M-9337, A NEW ANTISTREPTOLYSIN, PRODUCED BY *STREPTOMYCES* SP.

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A new biologically active substance, M-9337, was obtained from Streptomyces strain M-9337, a soil isolate. The producing organism was subsequently determined to be a new strain and named *Streptomyces antihaemolyticus* M-9337. The active substance was prepared as white yellow powder from culture broth by solvent extraction and silica gel thin-layer chromatography. It showed no antimicrobial activity and potent inhibitory activity against streptolysin, a type of hemolysin.

In the course of screening program for biologically active metabolites, a streptomycete, strain M-9337, was found to produce an antihemolytic substance which was designated as M-9337. The substance inhibited streptolysin O at low concentrations[1].

The present paper deals with the taxonomy of the producing strain and the production, isolation and physicochemical and biological properties of M-9337.

Taxonomy of the Producing Organism

Strain M-9337 was isolated from a soil sample collected at Niigata City, Japan. Most of the taxonomic studies of the culture were carried out in accordance with methods adopted by the International Streptomyces Project (ISP)[2]. Observations of the culture were made after incubation at 28°C for 2 weeks unless otherwise stated.

Morphology

Aerial hyphae are well grown on yeast - malt agar, tyrosine agar and oatmeal agar media, and have a diameter ranging 0.6 ~ 1.0 μm. The conidiophore is simple and has a wavy or spiral form, coiled

Plate 1. Aerial mycelium of strain M-9337. (×1,000)

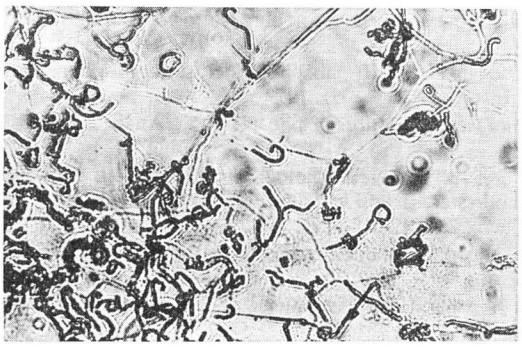


Plate 2. Electron micrograph of spores of strain M-9337. (×5,000)

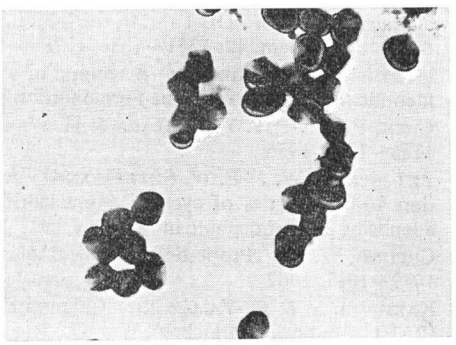


Table 1. Cultural characteristics of strain M-9337.

Medium	Growth	Aerial hyphae	Reverse	Soluble pigment
Sucrose - nitrate agar	Poor, moist, white	After 4 days, poor, white	White	—
Glucose - asparagine agar	Good, moist, pale yellow	Poor, initially white, after 10 days, becoming pale yellow	Pale yellow	Pale yellow
Glycerol - asparagine agar	Good, moist, whitish gray, after 4 days, yellowish gray	Poor, whitish gray	Whitish gray	Pale yellow
Starch agar	Poor, pale gray, clear	Moderate, white	White to pale yellow	—
Tyrosine agar	Good, moist, light gray	Good, whitish gray to gray	Blackish brown	Blackish brown
Nutrient agar	Good, moist, gray, later becoming pale grayish yellow	Poor, whitish gray	Pale brown	Brown
Yeast - malt agar	Good, dry, pale yellow	Good, whitish gray	Pale brown	Yellowish brown
Oatmeal agar	Good, moist, whitish gray, clear	Good, ash gray, abundant	Pale cream-yellow	

once or twice (Plate 1). The diameter of the spiral is in the range of 4.0~5.0 μm. Conidia are oval to spherical in shape and 0.7~1.1 by 1.1~1.2 μm in size. The surface appearance is smooth as observed by an electron microscope (Plate 2).

Cultural Characters

Growing state of strain M-9337 on various media is shown in Table 1.

Physiological Characters

The physiological characteristics of strain M-9337 and utilization of carbon sources are shown in Tables 2 and 3.

Table 2. Physiological properties.

Hydrolysis of starch	—
Reduction of nitrate	±
Decomposition of cellulose	—
Coagulation and peptonization of skim milk	+
Formation of hydrogen sulfide	±
Formation of indole	+
Formation of ammonia	+
VP test	+
Catalase	+
Formation of melanine-like pigment	+
Liquefaction of gelatin	+
Range of growth conditions:	
pH	5.0~7.5
Temperature	25~40°C

Table 3. Utilization of carbon sources.

Carbohydrate	Growth
L-Arabinose	++
D-Xylose	+++
D-Glucose	+++
D-Fructose	+++
Sucrose	++
Inositol	++
L-Rhamnose	+++
Raffinose	++
D-Mannitol	++

When the above-indicated mycological properties are examined with reference to ISP and BERGEY's Manual of Determinative Bacteriology, 8th edition, it has been found that the present strain belongs to the genus Streptomyces and resembles *Streptomyces robefuscus*, *S. albaduncus* and *S. naganishii*. However, the present strain differs from these species in that with *S. robefuscus*, the vegetative hyphae are a tint or shade of brown in color, that with *S. albaduncus* the utilization of sucrose is (±), and its spore silhouette is spiny, and that *S. naganishii* makes no use of sucrose and its conidiophore is not wavy or spiral. Accordingly, strain M-9337 was concluded to be a new species of the genus Streptomyces and designated as *Streptomyces antihaemolyticus* M-9337 which was deposited at the Fermentation Research Institute, Agency of Industrial Science and Technology, Japan, as FERM-P No. 4651.

