STUDIES ON THE MACROLIDE ANTIBIOTIC YL-704 COMPLEX. I

TAXONOMY OF THE PRODUCING STRAIN AND PRODUCTION OF THE COMPLEX

TAMOTSU FURUMAI, YOJI SHIMIZU, KATSUO TAKEDA,
NOBUO MATSUZAWA, KATO TANI
and TOMOHARU OKUDA

Microbial Chemistry Research Laboratory,
Tanabe Seiyaku Co., Ltd.,
Toda, Saitama, Japan

(Received for publication July 18, 1973)

Strain MCRL 0388 which produces a new complex of basic macrolide antibiotics designated YL-704 was identified as a new subspecies of *Streptomyces platensis* PITTENGER and GOTTLIEB, 1954, based upon its micromorphological and physiological characteristics. With ultra-violet irradiation, the original strain gave a mutant which produced dark green diffusible pigment. Fermentative production of the antibiotic YL-704 complex is described.

In the course of new antibiotics screening, a complex of new basic macrolide antibiotics collectively named YL-704 was isolated from the culture broth of a streptomycete designated MCRL 0388. This complex consisted of two major components designated YL-704 A_1 and YL-704 B_1 and eleven other minor components. The antibiotic YL-704 complex-producing strain was characterized by the formation of hygroscopic masses of smooth-walled spores on aerial mycelia and by the production of diffusible and substrate pigment behaving as a pH indicator in glycerol-asparagine agar, inorganic salts-starch agar and tyrosine agar media. Progeny of the type strain have been deposited in the collections of the Northern Regional Research Laboratory, Agricultural Research Service, U.S. Department of Agriculture, Peoria, Illinois, U.S.A. as NRRL 3761, and the Fermentation Research Institute Chiba, Japan, as FERM-P No. 289. In the course of selecting for mutant strains, a mutant (strain MCRL 0393) was obtained with ultra-violet irradiation. This mutant produced dark green diffusible pigment. In contrast to the wild-type strain, the pigment was not a pH indicator. The mutant strain did not form hygroscopic masses of spores on any agar medium. The present paper concerns taxonomic studies on strain MCRL 0388 and multant strain MCRL 0393. Based on the results, strain MCRL 0388 is considered as a new subspecies of *Streptomyces platensis*, and the name *Streptomyces platensis* subsp. *malvinus* FURUMAI and OKUDA subsp. nov. is proposed. The type strain is MCRL 0388 (NRRL 3761) by virtue of its being a single isolate. The fermentative production of antibiotic YL-704 complex also is dealt with in this paper. Isolation and characterization of the components of the complex will be reported in another paper[1].

Characterization of Strain MCRL 0388

The media used for morphological, cultural and physiological studies were those described by SHIRLING and GOTTLIEB[2], and WAKSMAN[3]. Taxonomic studies were generally carried out according to procedures described for the International Streptomyces Project (ISP)[2]. Color names

Fig. 1. Photomicrograph of *Streptomyces platensis* subsp. *malvinus*. (Yeast extract-malt extract agar, × 800)

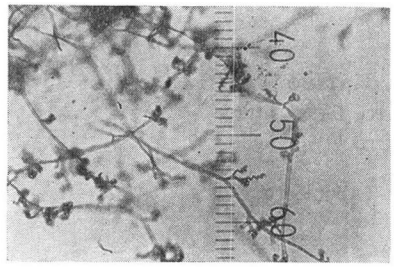
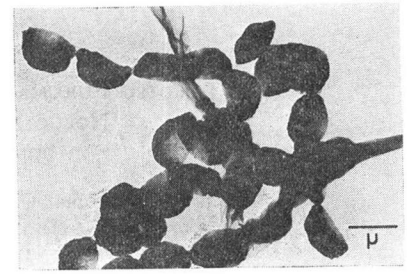


Fig. 2. Electronmicrogarph of *Streptomyces platensis* subsp. *malvinus*. (Yeast extract-malt extract agar, ×10,000)



and hue numbers are those of the Color Harmony Manual (4 th edition) published by Container Cooperation of America[4]. A detailed characterization of the new taxon follows:

Morphological Characteristics.

On various agar media, strain MCRL 0388 showed characteristics typical of streptomycetes and formed aerial mycelia of the Gray color-series. The spore chain morphology showed the presence of spirals (Section *Spirales*). Mature spore chains were formed in chains of not less than ten elliptical to cylindrical-phalangioform spores (Fig. 1). The surfaces of the spores were smooth (Fig. 2). On some agar media, the aerial mycelium frequently coalesced to form black hygroscopic masses of spores spreading over the whole surface.

