DC-86-M, A NOVEL ANTITUMOR ANTIBIOTIC

I. TAXONOMY OF PRODUCING ORGANISM AND FERMENTATION

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A novel antibiotic, DC-86-M was isolated from the culture broth of a new isolate, DO-86, from the soil sample collected in Machida-shi, Japan. The producing organism was found to belong to *Streptomycetes*, for it formed aerial mycelia and chains of spores and its cell wall analyses revealed the presence of LL-diaminopimelic acid. The morphological, cultural and physiological characteristics of the strain DO-86 resemble closely those of *Streptomyces luteogriseus* and we concluded that the strain DO-86 could be designated as *Streptomyces luteogriseus* DO-86. The antibiotic was produced in the fermentation medium consisting of lactose 20 g, glucose 10 g, Pharmamedia 15 g, yeast extract 5 g, meat extract 10 g and $CaCO_3$ 2 g per liter of tap water.

In the course of our screening program for new antitumor antibiotics, an actinomycete (strain DO-86) isolated from a local soil sample was found to produce novel antibiotics (DC-86-Y and DC-86-M). The taxonomical studies and their production were studied in this paper, and their isolation, purification and biological activities were studied in the following paper[1].

Materials and Methods

Cell Wall Analyses

Cell wall analyses were performed on cultures grown in SR-broth medium (glucose 10 g, starch 10 g, beef extract 3 g, yeast extract 5 g, $CaCO_3$ 2 g, pH 7.0, per liter of tap water) for 48 hours at 28°C. The mycelia were collected by centrifugation and were washed three times with distilled water. They were hydrolyzed in 6 N hydrochloric acid solution for 15 minutes at 121°C, and developed on a cellulose thin layer chromatogram with the solvent system, MeOH - H_2O - 1.0 N HCl - pyridine (160: 35: 5: 20).

Cultural and Physiological Characterization

The methods and media recommended by the International Streptomyces Project (ISP)[2] for characterization of the *Streptomyces* species were employed. Color codes were assigned to the reverse pigments and aerial mass pigments according to the Color Harmony Manual, 4th Ed., 1958 (Container Corporation of America, Chicago).

Taxonomy of the Producing Organism

Morphological Characteristics

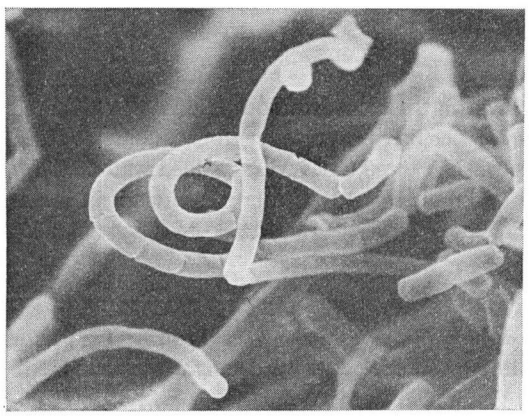
Strain DO-86 produced well developed aerial mycelia, branched but not fragmented, with the sporophores hooked to loose coils of one to three turns. It was therefore placed in the *Retinaculum-*

Table 1. Cultural properties of strain DO-86.

Medium	Growth	Aerial mass	Color of substrate mycelium	Soluble pigment
Sucrose - nitrate agar	Good	Abundant, white (a) to bisque (3ec)	Lt wheat (2ea) to bamboo (2gc)	None
Glucose - asparagine agar	Good	Fair, white (a) to biscuit ecru (2ec)	Lt ivory (2ca) to bamboo (2gc)	None
Inorganic salt - starch agar (ISP 4)	Good	Abundant, white (a) to silver gray (3fe)	Camel (3ie) to bamboo (2gc)	None
Nutrient agar	Good	None	Colorless to bamboo (2gc)	None
Tyrosine agar (ISP 7)	Good	Abundant, white (a) to lt wheat (2ea)	Natural string (2dc) to sepia brown (3pn)	Sepia brown (3pn)*
Oatmeal agar (ISP 3)	Good	Abundant, white (a) to natural (2dc)	Lt olive drab (1 1/2li) to mustard brown (2ni)	Mustard brown (2pi)
Glycerol - asparagine agar (ISP 5)	Good	Abundant, natural string (2dc) to silver gray (3fe)	Covert tan (2ge) to camel (3ge)	None
Yeast extract - malt extract agar (ISP 2)	Good	Abundant, white (a) to beige gray (3ih)	Camel (3ie) to topaz (3ne)	Adobe brown (3lg)*
Peptone - yeast extract - iron agar (ISP 6)	Moderate	None	Lt olive gray (1 1/2ge)	Sepia brown (3pn)
EMERSON's agar	Good	Scant, white (a)	Camel (3ie) to adobe brown (3lg)	Clove brown (3pl)

Incubation at 28°C for 2 weeks.
* Soluble pigment is produced but lighter than each color name.

Fig. 1. Scanning electron micrograph of 7-day growth of *Streptomyces luteogriseus* DO-86 on sucrose - nitrate agar.
Bar 1 μm.



Apertum (RA) or *Spira* (S) section of the classification by PRIDHAM *et al.*[3]. This morphology was readily observed on all media which supported the formation of aerial mycelia, for example sucrose - nitrate agar, starch agar, yeast extract - malt extract agar, oatmeal agar, and tap-water agar.

The sporophores bore chains of 10 to 20 or occasionally more spores. Scanning electron micrographs indicated that the spore was cylindrical, from 1.1 to 2.0 μm by 0.5 to 0.4 μm in size, and with smooth surfaces (Fig. 1). No sporangia, motile spore, vegetative mycelial spore, or sclerotium was observed.

