STREPTOMYCES SPADICOGRISEUS, A NEW SPECIES PRODUCING ANTHRAMYCIN

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The taxonomic description of *Streptomyces spadicogriseus*, a new species belonging to the Gray Series of streptomycetes as classified by PRIDHAM and TRESNER, is presented. This new species is distinguishable from the known members of the Gray Series. *Streptomyces spadicogriseus* produces anthramycin but bears no taxonomic relation to the known producer of the antibiotic: *S. refuineus* var. *thermotolerans*.

A new species of *Streptomyces* isolated from a soil sample collected in Fujiyoshida, Yamanashi Prefecture, Japan was found to produce anthramycin[1~3], an antibiotic having antitumor effects and antibacterial activities against both Gram-positive and Gram-negative bacteria. The name *Streptomyces spadicogriseus* KOMATSU is proposed for the organism. This paper deals with taxonomic studies of the organism. Description of production, isolation and identification of the antibiotic also is presented. The name previously was documented in patents[4,5].

The holotype strain of *Streptomyces spadicogriseus* KOMATSU sp. nov. has been deposited in the American Type Culture Collection under the accession number ATCC 31179 and in the Fermentation Research Institute (Chiba, Japan) with the accession number FERM-P 3275.

Taxonomic studies were carried out in accordance with the methods described by SHIRLING and GOTTLIEB[6], and by WAKSMAN[7]. The color designations were taken from the Japanese Guide to Color Standard[8]. The specific epithet "spadicogriseus" is derived from the color of mycelium, meaning "light brownish gray".

1. Microscopic Characteristics

The aerial and substrate mycelia develop well, and produce monopodially branched hyphae which

Plate 1. Photomicrograph of *S. spadicogriseus* on yeast extract-malt extract agar after 10 days at 27°C (×300).

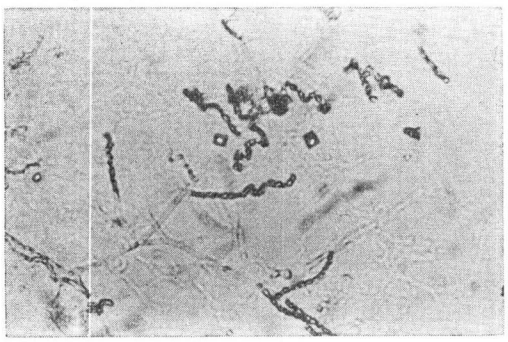


Plate 2. Electron micrograph of *S. spadicogriseus* (×5,000).

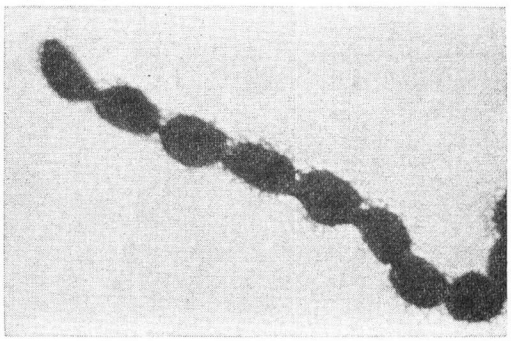


Table 1. Cultural characteristics of *S. spadicogriseus*.

