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A New Antifungal Antibiotic Produced by *Streptomyces galbus*

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ABSTRACT. A streptomycete producing an antibiotic having antifungal and antibacterial activity was isolated from a soil sample of West Bengal. It was characterized and identified as *Streptomyces galbus*. The antibiotic was isolated from the fermented broth by treatment with activated charcoal and purified by chromatography on alumina and paper. It is a colourless, odourless, hygroscopic, amorphous compound. Although homogenous by paper and thin-layer chromatography, the possibility of the presence of two components was indicated by gel filtration. Its physico-chemical characteristics and UV-absorption spectrum suggest a non-polyenic nature. It is active against a wide variety of fungi and bacteria. It has some phytotoxic effect, but is relatively non-toxic to rats.

In the course of a survey of antifungal actinomycetes from soils of West Bengal, 47 soil samples from different districts were analyzed. Altogether 127 strains were isolated, of which only 26 were antifungal. A *Streptomyces* sp. 5ME-13 was found to be the most active on the basis of its performance in cross-streak and agar-cup assay against fungi, yeasts and bacteria. Taxonomically, *Streptomyces* sp. 5ME 13 resembled closely *S. galbus* which has not so far been reported to produce any antifungal antibiotic but is known to produce streptomycin (Murase *et al.* 1959; Okami *et al.* 1959) and actinomycin (Umezawa 1967).

This paper deals with the characterization of the producing streptomycete, the isolation and purification of the antibiotic, the preliminary physico-chemical characterization and some biological properties of the antibiotic.

MATERIALS AND METHODS

Isolation and characterization of the streptomycete. Streptomycete strain 5ME-13 was isolated from a soil sample of Memari (Burdwan) West Bengal, India. The soil sample was enriched with $CaCO_3$ (Tsao *et al.* 1960), serially diluted in sterilized distilled water and the suspensions were plated on glucose–asparagine agar. After one week of incubation at 28 °C the streptomycete colonies were picked up in slants and purified by dilution streak method on glucose–asparagine plates. Single colonies were isolated in pure

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form. Their antifungal potency was determined by cross-streak method on glucose—asparagine agar and casein hydrolysate—peptone agar plates. The performance of the antibiotically active cultures in liquid medium was then tested by growing them in straw-infusion broth and assaying the culture filtrate by agar-cup method against test fungi.

The methods used for its characterization were those of the *International Streptomyces Project* (Shirling and Gottlieb 1966). Detailed morphological studies were made by the agar-cylinder culture technique of Nishimura and Tawara (1957) and the surface structure of the spores was studied with the aid of a Philips Scanning Electron microscope model PSEM 500 following the method of Dietz and Mathews (1971). The colour determinations were made using *Methuen Handbook of Colour* and *Munsell Book of Color*.

Production of the antibiotic. A suspension (absorbance 2.0 EEL units) of spores collected from 14-d-old slant cultures of the producer strain on glucose—asparagine agar in 0.1 % aqueous Tween 80 was used as inoculum.

The fermentation medium containing (g/L) $NaNO_3$ 3.4, K_2HPO_4 5.65, $KH_2PO_4.3H_2O$ 2.38, $MgSO_4.7H_2O$ 1.0, glycerol 30, trace salts solution 1.0 mL was adjusted to pH 6.8 and distributed to 50-mL quantities in 250-mL Erlenmeyer flasks before sterilization (103 kPa, 15 min). The flasks were cooled to room temperature, inoculated with 2.5 mL of spore suspension and incubated at 30 °C for 8 d. The antibiotic titer was measured daily by agar-cup with *Curvularia pallescens* as the test organism.

