

A NEW ANTITUMOR ANTIBIOTIC, CHROMOXYMYCIN

I. TAXONOMIC STUDIES ON THE PRODUCING STRAIN:
A NEW SUBSPECIES OF THE GENUS *STREPTOMYCES*MORITA IWAMI, YOSHIO KAWAI, SUMIO KIYOTO, HIROSHI TERANO,
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A new subspecies of the genus *Streptomyces*, for which the name *S. libani* subsp. *rubropurpureus* subsp. nov. is proposed, is described. Soil isolate, strain No. 6362, produces a new antitumor substance chromoxymycin. The strain has a gray or red spore mass color, spiral spore chains with smooth spores, is nonchromogenic, and utilizes most carbon sources. Strain No. 6362 is most closely related to *S. libani*, although there are differences in colorization of reverse colony and spore chain morphology between each other. Those differences are not enough to establish a new species. It is suitable to consider that strain No. 6362 is identified as a new subspecies in the species of *S. libani*.

In the course of a screening for new antitumor substances, chromoxymycin was discovered in the fermentation broth of actinomycete strain No. 6362 which was isolated from a soil sample. This report deals with the description of the producing organism and a discussion on its taxonomic position.

Materials and Methods

Bacterial Strain

Strain No. 6362 was isolated from a soil sample obtained from Kawachinagano-shi, Osaka Prefecture. *Streptomyces libani* subsp. *libani* IFO 13452 and *Streptomyces pulcher* IFO 13462 were obtained from the Institute for Fermentation, Osaka (IFO), Japan. *Streptomyces libani* subsp. *rufus* JCM 4325 was obtained from the Japan Collection of Microorganisms (JCM), Japan.

Morphological Characterization

The aerial mycelium of the organism grown on oatmeal agar, yeast extract - malt extract agar or inorganic salts - starch agar was examined directly under the optical microscope. Spore surface examination was observed with electron microscopes.

Cultural and Physiological Characterizations

For cultural and physiological characterizations, certain media recommended by WAKSMAN¹⁾ and the ISP²⁾ were used. Color names used in this study were based on the Color Standard of Nihon Shikisai, Co., Ltd.³⁾ Utilization of carbohydrates was determined by the method of PRIDHAM and GOTTLIEB⁴⁾. The cultures were incubated for 14 days at 30°C.

Growth-permissible temperature range and optimum growth temperature were determined on yeast extract - malt extract agar using a model TN-3 temperature gradient incubator (Toyo Kagaku Sangyo Co., Ltd.).

Cell Wall Analysis

Cell wall analysis was performed according to the method of BECKER *et al.*⁵⁾. Cell wall prepara-

tion was obtained by the method of YAMAGUCHI⁹⁾.

Results

Morphological Characteristics

The mature sporophores formed loose spirals and rather long chains (Fig. 1). The spores were short-cylindrical or oval, and $0.5\sim 0.7\times 0.7\sim 0.9\ \mu\text{m}$ in size. The spore surfaces were smooth by electron microscope examinations (Figs. 2 and 3). No fragmentation of substrate mycelium was observed in agar media or submerged culture. No sporangia or zoospores are observed.

Cultural Characteristics

Cultural characteristics of strain No. 6362 studied with various media are shown in Table 1. Aerial mycelium formed on oatmeal agar, yeast extract - malt extract agar or inorganic salts - starch agar was in the gray or red color series. Reverse side of colony was colored by red purple on inorganic salts - starch agar or potato - dextrose agar. Reverse mycelium pigment was a pH indicator, changing from red purple to brown with the addition of 0.05 N HCl. Soluble pigment was produced in sucrose - nitrate agar or nutrient agar.

Fig. 1. Aerial mycelium of strain No. 6362 on yeast extract - malt extract agar (incubated for 14 days at 30°C).

The organism was observed with an optical microscope ($\times 800$).

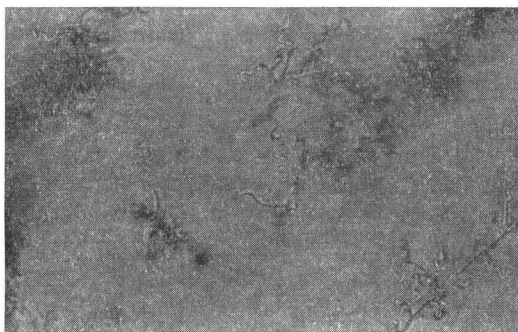
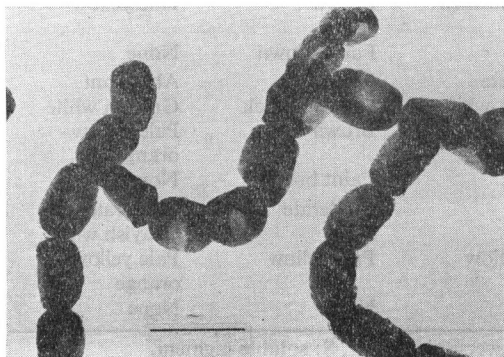


Fig. 2. Transmission electron micrograph of spore chains of strain No. 6362 on yeast extract - malt extract agar (incubated for 14 days at 30°C).