CZAPEK's solution agar (with sucrose)	G*: poor, thin A: light brownish gray R: light brownish gray S: none	Yeast extract-malt extract agar (I.S.P. No. 2)	G: abundant, thick A: light brownish gray, powdery R: brownish gray S: pale yellowish brown
Glucose CZAPEK's solution agar	G: good, thick A: light brownish gray, powdery R: dull yellow to light brownish gray S: pale yellow	Oatmeal agar (I.S.P. No. 3)	G: good A: pale brown, powdery R: light brownish gray S: none
Glycerol CZAPEK's solution agar	G: abundant, thick A: light brownish gray to light gray R: reddish brown to yellowish brown S: pale yellowish brown	Nutrient agar	G: poor, thin A: yellowish white R: pale yellow S: none
Glycerol-asparagine agar (I.S.P.** No. 5)	G: moderate, thin A: brownish white to pale yellowish brown, powdery R: pale yellow to dark yellow S: none	Glucose nutrient agar	G: abundant, thick A: light brownish gray R: pale yellow to dull yellow S: none
Inorganic salts-starch agar (I.S.P. No. 4)	G: good, thick A: pale yellowish brown to grayish yellow brown, powdery R: light brownish gray to pale yellowish brown S: none	Nutrient broth	G: poor, thin, settling to the bottom A: yellowish white to pale yellow R: yellowish white to pale yellow S: pale yellow, fading later
Tyrosine agar (I.S.P. No. 7)	G: moderate, thick A: light brownish gray, slightly powdery R: light brownish gray S: none	Glucose nutrient broth	G: abundant, wrinkled, settling to the bottom A: yellowish white R: dull yellow S: pale yellow
Peptone-yeast extract iron agar (I.S.P. No. 6)	G: good A: grayish white R: pale yellow to pale yellowish brown S: none	Potato plug	G: abundant, thick, wrinkled A: yellowish gray, powdery R: pale olive S: pale olive to olive gray
Tryptone-yeast extract broth (I.S.P. No. 1)	G: moderate A: white to grayish white R: pale olive to grayish olive S: none	Carrot plug	G: abundant, thick, folded A: pale yellowish brown, powdery R: olive to yellowish brown S: none

* G: Degree of growth A: Color of aerial mycelium
R: Color of reverse S: Color or presence of diffusible pigment

** Medium employed by International Streptomyces Project (I.S.P.).

are 0.9~1.3 μ in width. No whorl formation can be seen. When mature, spores occur in chains of more than 10. Open loops, hooks and coils are observed. In general, loosely extended open coils are predominant (Plate 1). Spores are oval, 0.9~1.4×1.5~2.0 μ in diameter. The spore surface is hairy (Plate 2).

2. Cultural Characteristics

Streptomyces spadicogriseus was cultivated on various media at 27°C, and cultural characteristics were observed at 7, 14 and 21 days. The results are shown in Table 1.

Growth is moderate to abundant on most media, but it is poor and thin on CZAPEK's solution agar, nutrient agar and nutrient broth. Color of sporulated aerial mycelium allows placement in the Gray Series of PRIDHAM and TRESNER[9], because light brownish gray is the most distinctive color. Pale yellow or pale yellowish brown diffusible pigments, but no melanoid pigments, are produced in some media.

3. Physiological Properties

The physiological properties of *S. spadicogriseus* were examined according to the methods described by SHIRLING and GOTTLIEB[6], WAKSMAN[7], and SKERMAN[10]. The results are shown in Table 2. It grows at 18~39°C, the preferred temperature range being 25~35°C. The culture shows positive reactions in peptonization of milk, liquefaction of gelatin, hydrolysis of starch and nitrate reduction, but negative reactions in melanin production, tyrosinase formation, cellulose decomposition and coagulation of milk. The microorganism belongs, therefore, to the mesophilic and nonchromogenic kind of *Streptomyces*.

Table 2. Physiological properties of *S. spadicogriseus*.

Temperature range for growth	18~39°C
Peptonization of milk	+
Liquefaction of gelatin	+
Hydrolysis of starch	+
Nitrate reduction	+
Melanin production	−
Tyrosinase reaction	−
Cellulose decomposition	−
Coagulation of milk	−

4. Carbon Utilization

The utilization of carbon sources was determined according to the procedures of SHIRLING and GOTTLIEB[6], and PRIDHAM and GOTTLIEB[11]. The results were comparable with both methods. As shown in Table 3, glucose, xylose, arabinose, fructose, galactose and mannitol were utilized by *S. spadicogriseus*. Utilization of salicin was doubtful, and there was no growth on rhamnose, raffinose, inositol or sucrose.