Isolation and purification of the antibiotic. After 8 d of incubation the fermented broth (4 L) was filtered first through absorbent cotton and then through Whatman (No. 1) filter paper to remove the mycelial fragments. Activated charcoal (E. Merck, 10 g/L) was added to the culture filtrate and kept on a mechanical shaker for 2 h. The charcoal with the adsorbed antibiotic was then filtered and washed thrice with distilled water. The charcoal cake was then transferred to a conical flask and the active principle was repeatedly extracted (5 times) by methanol on a shaker. The methanolic extracts were pooled and evaporated to dryness under reduced pressure at 60 °C. The died mass was then dissolved in a small quantity of methanol and the insoluble residue was discarded. On adding three volumes of chloroform to the methanolic solution, and antibiotically inactive precipitate was obtained and removed by centrifugation (5000 ***g***, 10 min). The supernatant was concentrated and the precipitation step was repeated. The precipitate was removed as before.

The concentrated supernatant (30 mL) was next placed on a 200 × 22 mm column of neutral alumina. The column was eluted at a rate of 0.2 mL/min with a butanol—methanol—water (4 : 1 : 2) mixture. Fractions of 5 mL each were collected and assayed microbiologically for antifungal activity. The active fractions (about 45 mL) were pooled and evaporated to dryness under vacuo.

The dried mass was dissolved in methanol and was applied on a 250 × 250 mm chromatographic paper (Whatman No. 1) in the form of a narrow band and developed with a chloroform—methanol (70 : 30) mixture. The site of the active component on the paper was detected by bioautography from the guide strip. The region of the paper containing the antibiotic was cut out as narrow strip and the active principle was extracted from the paper with methanol. Removal of methanol by evaporation yielded 33 mg of the colourless amorphous antibiotic.

TABLE I. Cultural characteristics of *Streptomyces* sp. 5ME-13

Culture media	Growth and colour		Diffusible pigment
	vegetative mycelium	aerial mycelium	
Tryptone—yeast extract	poor to moderate, oxide yellow, 5CY (9YR 6.0/8.3)	poor, white, without sporulation	light brown
Yeast extract—malt extract agar (ISP)	good, yellowish brown, 5D8 (9.5YR 5.0/8.6)	good, yellowish gray (putty), 4B2 (2Y 8.1/1.3)	light brown
Inorganic salts—starch agar (ISP medium 4)	moderate, silver white, 2B2 (6Y 8.0/1.2)	moderate, spreading, yellowish gray 3D2 (2.5 Y 5.9/0.7)	none
Glycerol—asparagine agar (ISP medium 5)	moderate, dark yellow to rust brown 6E8 (7YR 3.9/6.8)	moderate to abundant, grayish yellow, 1B3 (0.5 GY 8.0/2.5)	honey yellow or oak brown
Peptone—yeast extract iron agar (ISP medium 6)	poor, yellowish gray, 4B2 (2Y 8.1/1.3)	poor, white with grayish tinge, without sporulation	dark brown
Tyrosine agar (ISP medium 7)	moderate, dull, yellow, 3B3 (5.5Y 8.0/2.6)	moderate, silver gray, 4E2 (−4.9/0.5)	brown to dark brown
Glucose—asparagine agar	moderate, yellow to light brown, 6D8 (6.5 YR 4.7/8.0)	moderate, colonies raised and spreading, powdery, light gray to dark gray, 3D2 (2.5 Y 5.9/0.7)	yellow to brown
Benett's agar	moderate to abundant, olive brown to dark brown 7F7 (3YR 2.7/3.1)	moderate, brownich gray, 5D2 (6 YR 5.6/0.8)	honey yellow or oak brown
Sabouraud's maltose agar	profuse, greyish yellow, 4C6 (5Y 6.5/6.2)	profuse, spreading, poor sporulation, silver gray, 4E2 (−4.9/0.5)	yellowish to reddish brown
Glycerol Czapek-Dox agar	abundant brownish red, 8C7 (9R 5.0/7.9)	abundant, lead gray, 2D2 (2.5 Y 5.9/0.7)	brown
Sucrose Czapek-Dox	no vegetative growth	poor, few scattered masses of sporophores, very light gray	none