Cultural Characteristics.

The following characteristics were observed during cultivation of strain MCRL 0388 on various agar media for 3 weeks.

(1) Sucrose-nitrate agar (Waksman medium No. 1, at 27° C): poor, colorless to natural (2 dc) growth with light tan (3 gc) reverse; forming light gray (d) powdery aerial mycelium; producing light amber (3 ic) diffusible pigment.

(2) Glucose-asparagine agar (Waksman medium No. 2, at 27°C): good, colorless growth with nude tan (4 gc) reverse; forming white to silver gray (3 fe) powdery aerial mycelium; producing light brown (6 1/2 lg) diffusible pigment.

(3) Glycerol-asparagine agar (ISP medium No. 5, at 27°C): good, colorless growth with ivory (2db) reverse, later turning to dark brown mahogany (6 pn) growth with mauve wine (8 ni) to dark rose brown (7 nl) reverse. The reverse color changed from reddish purple to brownish yellow by addition of 0.1 N NaOH and again to light reddish purple with 0.1 N HCl; forming pussywillow gray (5 dc) powdery aerial mycelium; producing dark mauve taupe (8 nl) diffusible pigment. The diffusible pigment is a pH indicator, the color being light rose brown (71 g) on addition of 0.1 N HCl and brick red (5 ng) with 0.1 N NaOH.

(4) Glycerol-calcium malate agar (Waksman medium No. 7, at 27°C): good, colorless growth with ivory (2 db) reverse; forming white to beige (3 ge) powdery aerial mycelium, and later black moist areas on mature aerial mycelium; producing no diffusible pigment.

(5) Nutrient agar (Waksman medium No. 14, at 37°C): good, colorless to biscuit (2 ec) growth with ivory (2 db) reverse; forming oyster white (b) powdery aerial mycelium; producing no diffusible pigment.

(6) Glucose-nutrient agar (Waksman medium No. 14, at 37°C): good, ivory (2 db) to biscuit (2 ec) growth with topaz (3 ne) reverse; forming silver gray (3 fe) powdery aerial mycelium; producing no diffusible pigment.

(7) Inorganic salts-starch agar (ISP medium No. 4, at 27°C): good, colorless growth with cork tan (4 ie) reverse, substrate pigment being a pH indicator; forming silver gray (3 fe) powdery aerial mycelium, and later black moist areas on mature aerial mycelia; producing rose

wood (5 ge) diffusible pigment of pH indicator nature.

(8) Tyrosine agar (ISP medium No. 7, at 27°C): good, bisque (4 ec) to cocoa brown (5 lg) growth with cocoa brown (5 ni) reverse, substrate pigment being a pH indicator; forming white to ashes (5 fe) powdery aerial mycelium; producing rose wood (5 ge) diffusible pigment of pH indicator nature.

(9) Yeast extract-malt extract agar (ISP medium No. 2, at 27°C): good, light mustard tan (2 ie) growth with clove brown (3 ni) reverse; forming at first covert tan (2 ge) and, later, beige brown (3ig) powdery to velvety aerial mycelium, interspersed with moist, black patches which gradually spread over the surface; producing topaz (3 ne) diffusible pigment.

(**10**) BENNETT's agar (Waksman medium No. 30, at 27°C): good, colorless growth with dark brown (3 ni) reverse; forming, at the initial phase, beige (3 ge) powdery to velvety aerial mycelium, becoming ashes (5 fg) at the mature phase; forming black moist patches which gradually spread over the whole surface; producing light tan (3 ge) diffusible pigment.

(**11**) Oatmeal agar (ISP medium No. 3, at 27°C): good, colorless growth with clove brown (3 ni) reverse; forming gray (e) aerial mycelium with moist black patches; producing no diffusible pigment.

These characteristics place the strain in Section *Spirales*, color-series Gray.

Physiological Characteristics.

Starch hydrolysis (ISP medium No. 4), nitrate reduction (Difco nitrate broth) and milk coagulation and peptonization (Difco 10 % skimmed milk) tests gave positive results. Tests for tyrosinase (ISP medium No. 7 and Waksman medium No. 42), cellulase (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. Strain MCRL 0388 was aerobic, and showed good growth at 37°C in a pH range of 6.0 to 8.0 (Difco nutrient broth containing 2 % glucose). Also it grew at pHs of 5.0 or 9.0 at this temperature and at 27°C. No growth was observed at pH 4.0 irrespective of temperature, or at 5°C or 50°C irrespective of pH.