The above-stated taxonomic studies demonstrate that *S. spadicogriseus* can be classified into Table 17. 42i (S; C−; H)[9] in the Gray Series of PRIDHAM and TRESNER, because its spore chain morphology belongs to Section Spirales (S), melanoid pigments are not produced (C−), and spore surface is hairy (H). As summarized in Tables 3 and 4, *S. spadicogriseus* can be differentiated from all other members in Table 17. 42i in cultural characteristics, carbon utilization and antibiotic production. The differences between *S. spadicogriseus* and other known members in Table 17. 42i (*S. calvus, S. cyanoalbus, S. finlayi, S. flaveolus, S. geysiriensis, S. herbiferis* and *S. pactum*) are as follows:

Streptomyces calvus[9,12] shows sparse formation of aerial mycelium on most media and excellent growth on CZAPEK's solution agar, whereas *S. spadicogriseus* shows good formation of aerial mycelium

Table 3. Comparison of carbon utilization*.

Carbon source	*S. calvus*[9,12]	*S. cyanoalbus*[9,13]	*S. finlayi*[9,13]	*S. flaveolus*[9,14]	*S. herbiferis*[9]	*S. pactum*[9,17,18]	*S. spadicogriseus*
D-Glucose	+	+	+	+	+	+	+
D-Xylose	+	+	+	+	+	−	+
L-Arabinose	+	+	+	+	+	−	+
L-Rhamnose	+	+	+	+	+	−	−
D-Fructose	+	+	−	+		−	+
D-Galactose	+	+		+	+	+	+
Raffinose	+	+ or ±	−	+ or ±	+	−	−
D-Mannitol	+	+	−	+	+	−	+
i-Inositol	+	+ or −	−	+	−	−	−
Salicin	+	−		+		−	±
Sucrose	+	+	±	+	+	−	−

* Except for *S. spadicogriseus*, description is based on published data[9,12~14,17,18].

Table 4. Comparison of cultural characteristics and antibiotic production*.

Species	Cultural characteristics	Antibiotic produced
S. calvus[9,12]	Sparse formation of aerial mycelium on most media. Excellent growth on CZAPEK's solution agar.	Nucleocidin
S. cyanoalbus[9,13]	Grayed yellow to grayed yellow-green, or grayed yellow-green to blue substrate mycelium. Excellent growth on CZAPEK's solution agar.	
S. finlayi[9,13]	Color of substrate mycelium is grayed yellow to yellow-brown modified by green.	
S. flaveolus[9,14,15]	On synthetic agar, aerial mycelium white to ash-gray and substrate mycelium light sulfur-yellow to cadmium-yellow. No distinctive pigments on complex media at the reverse side of colony. Excellent growth on CZAPEK's solution agar.	Actinomycin (some strains)
S. geysiriensis[9,16]	Produces gray spores. Moderate growth on CZAPEK's solution agar.	Moenomycin
S. herbiferis[9]	Grass-green substrate mycelium on some media.	
S. pactum[9,17,18]	Gray to bluish-gray aerial growth and grayish-olive reverse on most media.	Pactamycin
S. spadicogriseus	Color of aerial and substrate mycelia is predominantly light brownish gray. Poor and thin growth on CZAPEK's solution agar.	Anthramycin

* Except for *S. spadicogriseus*, descriptions based on published data[9,12~18].

on most media and poor growth on CZAPEK's solution agar. The former utilizes rhamnose, raffinose, inositol and sucrose for growth, but the latter does not. *Streptomyces calvus* produces nucleocidin, an antitrypanosomal antibiotic.

Color of substrate mycelium of *S. cyanoalbus*[9,13] is grayed yellow to grayed yellow-green on oatmeal agar and yeast extract-malt extract agar, or grayed yellow-green to blue on inorganic salts-starch agar and glycerol-asparagine agar, while that of *S. spadicogriseus* is light brownish gray, brownish gray, pale yellowish brown and dark yellow respectively on these media, showing no green or blue color. The growth of the former on CZAPEK's solution agar is excellent and aerial mycelium is poorly developed on oatmeal agar, whereas that of the latter is poor on CZAPEK's solution agar and good on oatmeal agar. *Streptomyces cyanoalbus* utilizes rhamnose and sucrose, but *S. spadicogriseus* does not.