Emmerson's agar	good, light brown, 6D8 (6.5YR 4.7/8.0)	good, pale gray or grayish white, 1B1 (−8.2/−) with white margins, surface rough to powdery	Chinese yellow
Lindenbein's synthetic agar	moderate, reddish brown, 8E8 (0.5 YR 3.3/6.1)	good, powdery, light gray 3D2 (2.5 Y 5.9/0.7) not uniformly sporulated	dark brown
Casein hydrolysate−peptone agar	moderate, burnt sienna, 7D8 (2.5 YR 4.3/8.5)	moderate to profuse, papery, sporulation profuse in the middle, golden blonde, 4C4 (5Y 6.8/3.2)	reddish brown
Nutrient agar	poor, butter yellow, 4A5 (3Y 8.6/5.8)	poor, white, mostly non-sporulated	straw yellow
Cause mineral salts medium I	moderate, golden yellow to brown, 4B5 (3.5 Y 7.8/5.3)	moderate, cement colour, 4D2 (3.5 Y 5.9/0.8) less sporulated in the marginal region	none
Straw-infusion media	moderate, brown to dark brown in colour	profuse, powdery, cement colour, 4D2 (3.5Y 5.9/0.8)	brown to dark

Chromatography. For proper chromatographic separation one-dimensional ascending chromatography on Whatman No. 1 filter paper was performed at room temperature using a number of solvent systems. The culture filtrate or the partially purified product was applied in the form of spots or as a band on a line 80 mm away from the end of the chromatographic paper. The length of the paper was varied according to the requirement. The flow of the solvent was along the fibre direction. The spots were detected by iodine vapour and confirmed by bioautography.

Similarly thin layer chromatography was performed on silica gel plates at room temperature with a solvent run for 2—4 h, depending on the solvent system. Spots were detected with iodine vapour.

For Sephadex column chromatography, a 182×10 mm column was prepared with Sephadex G-25 powder. Void volume of the column was determined with the help of blue dextran; the flow rate was maintained at 0.4 mL/min and 2-mL fractions were collected.

UV-absorption spectra. The UV-absorption spectrum of the antibiotic in methanolic solution was determined in a Beckman recording spectrophotometer (Model 26) using 10-mm cuvette.

Determination of minimum inhibitory concentration. The minimum inhibitory concentrations of the antibiotic against fungi and bacteria were determined by agar dilution method using Czapek-Dox, Sabouraud's dextrose and nutrient agar. The inoculated plates were incubated for 2 d at 28—30 °C for fungi and at 37 °C for bacteria. The results were expressed as the minimum concentration required for total inhibition of fungal or bacterial growth.

Phytotoxicity and animal toxicity. Phytotoxicity of the antibiotic was determined by its effect on germination of seeds and permeability of plant cells. For the germination test, seeds of the following varieties were used: *Oryza sativa* (var. Jaya), *Triticum vulgare* (var. Sonalika), *Cucumis sativa* (var. CB Long green), *Trigonella corniculata* (var. Kesure) and *Cajanus cajan* (var. Prabhat). The seeds were surface sterilized with 0.1 % mercuric chloride solution for 2 min, washed repeatedly with sterile water and steeped in sterile water for 6 h. The seeds were then treated for a variable period with aqueous antibiotic solutions having different concentrations and were then allowed to germinate on germination plates at 30 °C for 4 d. Control sets were soaked in sterile distilled water in place of antibiotic solution for the same period.

The effect of antibiotic on the permeability of plant cells was determinated by testing the leaching out of betacyanin pigment from red beet root slices following the method of Nemec and Betina (1968).

Animal toxicity of the antibiotic was tested by injecting the aqueous antibiotic solution intravenously into albino rats of either sex having a body mass of 15—25 g following the method of Ghosh (1972).

