color of vegetative mycelium	Growth on Czapek's solution agar	Production of melanoid pigment on ISP medium ^b	Pigment of reverse side of colonies	Soluble pigment
Reference 15	Reference 7					
<i>S. antibioticus</i>	<i>S. lucensis</i>	ND ^c	ND	1, 6	—	—
<i>S. chromofuscus</i>	<i>S. chromofuscus</i>	ND	ND	1, 6	ND	ND
<i>S. cyaneus</i>	<i>S. afghaniensis</i>	ND	ND	1, 6, 7	+	+
<i>S. cyaneus</i>	<i>S. arenae</i>	ND	Excellent	1, 6, 7	—	+
<i>S. cyaneus</i>	<i>S. echinatus</i>	ND	Fair	1, 6, 7	+	—
<i>S. cyaneus</i>	<i>S. griseochromogenes</i>	ND	Excellent	1, 6, 7	—	—
<i>S. cyaneus</i>	<i>S. iakyrus</i>	Green or yellowish green	Excellent	1, 6	+	+
<i>S. filipinensis</i>	<i>S. filipinensis</i>	ND	Excellent	1, 6, 7	—	—
<i>S. filipinensis</i>	<i>S. durhamensis</i>	ND	Excellent	1, 6, 7	—	—
<i>S. fimbriatus</i>	<i>S. fimbriatus</i>	ND	Excellent	ND	ND	ND
SND ^d	<i>S. atrocyaneus</i>	Blackish blue	ND	ND	ND	ND
SND	<i>S. malachitofuscus</i>	Green	ND	1, 6, 7	+	—
SND	<i>S. malachitorectus</i>	Green	ND	1, 6, 7	+	—
MS16-10G and MS16-10W		None	Moderate	1, 6, 7	+	+

^a Data were compiled from references 6, 7, 9, 10, 11, 12, 13, and 15. The 13 species according to reference 7 were selected, because they have the same morphological characteristics as the isolates with spiral sporophores, a spiny spore surface, and gray aerial mass color.

^b Melanoid pigment was produced in tryptone-yeast extract broth (ISP medium 1), on peptone-yeast extract-iron agar (ISP medium 6), and/or tyrosine agar (ISP medium 7).

^c ND, not described.

^d SND, no descriptions for these species in reference 15.

two isolates with those of various species of *Streptomyces* described in *Bergey's Manuals* (7, 15) and with the ISP descriptions by Shirling and Gottlieb (9–13) by the classification key of Nonomura (6). The two isolates were found to be most closely related to the 13 species of the genus *Streptomyces* listed in Table 2 with respect to the formation of simple spiral spore chains and the spiny surface of the spores, which appeared as closed or compact spirals of two or more turns of spines, and also with respect to the formation of a gray aerial mass and production of melanoid pigment in tryptone-yeast extract broth, on tyrosine agar, and on peptone-yeast extract-iron agar. However, no known species showed characteristics similar to those of the two new isolates.

For more critical examination of the two strains, we used four strains of authentic *Streptomyces* species as reference strains, i.e., two *S. cyaneus* strains (one formerly named *S. afghaniensis* [7] IFO 12831 and the other formerly named *S. echinatus* IFO 12763), *S. antibioticus* (*S. lucensis*) IFO 13056, and *S. chromofuscus* JCM 4354 (Table 3). The other nine species of the genus *Streptomyces* according to reference 7 differed from the two isolates in the color of their vegetative mycelia, abundant growth on Czapek's solution agar, and/or pigment formation. The two isolates showed utilizations of carbon sources similar to those of the four reference strains, except that they were unable to utilize L-rhamnose, *i*-inositol, and salicin, whereas all four reference strains utilized these compounds. Another difference

TABLE 3. Biochemical characteristics of strains MS16-10G and MS16-10W and four species of the genus *Streptomyces*^a

Characteristic	MS16-10G	MS16-10W	<i>S. afghaniensis</i> IFO 12831	<i>S. chromofuscus</i> JCM 4354	<i>S. echinatus</i> IFO 12763	<i>S. lucensis</i> IFO 13056
Carbon-free control ^b	—	—	—	—	—	—
Utilization of:						
D-Glucose	+	+	+	+	+	+
L-Rhamnose	—	—	+	+	+	±
D-Fructose	+	+	+	+	+	+
D-Galactose	+	+	+	+	+	+
D-Xylose	+	+	+	+	+	±
L-Arabinose	+	+	+	+	+	+
Sucrose	±	±	+	+	—	+
Raffinose	—	±	+	—	+	+
D-Mannitol	+	+	+	+	+	+
<i>i</i> -Inositol	—	—	+	+	+	±
Salicin	—	—	+	±	±	+
NaCl tolerance	≥7%, but <10%	≥7%, but <10%	≥4%, but <7%	≥4%, but <7%	≥4%, but <7%	≥4%, but <7%
Streptomycin susceptibility	Sensitive	Sensitive	Resistant	Sensitive	Resistant	Sensitive

^a Species names are as in reference 7. All the characteristics described were confirmed with the test strains. Symbols: +, utilization of carbon compound; —, no utilization; ±, very slight utilization.