Utilization of carbon sources in PRIDHAM and GOTTLIEB's basal agar (ISP medium No. 9)

Arabinose, xylose, glucose, mannose, maltose, fructose, sucrose, inositol, raffinose, galactose, starch, glycerol and mannitol, each, are utilized. Lactose, rhamnose, sorbitol and salicin, each, are not utilized.

According to the literature[3] on streptomycetes, strains in which aerial mycelium becomes moist and forms dark hygroscopic masses of spores at maturity are classified as *Streptomyces hygroscopicus* (JENSEN) WAKSMAN and HENRICI, 1948. Based on the micromorphology of the spores observed on their carbon repligraphs, DIETZ and MATHEWS[5,6] further divided this taxon into two distinct types: Type I showing nonsegmented spore structure with an extremely wrinkled surface, and type II having a segmented spore chain with detailed surface structure resembling a basket weave. Later, TRESNER, BACKUS and HAYES[7] noted that type I organisms had short cylindrical and phalangioform spores, as typified by *S. hygroscopicus*, and type II organisms had eliiptical-shaped spores as typified by *Streptomyces platensis*. According to their criteria, strain MCRL 0388 resembles an *S. platensis*-type strain. Besides *S. platensis*, the following strains belong to this type: *Streptomyces hygroscopicus* var. *angustmyceticus* SAKAI, YÜNSTEN and ISHIKAWA, 1954,[8] *Streptomyces neyagawaensis* YAMAMOTO, NAKAZAWA, HORII and MIYAKE, 1960,[9,10] *Streptomyces hygroscopicus* var. *ossamyceticus* SCHMITZ, JUBUNSKI, HOOPER, CROOK and PRICE, 1965[11]. However, these strains can readily be differentiated from strain MCRL 0388 as follows:

Table 1. Differences in cultural characteristics between *Streptomyces platensis* subsp. *malvinus* MCRL 0388 and *Streptomyces platensis* IFO 12901.

	Streptomyces platensis subsp. *malvinus* MCRL 0388	*Streptomyces platensis* IFO12901
Yeast extract-malt extract agar	I: light mustard tan (2ie) II: clove brown (3ni) III: beige brown (3ig) with black moist patches VI: topaz (3ne)	I: cork tan (4ie) II: copper brown (5pi) III: beige brown (3ig) with black moist patches VI: light brown (4ng)
Glycerol-asparagine agar	I: dark brown mahogany (6pn) II: mauve wine (8ni) to dark rose brown (7nl) III: pussywillow gray (5dc) VI: dark mauve taupe (8nl)	I: ivory (2db) II: bamboo (2db) III: ashes (5fe) VI: none
Oatmeal agar	I: colorless III: gray (e) with black moist patches VI: none	I: ivory (2db) III: beaver (4li) with black moist patches VI: cork tan (4ie)
Inorganic salts starch agar	I: colorless II: cork tan (4ie) III: silver gray (3fe) with black moist patches VI: rose wood (5ge)	I: colorless II: aquash yellow (2ia) III: lead gray (3ih) with black moist patches VI: light lemon yellow (1ga)
Antibiotics produced	YL-704 complex	Oxytetracycline

I. Color of vegetative growth. II. Color of reverse. III. Color of aerial mycelium.
VI. Color of diffusible pigment.

S. hygroscopicus var. *angustmyceticus* produces ordinary white, occasionally gray, aerial mycelium and does not produce diffusible pigment in synthetic media and organic media. Arabinose and xylose are not utilized, and coagulation of milk and nitrate reduction are negative.

S. neyagawaensis forms melanoid pigments, and forms no pigment or only a trace of gray or olive brown diffusible pigment in yeast extract-malt extract agar, oatmeal agar, inorganic salts-starch agar and glycerol-asparagine agar. Rhamnose is utilized.

S. hygroscopicus var. *ossamyceticus* forms no diffusible pigment in synthetic media, but forms dark brown pigment in media containing tryptone. Rhamnose and lactose are utilized.

On the other hand, the type strain of *S. platensis*[12], most closely resembles strain MCRL 0388. Therefore, strain MCRL 0388 and *S. platensis*, strain IFO 12901 (ISP 5041), were compared. As shown in Table 1, strain MCRL 0388 was different from *S. platensis* in color of reverse and diffusible pigments on synthetic media. However, in spite of such differences, strain MCRL 0388 and *S. platensis* were quite similar in other morphological and physiological properties, including their ability to utilize various carbon sources. As a result, strain MCRL 0388 is considered a new subspecies of *S. platensis*, for which the name *S. platensis* subsp. *malvinus* subsp. nov. is proposed. The proposed subspecific epithet "*malvinus*" is the modern latin adjective meaning "mauve", and refers to the color of the diffusible pigment produced by this micro-organism in glycerol-asparagine agar medium.