RESULTS

Characterization and identification of the streptomycete

Morphological characteristics. Vegetative and aerial mycelia well developed, branched, hyphae 0.7—1.0 μm in width. Vegetative mycelium not fragmented, aerial mycelium with sporophores and spores. Spore chains loosely spiral, or in the form of loops or hooks, retinaculum apertum type, bearing chains of more than 10 spores. Spores oval to elongated, 1.7—2.0×0.8 μm

TABLE II. Physiological characteristics of *Streptomyces* sp. 5ME-13

Characteristics	Response[a]
Catalase production	+
Nitrate reduction	+
Indole production	−
Starch hydrolysis	+
Casein hydrolysis	+
Fat (Tween 80) hydrolysis	+
Gelatin liquefaction	+
Cellulose hydrolysis	±
H_2S production	−
Melanin production	+
NaCl tolerance	7 %
Streptomycin sensitivity	+
Optimum temperature of growth	28 °C
Optimum pH of growth	6.8
LD_{50} of spore at 55 °C	34 min

[a] + positive, ± weakly positive, − negative.

surface smooth. Their morphology was revealed by phase-contrast microscopy are shown in Plates 1 and 2, respectively.

Culture characteristics. The cultural characteristics were determined by growing it on *International Streptomyces Project* media and on some other media at 28 °C and the changes of growth, aerial mycelium and soluble pigment were observed after a period of 7, 14, and 21 d. The observations are recorded in Table I. Growth of the organism is abundant to profuse in Sabouraud's maltose agar, glycerol Czapek-Dox agar, casein hydrolyzate—peptone agar and straw-infusion agar. The media which supported poor growth included those with tryptone, peptone or meat extract. Sporulation was abundant to profuse on most of the synthetic and artificial media, but poor or feeble in the media containing peptone. Sporulating aerial mycelium colour mostly in various shades of gray, while the reverse of the colony varied from yellow to yellowish brown to dark brown. The yellow to dark brown diffusible pigment was pH sensitive and turned purple in dilute alkali but remained unchanged in acid.

Physiological characteristics. The physiological characteristics are summarized in Table II and the carbon source utilization pattern in Table III.

TABLE III. Carbon source utilization pattern of *Streptomyces* sp. 5ME-13

Response	Carbon source
Well utilized	D-glucose, L-arabinose, D-galactose, D-fructose, maltose, glycerol, D-mannitol, starch
Weakly utilized	D-xylose, D-mannose, lactose, cellulose
Not utilized	L-rhamnose, sucrose, raffinose, salicin, *myo*-inositol

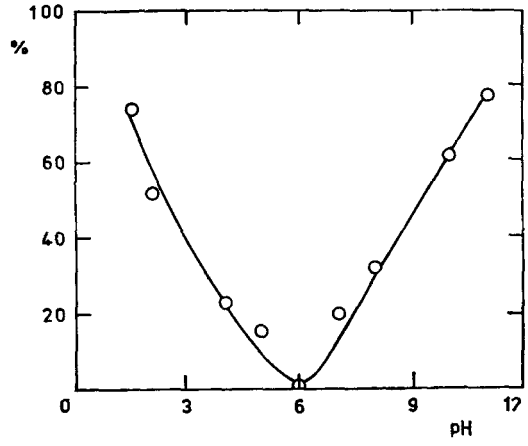

FIG. 1. Stability (loss of activity, %) of the antibiotic at different pH values.

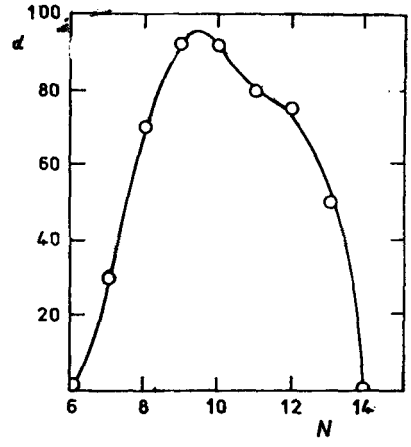

FIG. 2. Elution pattern of the antibiotic from a Sephadex G-25 column (bed volume 14 mL, eluent — distilled water); *N* fraction number, *d* diameter of inhibition zone (mm).

