MYCOPLANECINS, NOVEL ANTIMYCOBACTERIAL ANTIBIOTICS FROM *ACTINOPLANES AWAJINENSIS* SUBSP. *MYCOPLANECINUS* SUBSP. NOV.

I. TAXONOMY OF PRODUCING ORGANISM AND FERMENTATION

AKIO TORIKATA, RYUZOU ENOKITA, TAKAO OKAZAKI, MUTSUO NAKAJIMA, SEIGO IWADO, TATSUO HANEISHI and MAMORU ARAI

Fermentation Research Laboratories, Sankyo Co., Ltd.
2–58, 1-chome, Hiromachi, Shinagawa-ku, Tokyo 140, Japan

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Strain No. 41042, an actinomycete isolated from a soil sample, was found to produce 5-azacytidine and new antibiotics, mycoplanecins. Yellowish brown to yellowish orange color of colonies on agar media, formation of globose to subglobose sporangia bearing motile spores and presence of *meso*- and hydroxy-diaminopimelic acid and glycine in the cell wall ascribed this strain to genus Actinoplanes. From its morphological, cultural and physiological characteristics, this strain was determined to be a new subspecies of *Actinoplanes awajinensis* and designated as *A. awajinensis* subsp. *mycoplanecinus* subsp. nov. Production of mycoplanecins was carried out by conventional submerged culture: the highest antibiotic titer obtained was 145 μg/ml.

In the course of a screening program for new antibiotics produced by the organisms of the family Actinoplanaceae, new antibiotics, candiplanecin[1,2], isohematinic acid[3,4] and mycoplanecins were discovered in our laboratories.

This paper deals with the taxonomy of the producing organism and the fermentation of mycoplanecins. Isolation, physico-chemical characterization and structure elucidation as well as biological properties of the antibiotics are described in subsequent papers.

Taxonomic Studies of Strain No. 41042

Strain No. 41042 was isolated from a soil sample collected at Awaji Island in Hyogo Prefectre, Japan, by the selective isolation method using a novobiocin-containing medium. Most actinomycetes are sensitive to novobiocin, but some genera of Actinoplanaceae, such as Actinoplanes, Ampullariella and Amorphosporangium, and genus Micromonospora are resistant to the antibiotic[5,6].

Morphological and physiological properties of the organism were determined according to SHIRLING and GOTTLIEB[7]; several other tests were also used. Observation of the culture was made after incubation at 28°C for 2 weeks, unless otherwise mentioned. Color names were assigned according to "Guide to Color Standard" (a manual published by Nippon Shikisai Kenkyusho, Tokyo). The characteristics of the organism were compared with those of the known species of actinomycetes described in "The Actinomycetes, Vol. 2" by WAKSMAN, the "ISP Report" by SHIRLING and GOTTLIEB[8], "BERGEY's Manual of Determinative Bacteriology (8th edition)" and other recent references on the taxonomy of Actinoplanaceae.

Strain No. 41042 formed sporangia abundantly on inorganic salts - starch, sucrose - nitrate and potato extract - carrot extract agar. Sporangia were globose to subglobose in shape, and 7~17 μm

in size as shown in Plate 1. When drops of sterile water were placed on the surface of the culture sporangia-producing and the culture was covered with a cover slip, migration of sporangiospores was observed within 15 minutes. Sporangiospores with polar flagella were spherical to rod-shaped and 0.8×1.3 μm in size as shown in Plate 2. The cultural characteristics of the organism on various agar media are shown in Table 1. Development of aerial mycelium was not observed on all media tested. The color of

Plate 1. Scanning electron micrograph of sporangia of *A. awajinensis* subsp. *mycoplanecinus* No. 41042.
Potato extract - carrot extract agar, 7 days culture. Bar represents 10 μm.

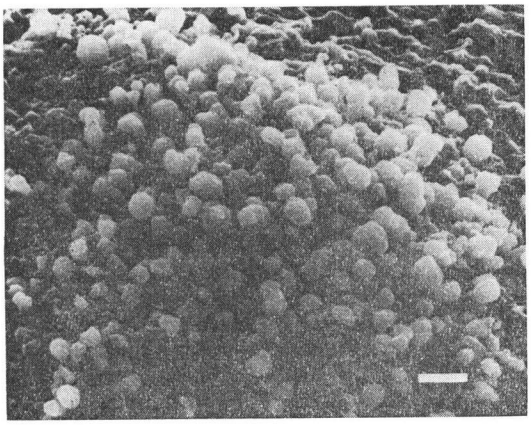


Plate 2. Scanning electron micrograph of a sporangiospore of *A. awajinensis* subsp. *mycoplanecinus* No. 41042.
Bar represents 1 μm.