Characterization of Mutant Strain MCRL 0393

In the course of mutation studies, a mutant designated MCRL 0393 with dark olive diffusible pigment was obtained by ultra-violet irradiation. The vegetative mycelium of this mutant was green to dark green and it formed grayish powdery aerial mycelium. The morphological properties were the same as those of the wild-type strain (MCRL 0388), but hygroscopicity (one of the most important characteristics of the wild-type strain) was not observed on any agar medium. The cultural characteristics of this mutant when grown at 27°C are as follows:

Cultural Characteristics

(1) Sucrose-nitrate agar: poor, dark green (24 pn) growth with dark green (24 pn) reverse; forming no aerial mycelium; producing ivy (24 ml) diffusible pigment.

(2) Glucose-asparagine agar: good, dark green (24 pn) growth with dark green (24 pn) reverse; forming gray (e) powdery aerial mycelium; producing dark green (24 pn) diffusible pigment.

(3) Glycerol-asparagine agar: moderate, dark green (24 pn) growth with dark green (24 pn) reverse; forming gray (e) powdery aerial mycelium; dark olive green (24 1/2 ml) diffusible pigment.

(4) Nutrient agar: good, dark bottle green (22 pn) growth with dark bottle green (22 pn) reverse; forming mistletoe gray (24 1/2 ih) powdery aerial mycelium; producing light olive gray (1 1/2 ge) diffusible pigment.

(5) Inorganic salts-starch agar; moderate, dark olive green (24 1/2 pn), becoming lamp black (p) growth with lamp black (p) reverse; forming oyster white (b) to, later, natural (2 be) powdery aerial mycelium; producing dark olive (1 pn) diffusible pigment.

(6) Tyrosine agar: good, dark olive green (24 1/2 pn) to, later, sepia brown (3 pn) growth with lamp black (p) reverse; forming gray (e) powdery aerial mycelium; producing chocolate brown (4 pn) diffusible pigment.

(7) Yeast extract-malt extract agar: good, dark bottle green (22 pn) growth with dark bottle green (22 pn) reverse; forming silver gray (3 fe) powdery aerial mycelium; producing lamp black (p) diffusible pigment.

(8) Oatmeal agar; good, dark bottle green (22 pn) growth with dark bottle green (22 pn) reverse; forming light olive gray (1 1/2 ge) powdery aerial mycelium; producing dark olive (1 nl) diffusible pigment.

Physiological Characteristics

Starch hydrolysis, nitrate reduction, milk coagulation, milk peptonization and serum liquefaction tests were positive. Formation of tyrosinase, cellulase, hydrogen sulfide and melanin were negative. The mutant strain did not grow in a 20 % gelatin medium. Optimum temperature and pH range were the same as for the original strain.

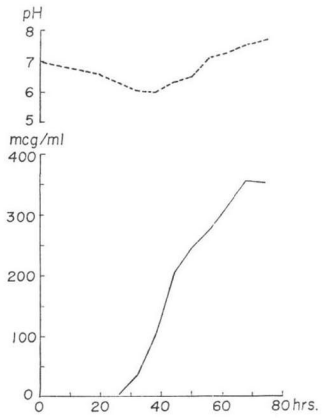
Utilization of carbon sources in PRIDHAM and GOTTLIEB's basal agar.

Xylose, glucose, maltose, fructose, inositol, raffinose, galactose, starch, glycerol and mannitol, each, are utilized. Rhamnose, sorbitol, salicin and mannose, each, are not utilized. Utilization of arabinose, lactose and sucrose, each, is doubtful.

Production of the Antibiotic YL-704 Complex

YL-704 complex production was monitored by a cylinder-plate assay method using *Bacillus subtilis* ATCC 6633 as the test organism in Penassay agar (Kyoei Pharmaceutical Co., Ltd.) at pH 8.0. Agar plates were prepared by pouring 10 ml of Penassay agar medium as the base

Fig. 3. Characteristics of YL-704 complex production in 2,000-liter fermentor.



layer, and overlaying with 5 ml of the above medium as a seed layer in sterile disposable Petri dishes. Crystalline antibiotic YL-704 A_1 (base) was used as a standard. The antibiotic activity of YL-704 complex in culture filtrates could be assayed using solutions of YL-704 A_1 at concentrations of 50 mcg/ml and 12.5 mcg/ml, respectively, dissolved in 0.2 M phosphate buffer (pH 8.0) as the standard solutions.

