THE ANTIBIOTIC YA-56 COMPLEX: TAXONOMY AND PRODUCTION OF THE PRODUCING STRAIN

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A new complex of phleomycin-bleomycin group antibiotics, YA-56, was isolated from the culture broth of a streptomycete designated as strain MCRL 0387 and identified as a new variety of *Streptomyces humidus* NAKAZAWA and SHIBATA in IMAMURA *et al.*, 1956. Ultra-violet irradiation of strain MCRL 0387 gave a high-yielding mutant which formed blue aerial mycelium instead of the grayish aerial mycelium of the original strain. Fermentative production of the antibiotics YA-56 complex is described.

In the course of screening for new antibiotics, a complex of antibacterial and antitumor antibiotics was obtained from the culture broth of a streptomycete. This complex, named YA-56, consisted of two main components designated YA-56 X and XA-56 Y and at least three other minor components. Further investigation indicated these belonged to the phleomycin-bleomycin group. The present paper deals with the taxonomy and antibiotic production of the producing strain. Isolation and characterization of the main components of the antibiotic YA-56 complex will be reported in a succeeding paper. The antibiotic YA-56 complex-producing strain was characterized by the formation of hygroscopic masses of smooth-walled spores in coils on aerial mycelia. As a result of taxonomic study, the strain was considered as a new variety of *Streptomyces humidus*, for which the name *Streptomyces humidus* var. *antitumoris* FURUMAI and OKUDA var. nov. is proposed. The type strain has been deposited in the Northern Utilization Research Division, Agricultural Research Service, U.S. Department of Agriculture, Peoria, Illinois, U.S.A. and the Fermentation Research Institute, Chiba, Japan, and accessioned as NRRL 3885 and FERM-P No. 365, respectively. Later, in the course of selecting for a strain with high productivity of the antibiotic YA-56 complex, a mutant (MCRL 0432) forming blue aerial mycelium was obtained with ultra-violet irradiation. Unlike the original strain, the mutant strain gave no hygroscpic masses of spores on any agar medium but did form coiled but did form coiled chains of smooth-walled spores.

Characterization of *Streptomyces humidus* var. *antitumoris* Strain MCRL 0387

Taxonomic studies were generally carried out in accordance with methods adopted

by the International Streptomyces Project (ISP)[1]. The media used in these studies were prepared according to the recommendations of SHIRLING and GOTTLIEB[1], and WAKSMAN[2]. The proposed varietal epithet "*antitumoris*" is the modern Latin adjective meaning "against tumor". A detailed characterization of the new taxon is as follows:

Morphological Characteristics.

Strain MCRL 0387 showed good growth and formed grayish powdery aerial mycelia on various agar media. On yeast extract-malt extract agar oatmeal agar and BENNETT's agar, hygroscopic masses were formed in spots on the wefts of aerial mycelium. The hygroscopic masses sometimes spread over the whole surface of the cultures. On observation with the microscope, the aerial mycelia showed the presence of open spirals. Formation of true whorls was not observed. Not less than ten oval, phalangioform spores, 0.8 to 1.0 μ by 1.2 to 1.4 μ, were formed in chains. The surfaces of the spores were smooth (Plates 1 and 2).

Plate 1. Photomicrograph of strain MCRL 0387. (Yeast extract-malt extract agar, ×800)

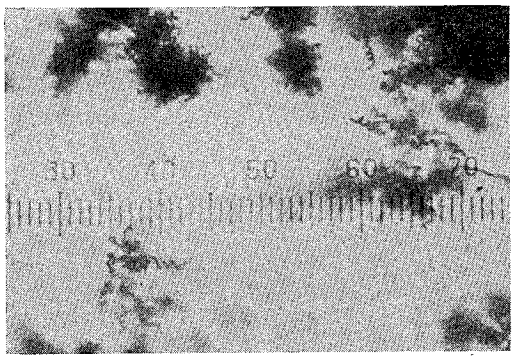
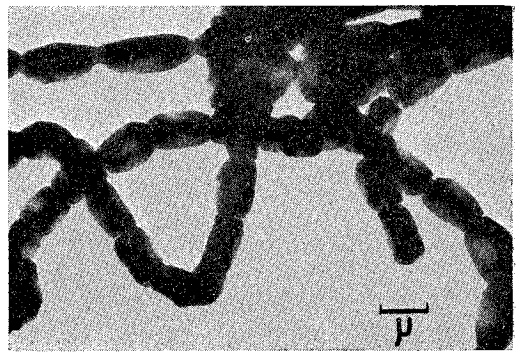


Plate 2. Electron micrograph of strain MCRL 0387. (Yeast extract-malt extract agar, ×10,000)



Cultural Characteristics.

The following characteristics were observed during cultivation of strain MCRL 0387 on various media for 3 weeks. The number in parentheses corresponds to the hue number used in the "Color Harmony Manual"[3].

