MARIDOMYCIN, A NEW MACROLIDE ANTIBIOTIC. I

TAXONOMY AND FERMENTATION

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Streptomyces sp. No. B-5050 was found to produce a new antibiotic maridomycin. The taxonomic study on strain No. B-5050 was carried out and this strain was found to be a strain of *Streptomyces hygroscopicus* (JENSEN, 1931) WAKSMAN *et* HENRICI, 1948. The cultural condition for the production of maridomycin was also investigated.

In the course of our screening program for new antibiotics active against Gram-positive bacteria and mycoplasma, *Streptomyces* strain No. B-5050 was found to produce antibiotic showing the cross-resistance against macrolide antibiotics.

Strain No. B-5050 was found to belong to *Streptomyces hygroscopicus* group and the antibiotic produced by the strain was assumed to be a new macrolide antibiotic. As a result of the isolation and characterization of this antibiotic from culture fluid, it was found to be a new member of the macrolide antibiotics showing no characteristic ultraviolet absorption and named maridomycin.[1,2]

Strain No. B-5050 is different from the typical strain of *Streptomyces hygroscopicus* (JENSEN, 1931) WAKSMAN *et* HENRICI, 1948, in some respects, but in broad concept it was regarded as belonging to this species. Present paper describes the taxonomic characteristics, the antibiotic activity and fermentation of strain No. B-5050.

Materials and Methods

I. Microbiological Properties

(1) *Streptomyces* sp. No. B-5050: The strain was isolated from a soil sample collected in Chichibu City, Saitama Prefecture, Japan.

(2) Morphological observation: The culture of strain No. B-5050 incubated on a glucose-asparagine agar at 28°C for 14 days was observed by a light microscope and an electron microscope (JEM-SS, Japan Electron Optics Laboratory, Co., Ltd.)

(3) Cultural characteristics: Each of the media used in this study was prepared according to the description of S. A. WAKSMAN.[3] Spores of strain No. B-5050 collected from the 7-day culture on a glucose-asparagine agar were suspended in sterile water and a loopful of the suspension was added to each of the media.

Strain No. B-5050 was incubated at 28°C for 14 days and observations were carried out on every 7 days after inoculation. The color names based on RIDGWAY's description were used.[4]

(4) Utilization of carbon sources: Utilization of carbon sources was investigated by the method of PRIDHAM and GOTTLIEB.[5]

II. Fermentation Procedure

(1) Fermentation in shake flasks: Fermentations were carried out with 20 ml of a medium in 200-ml Erlenmeyer flasks. The seed culture medium containing 2% glucose, 3% soluble starch,

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1 % soybean flour, 1 % corn steep liquor, 0.5 % Polypepton, 0.3 % NaCl and 0.5 % $CaCO_3$ (pH 7.0) was inoculated with spores from the slant culture. The microorganisms were grown at 28°C for 48 hours on a rotary shaker (220 rpm, 5 cm radius). The resultant culture was transferred to various media with the inoculum size of 5 %. Fermentations were conducted at 28°C for 96 hours on a rotary shaker.

(2) Fermentation in a 2,000-liter fermentor: The seed culture medium was inoculated with the organism and incubated at 28°C for 48 hours on a reciprocal shaker (115 spm) as pre-seed culture. One liter of the resultant culture fluid was used as the inoculum for 100 liters of the seed culture medium in a 200-liter fermentor, which was maintained at 28°C for 24 hours with stirring (280 rpm) under aeration (100 liters per minute). One hundred liters of the resultant seed culture were transferred to the main fermentor (1,000 liters of the production medium in a 2,000-liter fermentor). The production medium was composed of 3 % glucose, 0.5 % corn steep liquor, 1 % defatted soybean meal, 0.5 % NaCl, 0.05 % $MgSO_4 \cdot 7H_2O$ and 0.3 % $CaCO_3$. Fermentation was carried out at 28°C for 66 hours with stirring (120 rpm) under aeration (1,000 liters per minute). As antifoam agent 0.05 % Actcol was used.

III. Assay Methods

(1) Agar streak method: Agar streak method and cross-streak agar disc method[6] were used for the estimation of antibiotic activities of the organism.

(2) Paper disc method: *Bacillus subtilis* PCI 219 was used as test organism. It was grown on nutrient agar at 37°C for 18 hours. Maridomycin was used as standard.