Fermentation

A well sporulated agar slant of *S. antihaemolyticus* M-9337 was inoculated into a 500-ml Sakaguchi flask containing 100 ml of the medium composed of glucose 2.0%, $NaNO_3$ 0.2%, K_2HPO_4 0.1%, KCl 0.05%, $MgSO_4 \cdot 7H_2O$ 0.05%, $FeSO_4 \cdot 7H_2O$ 0.001%, meat extract (Difco) 0.2%, yeast extract (Difco) 0.2%, peptone 0.2%, defatted soybean 2%, and Adecanol LG-109 (Asahi-Denka) 0.01%. The pH of the medium was adjusted to pH 7.0 before sterilization. The flask was incubated on a rotary shaker at 30°C for 48 hours. Two hundred ml of the culture broth was inoculated into 16 liters of the above-mentioned medium in a 30-liter fermenter. The fermentation was conducted for 96 hours under the following conditions: temperature 30°C, aeration 8 liters/minute, and agitation 250 rpm.

Isolation and Purification

The culture broth (about 60 liters) was centrifuged to remove the microorganisms therefrom. To the supernatant was added 1 M hydrochloric acid to adjust its pH to 3.0 and the resulting precipitate was collected. The precipitate was washed with cold acetone and then ether, followed by extracting with a mixed solvent of chloroform and methanol (2: 1) and evaporating the extract to dryness to obtain crude M-9337.

Further purification was carried out by ion exchange chromatography using DEAE-Sephadex A-50, which was equilibrated with a mixed solvent of chloroform, methanol and 0.8 M sodium acetate (30: 60: 8). The crude powder was dissolved in a small amount of 85% ethanol and applied on the DEAE-Sephadex column, followed by eluting with a mixed solvent of chloroform, methanol and 0.8 M sodium acetate (30: 60: 8). An active fraction was collected and concentrated *in vacuo*. After evaporating the solvent layer the powder was dissolved in methanol, subjected to preparative silica gel thin-layer chromatography and developed with a mixed solvent of chloroform, methanol and water (65: 25: 4). A single spot of an active fraction was scraped off and extracted with methanol. Evaporation of the solvent under vacuum yielded purified M-9337.

Physicochemical Properties

M-9337 was obtained as a white or light yellow powder which melted at 170~175°C with decomposition. The elemental analysis was as follows: C 52.56, H 7.31, O 40.13%. M-9337 is readily soluble in dimethyl sulfoxide and pyridine, soluble in methanol and 1-butanol, slightly soluble in ethanol, chloroform and water, and insoluble in ethyl acetate, ethyl ether and acetone. It gave positive reactions to anthrone, ammonium molybdate-perchloric acid and anisaldehyde tests, and gave negative reactions to ninhydrin and DRAGENDORFF tests. The UV spectrum showed no absorption maximum below 220 nm

Fig. 1. UV spectrum of M-9337 (MeOH).

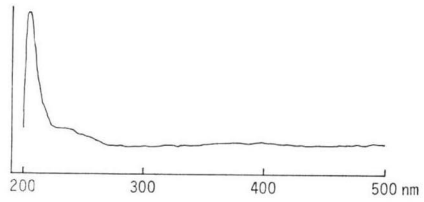
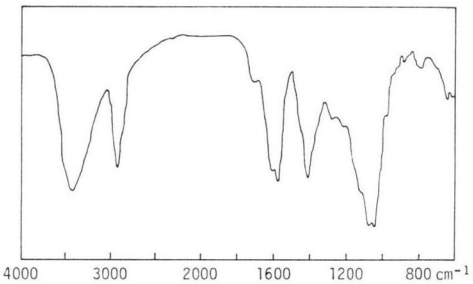


Fig. 2. IR spectrum of M-9337 (KBr).



(Fig. 1). The IR spectrum measured in KBr disk showed the presence of hydroxyl (3400(br) cm^{-1}) and carboxylate (1580 and 1410 cm^{-1}) groups (Fig. 2).

Biological Properties

M-9337 showed strong inhibitory activity against streptolysin. During the course of culture, extraction and purification, the antihemolytic activity was measured with the use of streptolysin O produced by a group A streptococcus. That is, each 1 ml of the sample which has been diluted stepwise was placed in a test tube, to which was added 0.5 ml of a solution of streptolysin O (Eiken) followed by mixing well and incubating for 15 minutes at 37°C. Then 0.5 ml of defibrinated rabbit red cell (5%) was added and incubation was continued for additional 45 minutes at 37°C. The end point was considered to be the last tube showing no hemolysis of the supernatant fluid and the result was expressed in Todd units. The purified M-9337 showed 32 units/mg.

Antimicrobial activity of M-9337 was assayed by a standard 2-fold tube dilution method. As the result, M-9337 displayed no antibacterial and no antifungal activity at 1 mg/ml.

The acute toxicity of M-9337 was determined from the number of survivors at 14 days after a single intraperitoneal injection into *dd* mice. The LD_{50} was 290 mg/kg.

Discussion

M-9337 was obtained from a soil microorganism by a screening method using streptolysin which is a type of hemolysin and is a kind of bacterial toxin. Toxins discharged from streptococci, staphylococci and others cause various diseases. Therefore, the microbial antitoxic substance may have a special significance in therapeutic treatment of toxigenic diseases.

References

1) MIYAMURA, S.: A new antistreptolysin, M-9337, from actinomycetes. J. Antibiotics 34: 472~473, 1981
2) SHIRLING, E. B. & D. GOTTLIEB: Method for characterization of *Streptomyces* species. Intern. J. Syst. Bacteriol. 16: 313~340, 1966