Identification of the strain. The gray colour of the aerial mycelium, retinaculum apertum type of sporophore, melanoid pigment and the smooth spore surface, indicated that the organism belonged to the gray chromogenic series of the genus *Streptomyces* with spiral sporophores. The pattern of carbon source utilization pointed to its close similarity with *Streptomyces galbus*. It was found that the similarity of the strain 5ME-13 with *S. galbus* (Shirling and Gottlieb 1969; Pridham and Tresner 1974) was in the order of 65—70 %.

Physico-chemical properties of the antibiotic

The purified sample of the antibiotic is a colourless, odourless, hygroscopic compound, having a slightly acidic reaction in aqueous colution. It is highly soluble in water, methanol, ethanol. acetone, propanol and dimethylformamide; insoluble in butanol, chloroform, diethyl ether, benzene and ethyl-, methyl-, and pentyl acetate.

The antibiotic is thermostable and can withstand heating at 100 °C for more than 20 min. Even at 121 °C it loses only 11.4 % of its activity in 15 min. The methanolic solution retains full antibiotic activity when kept at 5—10 °C even after one year of storage. However, the antibiotic is very susceptible to degradation by changes in pH. In aqueous solution a shift of pH either towards the acidic side or towards the alkaline side leads to decrease in activity (Fig. 1). The antibiotic is also insensitive to photooxidation. The UV-absorption spectrum of the antibiotic in methanolic solution exhibits a single peak at 210 nm.

The purified product was found to be homogenous by paper chromatography and thin-layer chromatography using a large number of solvent systems (Table IV). The mobility of the antibiotic compound on chromatograms, both paper and thin-layer, indicated clearly an affinity towards polar solvents.

TABLE IV. R_F values of the antibiotic[a]

Solvent system	$R_F \times 100$	
	paper chromatography	TLC
Butanol—acetic acid—water (2 : 1 : 1)	67	70
Acetone—water (1 : 1)	84	—
Butanol—ethanol—water (3 : 3 : 2)	71	74
Methanol—ammonia—water (2 : 1 : 4)	84	—
Propanol—water (4 : 1)	71	75
Ammonium chloride in water (3 %)	79	—
Butanol saturated with water	41	51
Pentanol—methanol—water (10 : 5 : 4)	45	59
Butanol—methanol—water (4 : 1 : 2)	57	63
Ethyl acetate saturated with water	9	—
Benzene saturated with water	0	—
Methanol—water (4 : 6)	76	90
Chloroform—ethanol—water (50 : 50 : 8)	67	—
Chloroform—methanol (4 : 6)	78	—
Chloroform—methanol (7 : 3)	23	48
Butanol—pyridine—water (6 : 1 : 7)	31	72
Ethyl acetate—pyridine—water (12 : 5 : 14)	61	64

[a] Whatman No. 1 filter paper, ascending chromatography at room temperature, detection by iodine vapour and bioautography using *Curvularia pallescens* as test organism.
[b] No inhibition zone corresponding to this spot was detected by bioautography.

The elution profile from Sephadex G-25 showed a main peak, between the 9th and the 10th fraction and a shoulder at the 12th fraction (Fig. 2) which indicate the possibility of the presence of two components in the purified sample.

Biological properties of the antibiotic

Antimicrobial property. The minimum inbibitory concentration of the antibiotic for some selected fungi and bacteria was determined by agar dilution method (Table V). Yeasts and dimorphic fungi, like *Candida*, were in general, more resistant than the filamentous fungi. Bacteria also differed in susceptibility, but the difference was not correlated with their gram reaction.

Seed germination. The effect on seed germination was variable in the different seeds tested. The antibiotic was without any adverse effect in rice and cucumber seeds. But it inhibited germination of *Cajanus cajan* to a significant extent and of wheat to a lesser extent. On the other hand, germination of *Trigonella corniculata* seeds was stimulated by the antibiotic.