Cultural Characteristics

Cultural characteristics of strain DO-86 on various agar media are shown in Table 1. The color of the aerial mass abundantly produced on most media was identified as belonging to the gray color-series of TRESNER and BACKUS[4]. Strain DO-86 produced branched substrate mycelium which varied from cream to olive or brownish color, depending on the medium. A brown water-soluble pigment was occasionally produced.

Table 2. Physiological characteristics of strain DO-86.

Liquefaction of gelatin	Negative
Peptonization of milk	Positive
Coagulation of milk	Negative
Starch hydrolysis	Positive
Melanine formation	Positive
Temperature range	27~42°C
Optimum temperature	34°C

Table 3. Utilization of carbon sources by strain DO-86.

Carbon source	Response
No carbon source	−
D-Glucose	++
D-Xylose	++
D-Mannitol	++
D-Fructose	+
L-Arabinose	+
Sucrose	+
i-Inositol	++
L-Rhamnose	+
Raffinose	++
Cellulose	±

Physiological Characteristics

Various carbon sources were utilized, except only a trace of growth on cellulose in PRIDHAM and GOTTLIEB's basal medium. The optimum growth temperature of the strain was around 34°C. The growth-permissible temperature range was from 27°C to 42°C. A melanoid pigment was produced in tyrosine agar and peptone - yeast extract - iron agar medium.

Analysis of the whole cell hydrolysate by thin-layer chromatography demonstrated the presence of LL-diaminopimelic acid. No *meso*-isomer was detected. This indicated the cell wall belonged to cell wall type I[5].

Species Determination

The morphology and chemical characteristics of strain DO-86 permitted a clear assignment of the organism to the genus *Streptomyces*.

The strain DO-86 was compared with *Streptomyces* species recognized in the approved list of

Table 4. Comparison of characteristics by strain DO-86 and related species.

	Species				
	S. regensis	*S. bottropensis*	*S. luteogriseus*	*S. violaceo-chromogenes*	DO-86
Aerial mass color	Gy Y	Gy	Gy	RGy	Gy (W)
Melanoid pigment	+	+	+	+	+
Soluble pigment	+	+	+	+	+
Spore chain	SRF	S	S	SRA	S
Spore surface	sm	sm	sm	sm	sm
Arabinose	+	+	+	+	+
Xylose	+	+	+	+	+
Inositol	+	+	+	+	+
Mannitol	+	+	+	+	+
Fructose	+	+	+	+	+
Rhamnose	+	+	+	+	+
Sucrose	+	+	+	+	+
Raffinose	+	+	+	+	+
Color of substrate mycelium*	st-dk-Y, st-Br	Y to st-Br, R-Br to dk-Br	st-Y-Br, dk-Br	Gy-Y to st-Br	Y-Br, Br

This table is quoted from H. NONOMURA[11].

Gy; Gray, Y; yellow, R; red, W; white, st; strong, dk; dark, Br; brown, S; *Spira*, RA; *Retinaculum-Apertum*, RF; *Rectiflexibiles*, sm; smooth.

* These characters were shown on glycerol - asparagine agar, yeast extract - malt extract agar, oatmeal agar and/or inorganic salt - starch agar medium.

bacterial names[6] and subsequently validly published species, depending upon the description in ISP reports by SHIRLING and GOTTLIEB[7~10] and NONOMURA's key for classification[11].

The four *Streptomyces* species listed in Table 4 were reported as belonging to the gray color series, with flexuous to spiral sporophore morphology, smooth surface ornamentation, production both of soluble pigment and melanoid pigment, and a carbon utilization pattern similar to that of DO-86 as shown in Table 3. Strain DO-86 is different from three species in the following properties as summarized in Table 4.

S. regensis and *S. violaceochromogenes*: The color of the substrate mycelium contains yellowish or reddish color on starch agar, glycerol - asparagine agar, yeast extract - malt extract agar, and oatmeal agar.

S. bottropensis: The color of reverse side of colony sometimes shows reddish brown.

Characters of DO-86 were virtually identical to those of *S. luteogriseus* D. SCHMITZ, K. E. CROOK, Jr. and I. R. HOOPER, 1964.

Therefore, DO-86 was classified as a strain of *Streptomyces luteogriseus* and named *Streptomyces luteogriseus* DC-86.

Fermentation

Seed flasks were inoculated with stock cultures maintained in a deep freezer (−70°C) and grown for 48~72 hours at 28°C. The seed medium consisted of glucose 10 g, soluble starch 10 g, yeast extract 5 g, Bacto-Tryptone 5 g, beef extract 3 g and $CaCO_3$ 2 g per liter of tap water. A 5% vegetative seed was used to inoculate the fermentation medium consisting of lactose 20 g, glucose 10 g, Pharmamedia 15 g, yeast extract 5 g, meat extract 10 g and $CaCO_3$ 2 g per liter of tap water. The pH of media was adjusted to 7.2 prior to sterilization. Total antibacterial activity reached a maximum at 65 hours of incubation. The activity was determined by the following procedure. After 10 ml of culture broth was centrifuged at $1,200 \times g$ for 15 minutes, the clarified solution was decanted. To packed cells in a test tube, 5 ml of acetone was added and mixed vigorously. After centrifugation at $1,200 \times g$ for 10 minutes, the acetone extract was obtained. Activities were measured by paper disc method on nutrient agar using *Bacillus subtilis* as the test organism.

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