Bar represents $1\ \mu\text{m}$.



Physiological Characteristics

Physiological characteristics of strain No. 6362 are summarized in Table 2. The growth-permissible temperature range was from 14°C to 42°C. The optimum growth temperature of the strain was 32°C. Melanoid pigment was not produced in tyrosine agar, peptone - yeast extract - iron agar or Tryptone - yeast extract broth. The organism could grow on yeast extract - malt extract agar supplemented with 10% NaCl. Nitrate reduction of the strain was positive.

Fig. 3. Scanning electron micrograph of spore chains of strain No. 6362 on yeast extract - malt extract agar (incubated for 14 days at 30°C).

Bar represents $5\ \mu\text{m}$.



Table 1. Cultural characteristics of strain No. 6362, *Streptomyces libani* subsp. *libani* IFO 13452, *Streptomyces libani* subsp. *rufus* JCM 4325 and *Streptomyces pulcher* IFO 13462.

	No. 6362	IFO 13452	JCM 4325	IFO 13462
Oatmeal agar	G: Moderate	Moderate	Poor	Poor
	A: Pale reddish brown	Pale reddish brown	Reddish brown	Olive gray
	R: Colorless	Pale yellow	Colorless to pale pink	Colorless
	S: None	None	None	None
Yeast extract - malt extract agar	G: Abundant	Abundant	Abundant	Abundant
	A: Pale reddish brown	Grayish white to pale brown	Gray to black	Gray
	R: Pale yellowish brown	Dark yellow orange	Dark orange	Yellowish gray
	S: None	None	Faint brown	None
Inorganic salts - starch agar	G: Abundant	Abundant	Moderate	Moderate
	A: Grayish yellow brown	Pale reddish brown	Brown to black	Dark olive gray
	R: Grayish red purple	Pale yellow	Pale yellow orange	Colorless
	S: None	None	None	None
Glucose - asparagine agar	G: Abundant	Moderate	Moderate	Moderate
	A: Pale reddish brown	Grayish white	Gray to brown	Grayish white
	R: Pale yellow orange	Pale yellow orange	Brown	Pale yellow
	S: None	None	None	None
Glycerol - asparagine agar	G: Abundant	Abundant	Moderate	Moderate
	A: Pale yellow orange to light gray	Grayish white to grayish yellow	Grayish brown	Olive gray
	R: Reddish orange	Pale yellow orange	Pale yellowish brown	Pale yellow
	S: None	Pale yellow orange	Faint brown	None
Sucrose - nitrate agar	G: Abundant	Abundant	Moderate	Poor
	A: Pale pink to grayish white	White	Gray to light purplish gray	Grayish white
	R: Dark brown	Yellowish white	Black	Pale yellow
	S: Faint brown	None	None	None
Nutrient agar	G: Moderate	Moderate	Moderate	Moderate
	A: Pale reddish brown	Grayish white	Poor, grayish white	Grayish white
	R: Pale yellow orange	Pale yellow	Pale yellow	Pale pink
	S: Pale yellow orange	None	None	None
Potato - dextrose agar	G: Moderate	Moderate	Abundant	Moderate
	A: Poor, light gray	Grayish white	Gray to black	None
	R: Deep red purple	Pale yellowish brown	Brown	Pale yellow
	S: None	None	Faint brown	None
Tyrosine agar	G: Abundant	Moderate	Moderate	Abundant
	A: Pale cinnamon pink	Grayish white	Gray to black	Grayish white
	R: Dark red	Pale yellow brown	Brown	Pale yellow orange
	S: None	None	Faint brown	None
Peptone - yeast extract - iron agar	G: Moderate	Poor	Moderate	Moderate
	A: None	None	None	Grayish white
	R: Pale yellow	Pale yellow	Pale yellow	Pale yellow orange
	S: None	None	None	None

Abbreviation: G; Growth, A; aerial mass color, R; reverse side color, S; soluble pigment.

Table 2. Physiological characteristics of strain No. 6362, *Streptomyces libani* subsp. *libani* IFO 13452, *Streptomyces libani* subsp. *rufus* JCM 4325 and *Streptomyces pulcher* IFO 13462.

	No. 6362	IFO 13452	JCM 4325	IFO 13462
Temperature range for growth	14~42°C	13~36°C	15~37°C	14~43°C
Optimum temperature	32°C	28°C	29°C	33°C
Nitrate reduction	+	—	—	—
Starch hydrolysis	—	+	+	+
Milk coagulation	—	—	—	—
Milk peptonization	—	—	—	—
Melanin production	—	—	—	—
Gelatin liquefaction	—	—	—	—
H ₂ S production	—	—	—	—
Urease activity	+	—	—	—
NaCl tolerance	>10%	>10%	>7%, <10%	>7%, <10%

— Negative, + positive.