Permeability. There was no significant leaching of betacyanin pigment from beet root slices when kept immersed in aqueous solutions of the antibiotic at various concentrations for 4 h, however, leaching of betacyanin pigment was observed when they were subjected to a prolonged treatment for 18 h.

To determine the effect on whole plants, young sunflower plants with intact root system were dipped into aqueous antibiotic solution (100 μg/mL). The treated plant showed symptom of wilting within 2—2.5 h, while control

TABLE V. Antimicrobial activity of the antibiotic

	Test organism	Medium[a]	Minimum inhibitory concentration, μg/mL
Fungi	*Alternaria solani*	CZ	20
	Aspergillus niger	CZ	30
	Candida albicans	SD	50
	Curvularia pallescens	CZ	15
	Epidermophyton floccosum	SD	20
	Helminthosporium oryzae	CZ	30
	Macrophomina phaseolina	CZ	15
	Microsporium gypseum	SD	50
	Saccharomyces cerevisiae	SD	50
	Trichophyton rubrum	SD	30
	T. mentagrophytes	SD	20
Bacteria	*Alcaligenes faecalis*	NA	30
	Bacillus subtilis	NA	30
	B. cereus	NA	30
	B. megaterium	NA	30
	Citrobacter sp.	NA	50
	Escherichia coli	NA	50
	Serratia sp.	NA	20

[a] CZ Czapek-Dox agar, SD Sabouraud's dextrose agar, NA nutrient agar.

plants maintained their normal turgidity. When the antibiotic was tested as foliar spray (50 and 100 μg/mL) they developed yellowing of the leaves after 2 d.

Animal toxicity. When injected intravenously in albino rats there was no mortality up to a concentration of 150 mg/kg of body mass within 1 d.

DISCUSSION

The physico-chemical characteristics indicate strongly a non-polyenic nature of the compound. From the UV-absorption spectrum also it seems justified to conclude that the compound is saturated which is in keeping with the non-polyenic nature of the antibiotic compound.

Comparatively small number of antibiotics exhibit single peaks at 200 to 220 nm in their UV-absorption spectrum. Among these only a few possess antifungal activity, the rest are exclusively antibacterial and show antagonistic action mainly against gram-positive bacteria (Umezawa 1967). The antifungal antibiotics showing absorption peaks between 200 to 300 nm include blasticidin (Takeuchi *et al.* 1958) with a single peak at 275 nm (in 0.1 M HCl); ossamycin (Umezawa 1957) having a single peak at 214 nm (in chloroform); antibiotic U-20904 (Umezawa 1967) with a single peak at 211 nm (in water); and eumycetin (Arai and Takamizawa 1934) with a single peak at 302 nm (in methanol). Among the more recently discovered non-polyenic antifungal antibiotics, nanaomycin (Tanaka *et al.* 1975) and trichostatin (Tsuji *et al.* 1976) have more than one peak in the UV-range. Their solubility properties also differ from those of the antibiotic described here.

From the solubility properties and the UV-absorption spectrum, it seems that the antibiotic is distinct from the previously described antifungal agents.

Such a conclusion seems justified also from the taxonomic consideration of the producing organism. The producing strain identified as *Streptomyces galbus* has been reported to produce streptomycin (Okami *et al.* 1959; Murase *et al.* 1959), and actinomycin (Umezawa 1967), but no antifungal antibiotic.

As regards the purity of the isolated antibiotic, it was observed that on paper or thin layer chromatograms using a large number of solvent systems always only single active spots appeared (Table IV). However, the elution profile from Sephadex G-25 showing a main peak followed by a subsidiary one (Fig. 2) suggested that there might be two antifungal components.

The antibiotic in its present state of purity possesses both antifungal and antibacterial properties (Table V). The filamentous fungi including the human pathogenic ones were in general more sensitive than the yeasts and dimorphic fungi. The pattern of antifungal spectrum differs characteristically from those of polyene antibiotics which are more active against yeasts than they are against filamentous fungi (Arai and Mikami 1969; Hamilton-Miller 1973; Thomas 1976).