(3) Agar dilution method: The serial agar dilution method was applied for the estimation of the antibacterial spectrum of antibiotic produced by strain No. B-5050. Most of the bacteria were grown on nutrient agar at 37°C for 18 hours. The acid-fast bacteria were grown on glycerol nutrient agar for 40 hours. The fungi and the yeasts were grown on glucose nutrient agar at 28°C for 40 hours.

Results and Discussion

I. Taxonomical Characteristics of *Streptomyces* sp. Strain No. B-5050

(1) Morphological characteristics

The aerial mycelium of strain No. B-5050 is simply branched and the chains of spores form loops or spirals of 3 to 5 turns (Plate 1).

Spores are oval or cylindrical (0.7~1.7 μ × 0.9~1.4 μ) with smooth surface (Plate 2).

(2) Cultural characteristics

Plate 1. Microphotograph of sporophores of *Streptomyces hygroscopicus* strain No. B-5050. (× 2250)

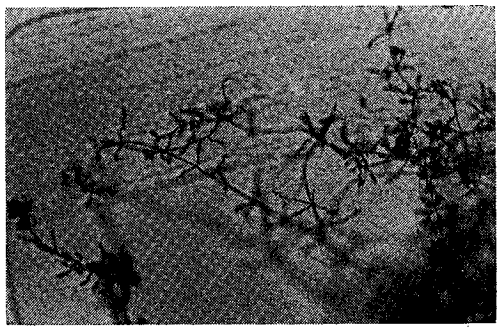


Plate 2. Electronmicrograph of spores of *Streptomyces hygroscopicus* strain No. B-5050. (× 11200)

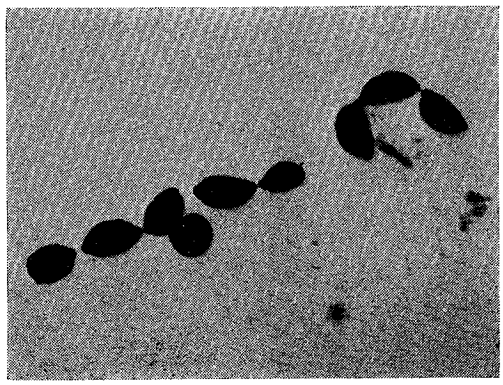


Table 1. Cultural characteristics of *Streptomyces hygroscopicus* No. B-5050

Medium	Cultural characteristics
CZAPEK's agar	G*: good, colorless to pale yellowish brown A*: good, velvety, white to Pale Olive Gray (Rdg.,** LI, 23″″′-f) or Light Olive Gray (Rdg., LI, 23″″′-d), with black patches R*: Smoke Gray (Rdg., XLVI, 21″″-d) SP*: none
Glucose-CZAPEK's agar	G: moderate, colorless A: moderate, white to Pale Drab Gray (Rdg., XLVI, 17″″-f) R: colorless to Pale Drab Gray (Rdg., XLVI, 17″″-f) SP: Pale Vinaceous-Fawn (Rdg., XL, 13″′-f)
Glycerol-CZAPEK's agar	G: moderate, colorless A: moderate, powdery, white to Pale Drab Gray (Rdg., XLVI, 17″″-f) R: Cream Color (Rdg., XVI, 19′-f) SP: none
Glucose-asparagine agar	G: moderate, colorless to pale yellow A: moderate, white to Mouse Gray (Rdg., LI, 15″″′) with black patches R: Cream Color (Rdg., XVI, 19′-f) to Smoke Gray (Rdg., XLVI, 21″″-d) SP: none or pale yellowish brown
Nutrient agar	G: moderate, spreading, colorless to faint yellow A: moderate, white R: Cream Color (Rdg., XVI, 19′-f) SP: faint yellow
Nutrient broth	G: moderate, colorless, sink to the bottom A: none SP: none
Glycerol-nutrient agar	G: good, wrinkled, colorless to faint yellow A: good, white R: Cream Buff (Rdg., XXX, 19″-d) SP: yellow to golden yellow
Glucose-nutrient agar	G: good, wrinkled, colorless to faint yellow A: good, white to Pallid Mouse Gray (Rdg., LI, 15″″′-f) R: Sorghum Brown (Rdg., XXXIX, 19″′-i) SP: Fawn Color (Rdg., XL, 13″′)
Starch agar	G: poor, colorless A: none or poor, white R: pale yellow SP: none
Yeast extract agar	G: good, wrinkled, colorless to faint yellow A: good, white R: Cream Color (Rdg., XXX, 19″-d) SP: yellow to golden yellow
Egg (cultivated at 37°C)	G: moderate, spreading, colorless A: moderate, powdery, white SP: none
Potato plug	G: good, wrinkled, raised, pale brown A: poor, white to Pallid Mouse Gray (Rdg., LI, 15″″′-f) SP: none