Fermentation studies were carried out in a 2,000-liter fermentor. The medium for inoculum preparation was composed of glucose 2 %, Polypeptone 1 %, meat extract 0.75 %, yeast extract 0.3 % and NaCl 0.3 %. The pH was not adjusted. The production medium was composed of corn starch 1.5 %, glucose 0.5 %, soy bean meal 1.5 %, corn steep liquor 0.1 %, yeast extract 0.2 %, NaCl 0.5 %, and $CaCO_3$ 0.2 %. The pH of the medium was not adjusted. As an antifoam agent, Silicone KM-75 (Shin-Etsu Chemical Industry Co., Ltd.) was used. In a 2,000-liter fermentor, 1,200 liters of production medium was prepared and sterilized at 120°C for 20 minutes. After cooling, the medium was inoculated with 15 liters of inoculum culture of *S. platensis* subsp. *malvinus*, strain MCRL 0388 obtained by cultivation for 50 hours. Fermentation was carried out under the following conditions: temperature 23.5~26°C, aeration 550~650 liters/minute, agitation 125 r.p.m., internal pressure 0.45~0.6 kg/cm^2. As shown in Fig. 3, production of 300~400 mcg of YL-704 complex per ml was attained after about 60 hours.

Productivity of the antibiotic complex by the mutant, strain MCRL 0393 is less than that of the wild-type strain (100~200 mcg/ml). The major components in the broth are the same as obtained with the original strain.

Acknowledgment

The authors are indebted to Dr. T. HASEGAWA of the Institute for Fermentation, Osaka for providing a culture of the type strain of *S. platensis*.

Reference

1) KINUMAKI, A.; I. TAKAMORI, Y. SUGAWARA, N. NAGAHAMA, M. SUZUKI, Y. EGAWA, M. SAKURAZAWA & T. OKUDA: Studies on the macrolide antibiotic YL-704 complex. II. Isolation and physicochemical properties of YL-704 components. J. Antibiotics 27: 102~106, 1974
2) SHIRLING, E B. & D. GOTTLIEB: Methods for characterization of *Streptomyces* species. Intern. J. Syst. Bact. 16: 313~340, 1966
3) WAKSMAN, S. A.: The actinomycetes. Vol II. Classification, identification and description of genera and species, pp. 328~334. The Williams & Wilkins Co., Baltimore, 1961
4) Container Corporation of America: Color Harmony Manual, 4th edition, Chicago, U.S.A.
5) DIETZ, A. & J. MATHEWS: Taxonomy by carbon replication. I. An examination of *Streptomyces hygroscopicus*. Appl. Microbiol. 10: 258~263, 1962
6) DIETZ, A. & J. MATHEWS: Taxonomy by carbon replication. II. Examination of eight additional cultures of *Streptomyces hygroscopicus*. Appl. Microbiol. 16: 935~941, 1968

7) TRESNER, H.D.; E. J. BACKUS & J.A. HAYES: Morphological spore tpyes in the *Streptomyces hygroscopicus*-like complex. Appl. Microbiol. 15: 637~739, 1967
8) SAKAI, H.; H. YÜNTSEN & F. ISHIKAWA: Studies on a new antibiotic angustmycin. II. J. Antibiotics, Ser. A 7: 116~119, 1954
9) YAMAMOTO, H.; K. NAKAZAWA, S. HORII & A. MIYAKE: Studies on agricultural antibiotic folimycin, a new antifungal antibiotic produced by *Streptomyces neyagawaensis* nov. sp. J. Agr. Chem. Soc. 34: 268~272, 1960
10) SHIRLING, E.B & D. GOTTLIEB: Cooperative description of type strains of *Streptomyces*. V. Additional descriptions. Intern. J. Syst. Bact. 22: 323~325, 1972
11) SCHMITZ, H.; S.D. JUBINSKI, I.R. HOOPER, K.E. CROOK, Jr., K. E. PRICES & J. LEIN: Ossamycin, a new cytotoxic agent. J. Antibiotics, Ser. A 18: 82~88, 1965
12) MCGUIRE, J.M.: Improvments in or relating to terramycin. Brit. Pat. 713795, Aug. 18, 1954