(1) Sucrose-nitrate agar (WAKSMAN medium No. 1, at 27°C): Colorless, transparent growth with light ivory (2 ca) reverse; forming ashes (5 fe) powdery aerial mycelium with white patches; producing no soluble pigment.

(2) Glucose-asparagine agar (WAKSMAN medium No. 2, at 27°C): Colorless, transparent growth with light ivory (2 ca) reverse; forming gray (g) to ashes (5 fe) powdery aerial mycelium; producing no soluble pigment.

(3) Glycerol-asparagine agar (ISP medium No. 5, at 27°C): Colorless to pale yellow (1 ca) with bamboo (2 fb) reverse; forming light silver gray (3 fe) powdery aerial mycelium; producing no soluble pigment.

(4) Glycerol calcium-malate agar (WAKSMAN medium No. 7, at 27°C): Colorless, transparent growth with light ivory (2 ca) reverse; forming white (b) powdery aerial mycelium, and later turning ashes (5 fe) on the margin of colony; producing no soluble pigment.

(5) Nutrient agar (WAKSMAN medium No. 14, at 37°C): Pastel yellow (1 db) with pastel yellow (1 1/2 fb) to bamboo (2 fb) reverse; forming ashes (5 fe) powdery aerial mycelium with white patches; producing no soluble pigment.

(**6**) Glucose-nutrient agar (WAKSMAN medium No. 14, at 37°C): Yellow tint (1 ba) growth with bamboo (2 fb) to amber (3 nc) reverse; forming ashes (5 fe) powdery aerial mycelium with white patches; producing no soluble pigment.

(**7**) Inorganic salts-starch agar (ISP medium No. 4, at 27°C): Colorless, transparent with parchment (1 1/2 db) reverse; forming rose wood (5 ge) aerial mycelium with white patches; producing no soluble pigment.

(**8**) Tyrosine agar (ISP medium No. 7, at 27°C): Pearl (2 ba) growth with dark olive green (24 1/2 pn) color at the middle of the colony, with mistletoe green (24 1/2 li) reverse; forming ashes (5 fe) powdery aerial mycelium; producing no soluble pigment.

(**9**) Yeast extract-malt extract agar (ISP medium No. 2, at 27°C): bamboo (2 gc) growth with bamboo (2 fe) reverse; forming ashes (5 fe) powdery aerial mycelium, forming moistened black patches; producing no soluble pigment.

(**10**) BENNETT's agar (WAKSMAN medium No. 30, at 27°C): Colorless to bamboo (2 gc) growth with light ivory (2 ca) to bamboo (2 gb) reverse; forming ashes (5 fe) to pussywillow gray (5 dc) powdery aerial mycelium, forming moist black patches which gradually spread over the whole surface; producing no soluble pigment.

(**11**) Oatmeal agar (ISP medium No. 3, at 27°C): Colorless to pale yellow (1 ca) growth with light wheat (2 ea) reverse; forming ashes (5 fe) powdery aerial mycelium with moist black patches; producing no soluble pigment.

Concomitantly, it was to be noted that, as in the case of *S. humidus*, the characteristic of strain MCRL 0387 to produce moist (hygroscopic) areas on mature aerial mycelium readily disappeared by successive cultivation for several generations, though this characteristic was clearly observed in the original strain isolated from soil.

Utilization of Carbon Sources in PRIDHAM and GOTTLIEB's Agar.

Arabinose, xylose, glucose, fructose, sucrose, inositol, rhamnose, mannitol, glycerol, galactose, lactose, maltose, mannose and starch were utilized. Raffinose, salicin and sorbitol were not utilized.

Physiological Characteristics.

Starch hydrolysis (ISP medium No. 4), nitrate reduction (Difco nitrate broth), milk coagulation and peptonization (Difco 10 % skimmed milk), gelatin liquefaction (20 % gelatin), serum liquefaction (Difco LOEFFLER blood serum) and haemolysis (nutrient agar, WAKSMAN medium No. 14 containing 10 % horse blood) all were positive. The tyrosinase reaction (ISP medium No. 7 and WAKSMAN medium No. 42), cellulase reaction (CZAPEK's solution with a strip of filter paper as the sole carbon source) and hydrogen sulfide production (Difco peptone iron agar containing 0.1 % yeast extract) were negative. The calcium malate solubilization test gave doubtful results. Strain MCRL 0387 was aerobic and showed good growth at 37°C in the pH range of 6.0 to 8.0 (Difco nutrient broth containing 2 % glucose). No growth was observed either at pH 4.0, irrespective of temperature, or at 10°C and 50°C, irrespective of pH.