^b Cell growth on the carbon utilization medium (ISP medium 9) (9).

TABLE 4. G+C contents and DNA-DNA similarities of strains MS16-10G and MS16-10W and four strains of authentic species of the genus *Streptomyces*^a

Strain	% Reassociation value ^b with ³² P-labeled DNA from strain:						G+C content (mol%)
	MS16-10G	MS16-10W	<i>S. afghaniensis</i> IFO 12831	<i>S. chromofuscus</i> JCM 4354	<i>S. echinatus</i> IFO 12763	<i>S. lucensis</i> IFO 13056	
MS16-10G	100	85.2	33.1	35.1	26.0	31.3	70.3
MS16-10W	89.5	100	24.1	33.2	26.1	28.7	70.5
<i>S. afghaniensis</i> IFO 12831	24.8	20.4	100	26.6	26.5	28.2	72.2
<i>S. chromofuscus</i> JCM 4354	35.2	24.1	27.6	100	25.3	36.7	69.6
<i>S. echinatus</i> IFO 12763	24.7	19.1	26.8	35.1	100	36.3	69.3
<i>S. lucensis</i> IFO 13056	32.3	24.4	34.3	37.4	36.8	100	71.2

^a Species names of the authentic *Streptomyces* strains are as in reference 7.^b Reassociation is expressed as a percentage of reassociation of labeled and unlabeled DNA from the same strain.

was that the isolates grew on yeast extract-malt extract agar supplemented with 7% NaCl, whereas the four reference strains did not.

DNA base compositions and similarities of the two isolates. To clarify the relationships between the two isolates and the four reference strains, we compared the guanine-plus-cytosine (G+C) contents of their DNAs and their abilities to form duplexes by DNA-DNA reassociation. Although the DNAs of the two isolates had G+C contents similar to those of the four reference strains (Table 4), DNA-DNA reassociation experiments indicated that they differed from the four reference strains of *Streptomyces* species in that both isolates belonged to the same species, because their DNAs showed reassociation values of 85.2 and 89.5%. It is noteworthy that strains IFO 12831 and IFO 12763, formerly named *S. afghaniensis* and *S. echinatus*, respectively, in reference 7, but later described as synonyms of *S. cyaneus* (15), should be classified into two different species, because the DNA-DNA reassociation values between them were 26.5 and 26.8%.

From the above observations, we concluded that MS16-10G and MS16-10W are members of a single new species of *Streptomyces*, for which we propose the name *Streptomyces bungoensis* sp. nov. Strain MS16-10G, the type strain of *S. bungoensis*, and strain MS16-10W have been deposited in the Fermentation Research Institute, Agency of Industrial Sciences and Technology, Tsukuba, Japan, under the accession nos. FERM 8432^T and FERM 8433, respectively.

Description of *Streptomyces bungoensis* sp. nov. *Streptomyces bungoensis* (bun.go.en'sis. M.L. masc. adj. *bungoensis*, indicating their geographic origin in Bungo, region of Japan).

Substrate and aerial mycelia are produced. Mature spore chains are spiral, with 20 or more spores per chain. Spores are ellipsoidal, 0.5 by 0.7 µm in diameter. The spore surface is spiny. Mycelia do not fragment into coccoid or bacillary structures.

The aerial mass on glucose-asparagine agar, glycerol-asparagine agar, inorganic salts-starch agar, tyrosine agar, yeast extract-malt extract agar, and oatmeal agar is grayish brown or gray. The reverse sides of colonies are brownish yellow on glucose-asparagine agar and glycerol-asparagine agar, gray on inorganic salts-starch agar and oatmeal agar, yellow on tyrosine agar, and reddish brown on yeast extract-malt extract agar. The melanoid pigment produced is pale brown on tyrosine agar and brownish black on peptone-yeast extract-iron agar and in tryptone-yeast extract broth. A reddish brown soluble pigment is produced on nutrient agar, and a brownish black pigment is produced on yeast extract-malt extract agar. No soluble pigment is produced on su-

crose-nitrate agar, glucose-asparagine agar, glycerol-asparagine agar, inorganic salts-starch agar, oatmeal agar, or Czapek's solution agar.

Starch is not hydrolyzed, gelatin is liquefied, and milk is peptonized but not coagulated. D-Glucose, D-fructose, D-galactose, D-xylose, L-arabinose, and D-mannitol are utilized for growth, but L-rhamnose, *i*-inositol, and salicin are not. Little if any growth is observed with raffinose, and scant growth is observed with sucrose. The cell wall composition is chemotype I containing LL-diaminopimelic acid and glycine but no galactose, arabinose, or *meso*-diaminopimelic acid. The G+C content of the DNA is 70.3 mol%.

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