Color of substrate mycelium of *S. finlayi*[9,13] is grayed yellow to yellow-brown modified by green on oatmeal agar, yeast extract-meat extract agar, inorganic salts-starch agar and glycerol-asparagine agar. In contrast, that of *S. spadicogriseus* is light brownish gray, brownish gray, pale yellowish brown and dark yellow respectively on these media. Rhamnose is utilized by *S. finlayi*, but not by *S. spadicogriseus*; fructose and mannitol are utilized by *S. spadicogriseus*, but not by *S. finlayi*.

On CZAPEK's solution agar, the growth of *S. flaveolus*[9,14,15] is excellent; color of aerial mycelium is white to ash-gray and that of substrate mycelium is light sulfur-yellow to cadmium-yellow. Aerial mycelium is poorly developed on yeast extract-meat extract agar, inorganic salts-starch agar, or glycerol-asparagine agar. On the contrary, the growth of *S. spadicogriseus* is poor on CZAPEK's solution agar and color of mycelia is light brownish gray, and the development of aerial mycelium is abundant and powdery on yeast extract-meat extract agar, inorganic salts-starch agar, or glycerol-asparagine agar. *Streptomyces flaveolus* utilizes rhamnose, raffinose, inositol and sucrose, but *S. spadicogriseus* does not.

Color of spores of *S. geysiriensis*[9,16] is gray, and the growth is moderate on CZAPEK's solution agar. *Streptomyces geysiriensis* produces moenomycin, an antibacterial antibiotic.

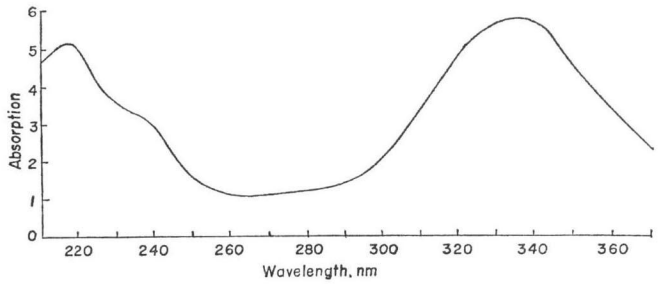
Streptomyces herbiferis[9] forms grass-green vegetative mycelium on some media and utilizes rhamnose, raffinose and sucrose, but *S. spadicogriseus* does not.

Aerial mycelium of *S. pactum*[9,17,18] is gray to bluish gray on most media. No distinctive pigments can be noted on the reverse side of colonies on yeast extract-malt extract agar, oatmeal agar, inorganic salts-starch agar, or glycerol-asparagine agar. Xylose, arabinose, fructose and mannitol are not utilized by *S. pactum*, while these sugars are utilized by *S. spadicogriseus*. *Streptomyces pactum* produces pactamycin, an antibacterial and antitumor antibiotic, which is different from anthramycin.

5. Production and Isolation of Anthramycin

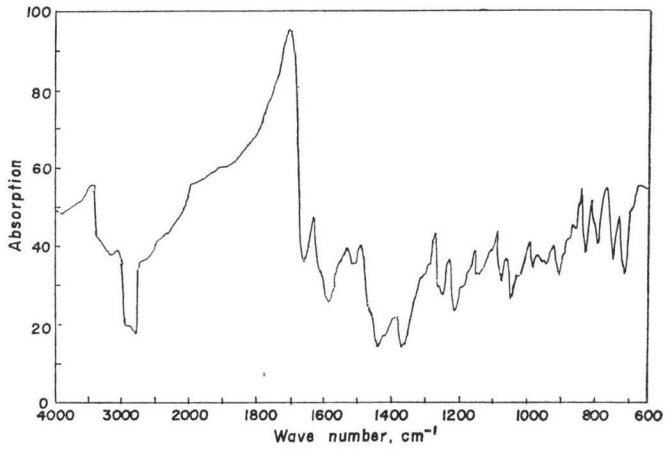
A stock culture of *S. spadicogriseus* was used to inoculate a seed medium composed of 0.5% meat extract, 0.5% peptone, 2.0% glucose and 0.5% sodium chloride (pH 7.2), and grown on reciprocal shaker at 27°C for 72 hours. The culture was transferred into a 30-liter jar fermentor containing 20 liters of the same medium and grown at 30°C for 3 days under submerged aerated conditions. The resulting fermented broth was filtered. The filtrate (15 liters) was treated with 750 g of activated charcoal to adsorb the antitumor substance. Assays were carried out according to the method described by STEFANOVICH and CEPRINI[19]. The carbon cake was collected by filtration and eluted with 80% aqueous acetone (750 ml). The eluate was concentrated to about one-fifth (150 ml) of the eluate under