Based on the characteristic formation of aerial mycelium which gradually became moist during cultivation and turned to dark and hygroscopic masses of spores, strain MCRL 0387 was classified in the *Streptomyces hygroscopicus* (JENSEN) WAKSMAN and HENRICI, 1948 group. According to TRESNER *et al.*[4] and DIETZ and MATHEWS[5,6], strain of this group may further be divided into 2~4 types, *i.e.*, *Streptomyces hygroscopicus*-type strains and *Streptomyces platensis*-type strains based on the micromorphology of

their spores. According to their criteria, strain MCRL 0387 was considered to represent a *Streptomyces platensis*-type strain. The following taxa are reported a to belong to this type: *S. platensis* PITTENGER and GOTTLIEB, 1954[7], *S. hygroscopicus* var. *angustmyceticus* SAKAI, YÜNSTEN and ISHIKAWA, 1954[8], *S. hygroscopicus* var. *decoyicus* VAVRA, DIETZ, CHURCHILL, SIMINOFF and KOEPSELL, 1959[9], *S. hygroscopicus* forma *glebosus*, OHMORI, OKANISHI and KAWAGUCHI, 1962[10] and *S. hygroscopicus* var. *ossamyceticus* SCHMITZ, JUBUNSKI, HOOPER, CROOK, PRICE and LEIN, 1965[11]. According to IMAMURA *et al*[12]., *S. humidus* belongs to the *S. hygroscopicus* group. In their publication, no description was made as to the surface structure of spores. However, SHIRLING and GOTTLIEB[13] later reported that *S. humidus* formed smooth-walled spores but no hygroscopic masses of spores. Con sequently, *S. humidus* also was considered to represent a *Streptomyces platensis*-type strain.

Among the strains of *S. platensis*-type mentioned above, *S. humidus* most closely resembles strain MCRL 0387. Accordingly, the microbiological characteristics of strain MCRL 0387 were compared with those obtained for *S. humidus*, strain IFO 12877 (ISP 5263) kindly provided by the Institute for Fermentation, Osaka. The results are shown in Table 1. It was found that strain MCRL 0387 and IFO 12877 are quite similar in their microbiological characteristics except that strain MCRL 0387

Table 1. Differences in cultural characteristics and utilization of carbon sources between *S. humidus* var. *antitumoris* MCRL 0387 and *S. humidus* IFO 12877

	S. humidus var. *antitumoris* MCRL 0387	*S. humidus* IFO 12877
Yeast extract-malt extract agar	I : bamboo (2 gc) II : bamboo (2 fb) III : ashes (5 fe) with black moist patches IV : none	I : bamboo (2 gc) II : mustard brown (3 li) III : pussywillow gray (5 dc) IV : none
Glycerol asparagine agar	I : colorless to pale yellow (1 ca) II : bamboo (2 fb) III : silver gray (3 fe) IV : none	I : light ivory (2 ca) II : light ivory (2 ca) III : none IV : none
Inorganic salts-starch agar	I : colorless II : pale greenish yellow (1 1/2 db) III : ashes (5 fe) with white patches IV : none	I : colorless to light ivory (2 ca) II : light ivory (2 ca) III : pussywillow gray (5 dc) VI : none
Oat meal agar	I : colorless to pale yellow (1 ca) II : light wheat (2 ea) III : ashes (5 fe) with black moist patches IV : none	I : colorless II : covert brown (2 nl) III : rouse wood (5 ge) IV : none
BENNETT'S agar	I : bamboo (2 gc) II : bamboo (2 fb) III : pussywillow gray (5 dc) IV : none	I : light ivory (2 ca) II : light ivory (2 ca) III : white IV : none
Utilization of sucrose	utilized	not utilized
Antibiotic(s) produced	YA-56 complex	Dihydrostreptomycin & humidin

I : Color of vegetative growth II : Color of reverse III : Color of aerial mycelium IV : Color of soluble pigment

does not produce dihydrostreptomycin or humidin. Several other differences, particularly in the color of aerial mycelium, utilization of sucrose and formation of hygroscopic masses of spores were noted. Strain MCRL 0387 was clearly distingushed from *S. verticillus* OKAMI, SUZUKI and UMEZAWA, 1959[14,15] producing phleomycins, *S. verticillus* producing bleomycins and *S. bikiniensis* var. *zorbonensis* DIETZ, 1971[16,17] producing zorbamycin, zorbonomycin B and zorbonomycin C, zorbamycin being identical with YA-56 X as will be reported in the succeeding paper[18], because *S. verticillus* forms whorls and *S. bikiniensis* var. *zorbonensis* shows long straight (RF) spores chain morphology and is melanin-positive.