Table 1. (continued)

Medium	Cultural characteristics
Carrot plug	G: good, wrinkled, colorless A: moderate, white SP: none
Litmus milk (cultivated at 37°C)	G: ring formed, later sinks to the bottom A: none SP: none
LOEFFLER'S serum (cultivated at 37°C)	G: moderate, glistening, colorless A: poor, white SP: none
Gelatin (cultivated at 24°C for 25 days)	G: poor, restricted, pale yellow A: poor, white SP: none
Cellulose	No growth
Calcium malate agar	G: moderate, colorless A: moderate, powdery, white R: Buff Pink (Rdg., XXVIII, 11″-d) SP: Vinaceous-Cinnamon (Rdg., XXIX, 13″-b)

* G: Growth. A: Aerial mycelium. R: Reverse. S: Soluble pigment.
** Rdg.: R. RIDGWAY.[4]

Cultural characteristics of the strain No. B-5050 on the media for the taxonomical study are shown in Table 1. The vegetative mycelium is colorless or light yellow to light brown on almost all media.

The aerial mycelium is white at first, later becoming light brownish gray to gray and black moist patches or hygroscopic areas are observed. Light violet or faint yellowish brown diffusible pigment is observed on a few kinds of media but no diffusible pigment is observed on most of media.

As no soluble pigment is formed on nutrient agar, peptone agar and tyrosine agar, strain No. B-5050 is regarded to be non-chromogenic.

Physiological properties of strain No. B-5050 are shown in Table 2. Hydrolysis of starch, peptonization of milk and reduction of nitrate to nitrite are positive, while tyrosinase reaction, decomposition of cellulose and liquefaction of serum are negative. Gelatin is not liquefied or liquefied very weakly.

Table 2. Physiological properties of *Streptomyces hygroscopicus* No. B-5050

Temperature	growth occurs at 18°~40°C better growth at 28°~35°C no growth at 10°C and 45°C
pH range	growth occurs at pH 5~9 no or poor growth at pH 4 and 10 optimum range at pH 6~8
Gelatin	no liquefaction
Starch	hydrolysis diameter of hydrolyzed area/diameter of colony=27 mm/8 mm
Tyrosinase reaction	negative
Litmus milk	peptonization without coagulation
Reduction of nitrate to nitrite	weak positive in peptone solution and CZAPEK's solution
Cellulose decomposition	negative
Chromogenicity	negative
Liquefaction of serum	negative

Various carbon sources such as adonitol, D-sorbitol, D-mannitol, D-galactose, D-glucose, D-fructose, melibiose, maltose, sucrose, raffinose, treharose, D-mannose, starch, glycerol, Na-succinate.

Na-citrate are utilized for growth. On the other hand, erythritol, dulcitol, L-sorbose, rhamnose, salicin, esculin and inulin are not or slightly utilized for growth (Table 3).

(3) Comparison of *Streptomyces* sp. strain No. B-5050 with other known streptomyces species.

The microbiological characteristics of *Streptomyces* sp. strain No. B-5050 were compared with those of known species.[7,8,9] From the above-mentioned characteristics, strain No. B-5050 was considered to be closely related to *Streptomyces hygroscopicus* (JENSEN, 1931) WAKSMAN *et* HENRICI, 1948.