The tests performed for determination of phytotoxicity of the antibiotic compound did not give unequivocal evidence either for phytotoxicity or against it. Germination of seeds of some plants like *Oryza sativa* or *Cucumis sativa*, was unaffected, while that of others, like *Cajanus cajan* was significantly inhibited and that of still others was stimulated. Both inhibition and promotion of seed germination by different antifungal antibiotics have been reported earlier (Brian *et al.* 1949; Wright 1951, Nandi *et al.* 1975, Black 1968; Thind and Prakash 1975).

The wilting test performed with intact plants (sunflower), and yellowing of leaves after foliar application of the antibiotic however, gave positive indications for its phytotoxic nature. On the other hand, the compound appears to be relatively non-toxic to albino rats.

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The plates will be found at the end of the issue.

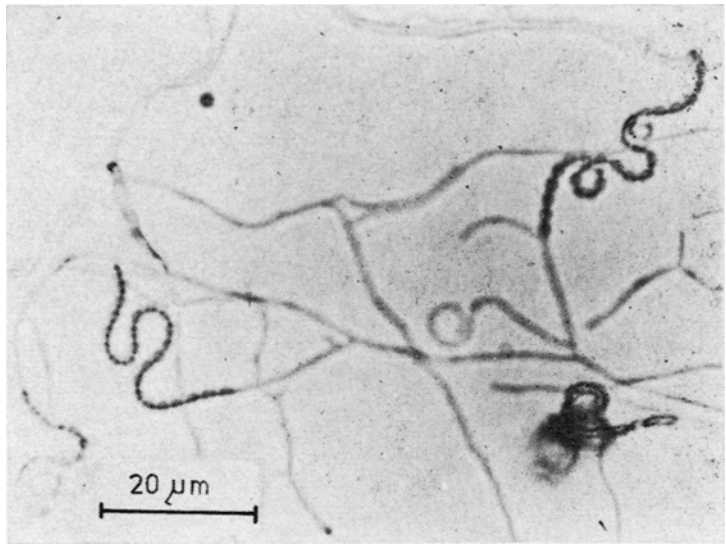

PLATE 1. Phase-contrast micrograph of *Streptomyces* sp. 5ME-13 showing aerial mycelium with sporophores (glucose—asparagine agar, 14 d at 28 °C).

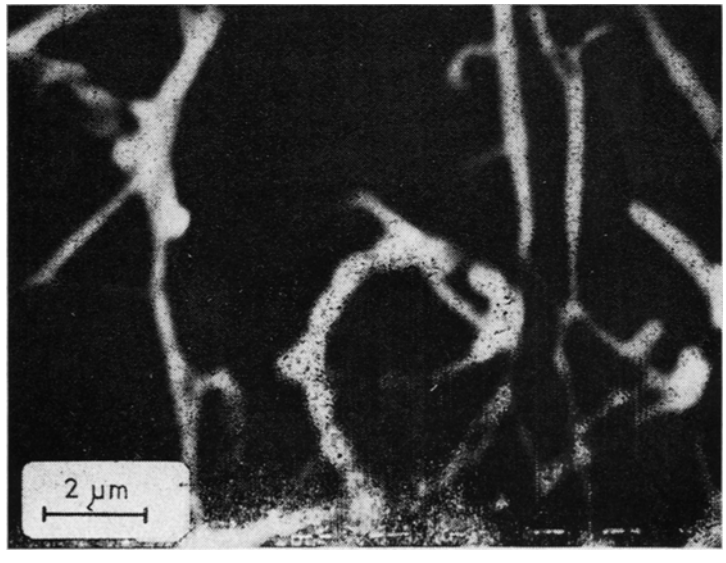

PLATE 2. Scanning electron micrograph of *Streptomyces* sp. 5ME-13 showing spore chains (glucose—asparagine agar, 14 d at 28 °C; Philips Scanning Electron Microscope Model PSEM 500).