Fig. 1. UV spectrum of antitumor substance produced by *S. spadicogriseus*.



reduced pressure. The concentrate was extracted with equal volume of butanol. The butanol layer was evaporated under reduced pressure, and the residue was dissolved in a methanol - chloroform (1 : 9) mixture. The solution was applied to a silica gel (Kieselgel G, Merck) column chromatography. The column was eluted with the chloroform - methanol mixture, and the active fractions were collected and concentrated. The residue was crystallized from methanol - water to yield 0.71 g of pure pale yellow needles, mp 198~201°C (dec.). Anal. calcd. for $C_{17}H_{19}N_3O_4 \cdot H_2O$: C 58.78, H 6.09, N 12.10. Found: C 59.07, H 6.10, N 12.20. Mol. wt.: 347. Specific rotation: $[\alpha]_D^{25}$ +990° (*c* 1, DMSO). The UV and IR spectra are shown Figs. 1 and 2. LD_{50}: 0.73 mg/kg (male ddY mice, ip).

Fig. 2. IR spectrum of antitumor substance produced by *S. spadicogriseus* (KBr).



Antitumor activity of the purified substance was tested against ascitic form of sarcoma 180. Sarcoma 180 cells (2×10^6 cells/0.2 ml) were transplanted intraperitoneally into ddY mice weighing about 25 g. Treatment was initiated 24 hours after tumor inoculation, and continued once daily for 5 days by the intraperitoneal administration. A marked prolongation in the survival period of treated mice could be observed by the administration of 0.04 mg/kg/day.

These data indicate that the antitumor substance isolated from culture filtrate of *S. spadicogriseus* is the same as anthramycin methyl ether isolated from those of *S. refuineus* var. *thermotolerans*[1~3].

Discussion

Streptomyces spadicogriseus can be differentiated from the known producer of anthramycin: *S. refuineus* var. *thermotolerans* (strains NRRL 3143 and NRRL 3144)[20]. The spore surface of the former is hairy, while that of the latter is irregularly rough to warty. *Streptomyces spadicogriseus* is mesophilic, and grows at 28°C but not at 45°C, the preferred temperature range being 25~35°C. On the contrary, *S. refuineus* var. *thermotolerans* reportedly is a facultative thermophile, and grows at both 28°C and 45°C, preferably from about 45°C to about 50°C. The aerial mycelium of *S. spadicogriseus* is predominantly light brownish gray, whereas that of *S. refuineus* var. *thermotolerans* is white, cream-yellow, or medium gray to deep gray-green. The former grows abundantly on carrot plug and potato plug, but the latter does not. Thus, *S. spadicogriseus* represents another species producing anthramycin.

Among known species of *Streptomyces*, *S. spadicogriseus* shares several primary key characteristics with the members listed in Table 17. 42i (Section Spirales; melanoid pigments not produced; spore surface hairy) in the Gray Series of PRIDHAM and TRESNER[9]. However, as already mentioned and summarized in Tables 3 and 4, *S. spadicogriseus* differs from those species in its carbohydrate utilization pattern, several cultural characteristics, and qualitatively in antibiotic production. *Streptomyces spadicogriseus* is, therefore, considered to be a new species.

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