As the species of *Streptomyces hygroscopicus* group, TRESNER and BACKUS[10] and WAKSMAN[11] described *Streptomyces hygroscopicus*, *Streptomyces violaceoniger*, *Streptomyces limosus*, *Streptomyces platensis* and *Streptomyces endus*. Color of aerial mycelium of *Streptomyces violaceoniger* (WAKSMAN *et* CURTIS, 1961) WAKSMAN *et* HENRICI, 1948, is carmin red to cinnamon-brown. *Streptomyces limosus* LINDENBEIN, 1952, forms lemon yellow soluble pigment on glucose-asparagine agar. *Streptomyces platensis* PITTENGER *et* GOTTLIEB, 1954, shows light brown to brown growth on synthetic media. The color of the vegetative mycelium of *Streptomyces endus* GOTTLIEB *et* CARTER, 1956, changes black. Therefore, these species were considered to be different from strain No. B-5050. On the other hand, properties of strain No. B-5050 are in good agreement with those of *Streptomyces hygroscopicus* (JENSEN, 1931) WAKSMAN *et* HENRICI, 1948, although the characteristics such as the ability of decomposition of cellulose, reduction of nitrate to nitrite and the formation of diffusible pigment on some media are slightly different from those described by WAKSMAN.[8]

Table 3. Utilizaiton of carbon sources by *Streptomyces hygroscopicus* No. B-5050

Carbon sources	Growth	Carbon sources	Growth
Erythritol	±	Melibiose	+++
Adonitol	+++	Sucrose	+++
D-Sorbitol	+++	Lactose	++
i-Inositol	++	Raffinose	+++
D-Mannitol	+++	Trehalose	+++
Dulcitol	±	Salicin	−
D-Xylose	+	Esculin	−
L-Arabinose	+	Inulin	±~+
L-Sorbose	±	D-Mannose	+++
D-Galactose	+++	Starch	+++
D-Glucose	+++	Glycerol	+++
D-Fructose	+++	Na-Acetate	+
D-Maltose	+++	Na-Citrate	++
Rhamnose	±	Na-Succinate	++
		None (Control)	−

+++: Abundant growth ++: Good growth
+: Moderate growth ±: Poor growth
−: No growth

From these results, strain No. B-5050 was regarded as a strain of *Streptomyces hygroscopicus* (JENSEN, 1931) WAKSMAN *et* HENRICI, 1948.

Table 4. Macrolide antibiotics produced by *Streptomyces hygroscopicus* group

Antibiotic	max (nm)	Producing organism
Tylosin	284	*S. violaceoniger*[13]
Carbomycin	238	*S. halstedii*[14]
Carbomycin B	278	*S. halstedii*[15]
Relomycin	282	*S. hygroscopicus*[16]
Antibiotic A6599	232, 278~282	*S. hygroscopicus*[12]
Antibiotic YL-704[2]	230, 280, end absorp.	*S. platensis*
Maridomycin	end absorp.	*S. hygroscopicus*

Strain No. B-5050 has been deposited in the Institute for Fermentation, Osaka and assigned accession number of IFO 12995. Comparison between strain No. B-5050 and other related macrolide antibiotic producing organisms is shown in Table 4.

It is reported that *S. hygroscopicus* produces carbomycin,[14] tylosin,[13] relomycin[16] and antibiotic A6599,[12] but these antibiotics are different from maridomycins produced by strain No. B-5050. Antibiotic YL-704C$_1$ produced by *S. platensis* will be mentioned in the following

Table 5. Antibacterial activity of *Streptomyces hygroscopicus* No. B-5050 by cross streak method

Test organism	Inhibition zone (mm)	
	Nutrient agar	Glycerol nutrient agar
Escherichia coli	0	0
Proteus vulgaris	0	0
Staphylococcus aureus	31	29.5
Staphylococcus aureus OM, EM-R*	0	—
Bacillus subtilis	>40	28.5
Bacillus cereus	28	27
Bacillus brevis	27	—
Sarcina lutea	37	34.5
Micrococcus flavus	35	35
Mycobacterium sp. Takeo	—	21.5

*: Resistant to oleandomycin and erythromycin.
—: Note tested.

Table 6. Antibacterial activity of *Streptomyces hygroscopicus* No. B-5050 against *Bacillus subtilis* by agar disc method

pH of test medium	Inhibition zone (mm)
6.0	15
8.0	23

Table 7. Fermentation medium for *Streptomyces hygroscopicus* No. B-5050

Glucose	3 %
Corn steep liquor	0.5
Soybean meal	1
NaCl	0.5
$MgSO_4 \cdot 7H_2O$	0.05
$CaCO_3$	0.3
Maridomycin	350 mcg/ml

course of a typical maridomycin fermentation is shown in Fig. 1. The reducing sugar was utilized almost completely within 40 hours. Maridomycin appeared in culture fluid at about 20 hours and its amount accumulated reached maximum at about 60 hours.