Characterization of Mutant Strain MCRL 0432

The mutant strain, MCRL 0432, which produces three times as much of the YA-56 complex as the original strain was obtained by irradiating the original strain with ultra-violet light. This mutant formed bluish powdery aerial mycelium. The aerial mycelium formed distinct spirals and spore surfaces were smooth. Although the aerial mycelium of the original strain (MCRL 0387) showed hygroscopic characteristics during cultivation, strain MCRL 0432 never exhibited this feature on any medium. The cultural characteristics of the mutant strain are as follows:

(1) Sucrose-nitrate agar: Colorless growth; forming white (b) powdery aerial mycelium; producing no soluble pigment.

(2) Glucose-asparagine agar: Ivory (2 db) growth with bamboo (2 fb) reverse; aqua gray (19 dc) powdery aerial mycelia; producing no soluble pigment.

(3) Glycerol-asparagine agar: Ivory (2 db) growth; gray (e) powdery aerial mycelium; producing no soluble pigment.

(4) Inorganic salts-starch agar: Colorless growth with pale yellow green (24 1/2 dc) reverse; pale yellowish green (24 1/2 dc) powdery aerial mycelium; producing no soluble pigment.

(5) Tyorsine agar: Light spice brown (41 g) growth with brick red (5 ng) reverse; pale yellowish green (24 1/2 dc) powdery aerial mycelium; producing no soluble pigment.

(6) Nutrient agar: Colorless to pearl (2 ba) growth with yellow tint (1 ba) reverse; yellow tint (1 ba) aerial mycelium; producing no soluble pigment.

(7) Yeast extract-malt extract agar: Colorless to ivory (2 db) growth with putty (1 1/2 ec) reverse; bayberry gray (22 fe) powdery aerial mycelium; producing no soluble pigment.

(8) Oatmeal agar: Ivory (2 db) growth with putty (1 dc) reverse; aqua gray (19 dc) powdery aerial mycelium; producing no soluble pigment.

Utilization of carbon sources: Arabinose, glucose, fructose, inositol and mannitol were utilized. Xylose, sucrose, rhamnose and raffinose were not utilized.

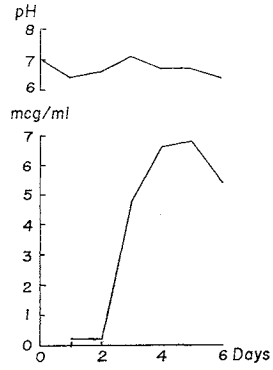
Physiological characteristics: Gelatin liquefaction, starch hydrolysis and milk coagulation were positive, while milk peptonization and tyrosinase reaction were negative. Optimum temperature and pH range were the same as for the original strain.

Production of the Antibiotic YA-56 Complex

Fermentation studies were carried out in a 2,000-literstain less steel fermentor. Antibiotic production was followed by a cylinder-plate method using *Escherichia coli* NIHJ as the test organism on a medium composed of glucose-peptone agar (glucose 0.2 %, peptone 0.5 % and agar 1.3 %, pH 8.0). The medium for inoculum preparation

was composed of potato starch 3%, Pharmamedia (Trader's Oil Mill Co., U.S.A.) 4%, yeast extract 0.2%, NaCl 0.5%, $CaCO_3$ 0.3%, $CuSO_4 \cdot 5H_2O$ 0.002% and an antifoam agent 0.2%. The pH was not adjusted (pH 6.5~7.5). As an antifoam agent, Klearol (Sonneborn Research U.S.A.)-Nikkol PBC 41 (Nikko Chemicals Co., Ltd.) (9:1) was used.

Fig. 1. Production pattern of YA-56 complex.



Streptomyces humidus var. *antitumoris*, strain MCRL 0387, was inoculated into 120 ml of inoculum medium prepared in a 500-ml flask and cultivated at 26~28°C for 2~3 days on a rotary shaker. A secondary inoculum culture was prepared in a 30-liter jar fermentor containing 15 liters of the inoculum medium. After sterilization and inoculation with 120 ml of the primary inoculum culture, fermentation was carried out under the following conditions: temperature 26°~28°C, aeration 7.5 liters/min., agitation 400 r.p.m. for 2~3 days.

The production medium (1,500 liters in 2,000-liter stainless steel fermentor) was composed of Malt Rup (a syrup containing 50~60% maltose, Hayashibarara Co., Ltd.) 10%, Pharmamedia 2%, NaCl 0.5%, $CaCO_3$ 0.3%, $CuSO_4 \cdot 5H_2O$ 0.002% and an antifoam agent 0.2%. The pH of the medium was not adjusted. The medium was sterilized at 120°C for 30 minutes. After cooling, followed by inoculation of 15 liters of the secondary inoculum culture, fermentation was carried out under the following conditions: temperature 26~28°C, aeration 700 liters/min., agitation 200 r.p.m., internal pressure 0.5 kg/cm². As shown in Fig. 1, YA-56 complex activity began to appear in culture filtrates after 2 days, and reached a maximum at about the 5th day, showing a potency of 6.8~7.5 mcg/ml.

Acknowledgment

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