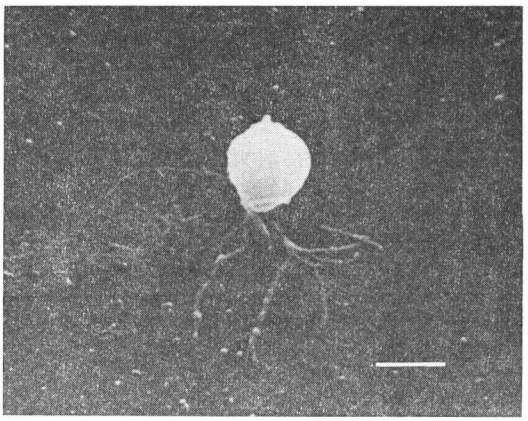


Table 1. Cultural characteristics of strain No. 41042.

Yeast extract - malt extract agar (ISP 2)	G:	Abundant
	SM:	Dull yellowish orange
	SP:	None
	SPR:	None
Oatmeal agar (ISP 3)	G:	Abundant
	SM:	Yellowish brown
	SP:	Pale yellowish brown
	SPR:	None
Inorganic salts - starch agar (ISP 4)	G:	Good
	SM:	Dull yellowish orange
	SP:	None
	SPR:	Abundant
Glycerol - asparagine agar (ISP 5)	G:	Abundant
	SM:	Pale yellowish brown
	SP:	None
	SPR:	None
Tyrosine agar (ISP 7)	G:	Abundant
	SM:	Light brown
	SP:	Pale yellowish brown
	SPR:	None
Hickey - Tresner's agar	G:	Abundant
	SM:	Yellowish brown
	SP:	None
	SPR:	None
Glucose - nitrate agar	G:	Good
	SM:	Dull yellowish orange
	SP:	Dull yellow
	SPR:	None
Sucrose - nitrate agar	G:	Moderate
	SM:	Pale orange
	SP:	None
	SPR:	Abundant
Glucose - asparagine agar	G:	Good
	SM:	Brownish white
	SP:	None
	SPR:	Poor
Nutrient agar (Difco)	G:	Moderate
	SM:	Pale orange
	SP:	None
	SPR:	None
Tomato paste - oatmeal agar	G:	Good
	SM:	Pale yellowish brown
	SP:	None
	SPR:	None
Glycerol -glycine agar	G:	Abundant
	SM:	Pale yellowish brown
	SP:	None
	SPR:	None
Water agar	G:	Poor
	SM:	Yellowish gray
	SP:	None
	SPR:	Good
Emerson's agar	G:	Good
	SM:	Pale yellowish brown
	SP:	Yellowish brown
	SPR:	None
Potato extract - carrot extract agar	G:	Good
	SM:	Pale yellowish brown
	SP:	None
	SPR:	Abundant

G: Growth, SM: Substrate mycelium, SP: Soluble pigment, SPR: Sporangium.

substrate mycelium was usually yellowish brown to yellowish orange and soluble pigment detected in the several agar media was pale yellowish brown in color. Physiological properties and utilization of carbon sources are summarized in Tables 2 and 3. Cell wall analysis of the organism was performed by the method described by BECKER *et al.*[9]: *meso*- and hydroxy-diaminopimelic acid and glycine were found to be the major constituents, and arabinose and xylose the whole-cell sugar constituents. This is in accordance with cell wall type II and whole cell sugar pattern D, respectively.

The results of taxonomic studies indicated that strain No. 41042 belongs to genus Actinoplanes. Furthermore, strain No. 41042 was found to be closely related to *A. awajinensis* ATCC 33917[6]; the only differences were noted for sporangium size, utilization of carbon sources and production of antibiotics, as shown in Table 4. These differences, however, are not sufficient to consider strain No. 41042 as a new species. From the above, strain No. 41042 was identified as a new subspecies and designated as *A. awajinensis* subsp. *mycoplanecinus* subsp. nov. ATCC 33919. The type strain of *A. awajinensis* subsp. *mycoplanecinus* ATCC 33919 was deposited in the culture collection of Northern Regional Research Laboratories of the U. S. Department of Agriculture, Peolia, Ill., under accession number NRRL 11462.