Table 3. Carbohydrate utilization of strain No. 6362, *Streptomyces libani* subsp. *libani* IFO 13452, *Streptomyces libani* subsp. *rufus* JCM 4325 and *Streptomyces pulcher* IFO 13462.

	No. 6362	IFO 13452	JCM 4325	IFO 13462
D-Glucose	+	+	+	+
Sucrose	+	+	+	—
Glycerol	+	+	+	+
D-Xylose	+	±	+	+
D-Fructose	+	+	+	+
Lactose	+	+	+	+
Maltose	+	+	+	+
Rhamnose	+	—	±	+
Raffinose	+	+	+	—
D-Galactose	+	+	+	+
L-Arabinose	+	+	±	+
D-Mannose	+	+	+	+
D-Trehalose	+	+	+	+
Inositol	+	+	+	—
Mannitol	+	+	+	+
Inulin	+	±	—	—
Cellulose	—	—	—	—
Salicin	+	+	±	±
Chitin	—	—	—	—
Sodium citrate	+	—	+	+
Sodium succinate	+	+	+	+
Sodium acetate	+	—	—	±

Symbols: +; Utilization, ±; doubtful utilization, —; no utilization.

The pattern of carbohydrates utilization by the strain is shown in Table 3. The strain was able to utilize all carbohydrates with the exception of cellulose and chitin.

Cell Wall Analysis

Strain No. 6362 contained LL-diaminopimelic acid and glycine in the cell wall. According to the chemical composition the strain is cell wall Type I.

Discussion

The above mentioned morphological characteristics and cell wall analysis of strain No. 6362

indicate that the strain belongs to the genus *Streptomyces*. Furthermore, from these results, the organism was assigned to the gray (Gy), spiral (S), nonchromogenic (C⁻), and glabrous (SM) groups as given in BERGEY'S Manual of Determinative Bacteriology, 8th Ed.⁷⁾ and to the gray (Gy), melanoid pigments (none, O), reverse-side pigments (various, V), spirals (S), and smooth spore surface (sm) groups in NONOMURA'S key⁸⁾.

Cultural and physiological characteristics and carbon utilization pattern of the strain No. 6362 were compared with those of various species selected for a comparative study^{7,9,10,11)}. Strain No. 6362 was most similar to *Streptomyces libani* subsp. *libani* Baldacci and Grein 1966, *Streptomyces libani* subsp. *rufus* Baldacci and Grein 1966 and *Streptomyces pulcher* Routien 1967 (nonapproved), although there were some differences in cultural and physiological characteristics (see, Tables 1~3).

Cultural characteristics of strain No. 6362 are different from those of *S. libani* subsp. *libani* on inorganic salts - starch agar, glucose - asparagine agar, sucrose - nitrate agar or potato - dextrose agar. Nitrate reduction, starch hydrolysis and urease activity of strain No. 6362 are different from those of *S. libani* subsp. *libani*. Differences are also observed in the utilization of rhamnose, sodium citrate and sodium acetate.

Spore chains of *S. libani* subsp. *rufus* are shorter than those of strain No. 6362. *S. libani* subsp. *rufus* forms hygroscopic spore mass on yeast extract - malt extract agar, inorganic salts - starch agar, potato - dextrose agar or tyrosine agar; strain No. 6362 does not form this mass. Nitrate reduction, starch hydrolysis and NaCl tolerance of *S. libani* subsp. *rufus* are different from those of the strain No. 6362. In carbon utilization, *S. libani* subsp. *rufus* is not able to utilize inulin and sodium acetate, while strain No. 6362 can utilize those carbohydrates.

Aerial mass colors of *S. pulcher* are different from those of the strain No. 6362 on eight media examined. Reverse side colors of *S. pulcher* are different from those of the strain on inorganic salts - starch agar, sucrose - nitrate agar and tyrosine agar. In physiological characteristics, starch hydrolysis, nitrate reduction, urease activity and NaCl tolerance of *S. pulcher* are different from those of the strain No. 6362. Differences are also observed in carbon utilization of sucrose, raffinose, inositol and inulin.

Considering results of comparative studies of strain No. 6362 with the type cultures of the species described above, strain No. 6362 is most closely related to *S. libani* subsp. *libani*. However, there are some differences in those morphological, cultural and physiological characteristics between each other. Those differences are not enough to establish a new species in the genus *Streptomyces*. It is appropriate to consider that strain No. 6362 is identified as a new subspecies in the species *S. libani*, for which the name *Streptomyces libani* subsp. *rubropurpureus* subsp. nov. (*rub. ro. pur. pur'e. us. L. adj. ruber red; L. adj. purpureus purple; M.L. adj. rubropurpureus red purple colored.*, referring to the color of vegetative mycelium on inorganic salts - starch agar or sucrose - nitrate agar) is proposed.

Type strain: Strain No. 6362. A culture of the strain has been deposited at the Fermentation Research Institute, Agency of Industrial Science and Technology, Japan and assigned as *Streptomyces libani* subsp. *rubropurpureus* No. 6362 with the accession number FERM-P 7209. The description given above for the type strain also serves for the present as the subspecies description.

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