IV. Antimicrobial Activity of Maridomycin

Maridomycin was found to be composed of six components, maridomycins I, II, III, IV, V paper.[2]

II. Antimicrobial Activity of Strain No. B-5050

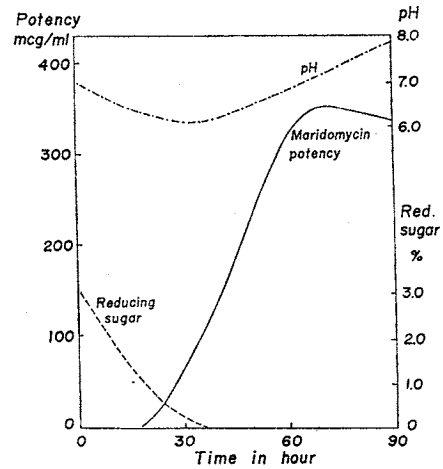
Antimicrobial activities of strain No. B-5050 estimated by the agar streak method and agar disc method are shown in Tables 5 and 6. From the results of agar streak method, it was found that strain No. B-5050 inhibited the growth of Gram-positive bacteria including acid-fast bacteria, but that oleandomycin- and erythromycin-resistant *Staphylococccus aureus* was not inhibited. By the agar disc method, it was confirmed that strain No. B-5050 produced physiologically basic antibiotic and it was suggested that strain No. B-5050 might produce some basic macrolide antibiotics.

III. Fermentation

The medium suitable for the production of maridomycin by strain No. B-5050 was investigated.

By shaking culture, a number of media were evaluated for the production of maridomycin and a representative medium is shown in Table 7. In this medium, 350 mcg/ml of maridomycin was accumulated in the culture fluid. This medium was applied to the fermentation in a 2,000-liter fermentor. The time

Fig. 1. Time course of maridomycin production in a 2,000-liter fermentor.



and VI.[2] Their structures are different from each other in acyl moieties at C_3 and $C_{4''}$ positions.

Antimicrobial activity of each component was estimated by agar dilution method. Maridomycins I, II, III, IV, V and VI showed similar antibacterial spectra against Gram-positive bacteria including acid-fast bacteria. Among the maridomycin components, maridomycin I showed the highest antibacterial activity, maridomycin IV medium, and maridomycin VI the lowest (Table 8).

Table 8. Antimicrobial activity of maridomycin components (I~VI)

Test organism	M. I. C. (mcg/ml)					
	I	II	III	IV	V	VI
Bacillus subtilis	0.2	0.2	0.5	1	2	2
B. cereus	0.5	0.5	1	2	5	5
B. brevis	0.2	0.2	0.5	5	10	10
B. megaterium	0.5	0.5	0.5	1	2	2
Staphylococcus aureus	0.5	0.5	1	2	5	5
S. aureus CM, EM-R*	20	20	50	>100	>100	>100
S. aureus SM, CP, TC, OM, EM-R**	20	20	50	>100	>100	>100
Sarcina lutea	0.1	0.1	0.2	0.5	0.5	0.5
Micrococcus flavus	0.1	0.1	0.2	0.5	0.5	0.5
Serratia marcescens	>100	>100	>100	>100	>100	>100
Escherichia coli	>100	>100	>100	>100	>100	>100
Proteus vulgaris	>100	>100	>100	>100	>100	>100
Pseudomonas aeruginosa	>100	>100	>100	>100	>100	>100
Mycobacterium smegmatis	50	100	>100	>100	>100	>100
M. sp. Takeo	50	100	>100	>100	>100	>100
M. sp. Takeo SM-R	50	100	>100	>100	>100	>100
M. sp. Takeo NM-R***	50	100	>100	>100	>100	>100
M. phlei	50	100	>100	>100	>100	>100
Mycobacterium sp. ATCC 607	50	100	>100	>100	>100	>100
Candida albicans	100	>100	>100	>100	>100	>100
Saccharomyces cerevisiae	100	>100	>100	>100	>100	>100
Penicillium chrysogenum	100	>100	>100	>100	>100	>100
Aspergillus niger	100	>100	>100	>100	>100	>100
Trichophyton mentagrophytes	100	>100	>100	>100	>100	>100

* Strain resistant to oleandomycin and erythromycin.
** Strain resistant to streptomycin, chloramphenicol, tetracycline, oleandomycin and erythromycin.
*** Strain resistaht to neomycin.

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