Fermentation

One loopful of strain No. 41042 growth was inoculated into a 500-ml Erlenmeyer flask containing

Table 2. Physiological properties of strain No. 41042.

Nitrate reduction	+
Starch hydrolysis	−
Gelatin liquefaction	+
Milk coagulation (26°C)	+
Milk peptonization (26°C)	+
Melanin formation : Medium 1	+
: Medium 2	+
: Medium 3	+
Growth temperature: Medium 4	9~32°C

Medium 1: Tryptone - yeast extract broth (ISP 1).
Medium 2: Peptone - yeast extract - iron agar (ISP 6).
Medium 3: Tyrosine agar (ISP 7).
Medium 4: Yeast extract - malt extract agar (ISP 2).

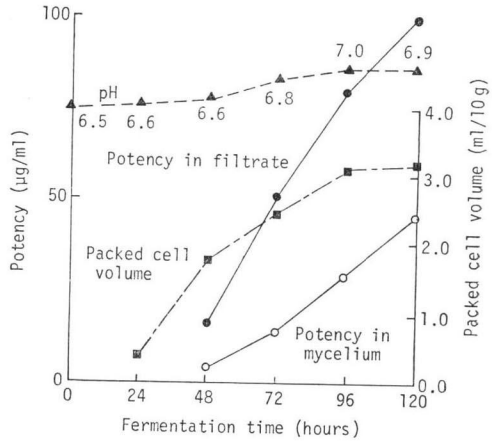
Table 3. Carbohydrate utilization by strain No. 41042.

Positive utilization:
D-Glucose, L-arabinose, D-xylose, D-fructose, L-rhamnose, D-galactose, D-mannose, sucrose, D-cellobiose, maltose, dextrin
Negative utilization:
Inositol, D-melibiose, β-lactose, trehalose, raffinose, D-mannitol, dulcitol, inulin, salicin, Na-acetate, Na-succinate, glycerol, cellulose

Table 4. Comparison of strain No. 41042 with *A. awajinensis* ATCC 33917.

	No. 41042	*A. awajinensis*
Sporangium formation	Only on poor nutritional media	On various media
Sporangium size	7~15 μm	4.5~10 μm
Carbohydrate utilization		
D-Xylose	+	−
D-Galactose	+	−
D-Cellobiose	+	−
Soluble starch	+	−
Antibiotic production		
5 Azacytidine	+	+
Mycoplanecin	+	−

Fig. 1. Time course of mycoplanecins production in 600-liter fermentor.



80 ml of a seed medium composed of 1.0% glucose, 1.0% glycerol, 1.0% sucrose, 0.5% oatmeal, 2.0% soybean meal, 0.5% Casamino Acids, 1.0% pressed yeast and 0.1% $CaCO_3$. The pH of the medium was adjusted to 7.0 before sterilization. The flask was incubated on a rotary shaker at 28°C for 120 hours. A 35-ml aliquot of the culture from the Erlenmeyer flask was inoculated into three 2-liter Erlenmeyer flasks each containing 700 ml of the medium described above and further incubated on a rotary shaker at 28°C for 72 hours. A 2-liter aliquot of the culture from the 2-liter Erlenmeyer flasks was transferred into a 100-liter fermentor containing 50 liters of the same seed medium and incubated at 28°C for 72 hours with agitation of 250 rpm and aeration of 25 liters per minute. After inoculation of 30 liters of the seed culture into a 600-liter fermentor containing 300 liters of a production medium composed of 0.5% glycerol, 2.0% sucrose, 1.0% soybean meal, 1.0% pressed yeast, 0.5% corn steep liquor and 0.001% $CoCl_2 \cdot 6H_2O$ (pH 7.0, before sterilization), fermentation was carried out for 120 hours under agitation of 200 rpm and aeration of 150 liters per minute at 28°C. Mycelial growth was expressed as packed cell volume (ml) after centrifugation of 10 g of the culture broth at 3,000 rpm for 15 minutes. The maximal potency approximately 100 μg/ml in supernatant, and 45 μg/ml in mycelium, was obtained after 120 hours of fermentation. Antibiotic production during fermentation was monitored by the paper-disc agar-diffusion method using the ethyl acetate extract from the culture filtrate and the methanol extract of the mycelium and *Micrococcus luteus* PCI 1001 as a test organism. A typical time course of the fermentation in 600-liter fermentor is shown in Fig. 1.

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