ANTIBIOTIC PRODUCTION BY NEW FORM-GENERA OF THE ACTINOMYCETALES. II.

ANTIBIOTIC A/672 ISOLATED FROM A NEW SPECIES OF ACTINOPLANES: *ACTINOPLANES BRASILIENSIS* NOV. SP.

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A new species of *Actinoplanes* designated as *Actinoplanes brasiliensis* nov. sp. is described. Its taxonomic relationship with other species of *Actinoplanes* as well as the cultural, physiological and biochemical characteristics, are also discussed. The antibiotic A/672 produced by the new isolate is described and its *in vitro* and *in vivo* data are given.

Reports on the antibiotic production by members of the Actinoplanaceae have been limited to the best of our knowledge, to sporaviridin[1], which can be considered as being the first antibiotic isolated from a member of the Actinoplanaceae[2], and to sporangiomycin an antibiotic produced by a member of the recently described genus *Planomonospora*[3]. Literature references do exist on the *in vitro* antibiotic activity of members of other new genera of the Actinomycetales, however, no efforts seem to have been made for the production of these antibiotics under submerged conditions and for their isolation[4,5,6,7].

In the present paper we wish to report on the taxonomy of a new species of *Actinoplanes* and on the antibiotic substance A/672 produced by the same.

The isolate, which received our collection number A/672 was isolated from a soil sample collected in the State of Bahia (Brazil), and showed to possess antibacterial activity when grown under submerged conditions. The abundant formation, on a number of culture media, of sporangia containing highly motile sporangiospores, places this isolate squarely in the family Actinoplanaceae and in the genus *Actinoplanes*. The various morphological, cultural and physiological characteristics presented by strain A/672, permit its differentiation from the other known species of *Actinoplanes*, and strain A/672 is considered to be a new species for which the name *Actinoplanes brasiliensis* nov. sp. is proposed.

For the taxonomic characterization of strain A/672, the procedures described by SHIRLING and GOTTLIEB[8] (supplemented by additional media recommended by WAKSMAN[9]) were followed. For comparative purposes, the three species of *Actinoplanes* described by COUCH[10,11] were included in the present study.

General Characteristics

Microscopic examination of strain A/672 grown on various media showed it to be consistently devoid of aerial mycelium. This lack of aerial mycelium is a general

Table 1. Cultural characteristics of *Actinoplanes* A/672 and related microorganisms

Culture media	*Actinoplanes* A/672	*A. missouriensis*	*A. utahensis*	*A. filippinensis*
Medium No. 2	Heavy growth, surface thick and slightly wrinkled; deep orange (P1.2; A 12)	Heavy growth, surface highly wrinckled; rose-orange (P1.9; C 9)	Good growth; surface smooth and glistening; deep orange (P1.9; A 12)	Good growth; surface slightly wrinkled; yellow amber (P1.11; I 7)
Medium No. 3	Heavy growth; surface thick and slightly wrinkled; deep orange (P1.2; A 12)	Moderate growth; surface smooth and flat; light rose orange (P1.9; B 7)	Moderate growth; surface smooth and thin; pale rose orange (P1.9; B 7)	Good growth; surface smooth; light orange (P1.11; G 5)
Medium No. 4	Moderate growth; surface flat and smooth; deep orange (P1.2; A 12)	Heavy growth, surface smooth, orange (P1.9; E 9)	Good growth; surface smooth; orange (P1.9; E 9)	Good growth, surface smooth; light orange (P1.10; G 10)
Medium No. 5	Moderate growth; surface flat and smooth; pale orange (P1.9; C 8)	Heavy growth, surface smooth, rose orange (P1.9; C 9)	Good growth; surface smooth deep orange (P1.9; E 10)	Good growth, surface smooth; light orange (P1.9; G 8)
Medium No. 6	Moderate growth; surface flat and smooth; deep orange (P1.2; A 12)	Heavy growth, surface smooth faint rose orange	Good growth; surface smooth and thin; brown (P1.15; C 11)	Moderate growth; surface crusty; amber (P1.12; J 8)
Medium No. 7	Heavy growth; surface thick and wrinkled; orange brown (P1.4; D 12)	Heavy growth; surface slightly wrinkled; orange with brown tinge (P1.11; B 10)	Good growth; surface wrinkled; brownish orange (P1.11; B 10)	Good growth; surface slightly crusty; brown caramel (P1.13; I 9)
Hickey and Tresner	Moderate growth, surface thin and wrinkled; light orange (P1.9; H 10)	Heavy growth, surface smooth pale rose orange (P1.10; B 7)	Good growth; surface wrinkled pale rose brown tinge (P1.12; A 7)	Moderate growth; surface smooth; hyaline to light cream
Bennett	Heavy growth; surface thick and wrinkled; deep orange (P1.2; A 12)	Heavy growth; surface smooth pale orange (P1.10; E 7)	Good growth; surface wrinkled and glistening; orange (P1.10; F 8)	Good growth; surface slightly wrinkled; yellow ochre (P1.11; J 7)
Czapek-Dox	Moderate growth; surface thin, wrinkled; deep orange (P1.2; A 12)	Moderate growth; surface smooth straw-color (P1.10; C 1)	Moderate growth; surface smooth; straw color (P1.10; C 1)	Good growth, surface smooth; orange brown (P1.11; K 12) to caramel (P1.13; A 12)
Skim milk	Good growth; surface wrinkled; deep orange rose (P1.10; A 9)	Good growth; surface wrinkled; orange (P1.4; D 12)	Good growth; surface slightly wrinkled; caramel (P1.13; A 12)	Good growth; surface smooth; deep orange (P1.11; D 11)
Glucose asparagine	Good growth; surface thin and smooth; pale rose (P1.9; B 6)	Moderate growth; surface smooth; deep orange (P1.2; A 12)	Good growth; surface smooth and glistening; deep orange (P1.9; A12)	Good growth; surface smooth; orange (P1.9; I 10)
Nutrient agar	Moderate growth; surface thin and smooth; pale orange rose (P1.9; E 7)	Good growth; surface thick and wrinkled; deep orange (P1.2; A 12)	Good growth; surface smooth; orange (P1.9; C 10)	Moderate growth; surface smooth and thin; pale orange (P1.10; F 8)
Potato agar	Good growth; surface wrinkled; rose orange (P1.9; B 8)	Good growth; surface thick and wrinkled; deep orange (P1.2; A 12)	Good growth; surface wrinkled and glistening rose orange (moist); (P1.9; B 9)	Good growth; surface smooth; brown amber (P1.12; I 9)
Calcium malate	Good growth; surface smooth; rose orange (P1.9; B 8)	Moderate growth; surface thin and wrinkled. Pale orange (P1.9; H 10)	Moderate growth; surface smooth and thin; pale orange (P1.9; B 6)	Moderate growth; surface smooth; yellow brown (P1.9; H 5)

* Letters and numbers refer to the color determined according to Maerz and Paul[14].

Plate 1. *Actinoplanes brasiliensis.* Typical umbrella-shaped sporangium. Internal differentiation of spores prior to the sporangiospore liberation noticeable (×1520×1/1.5)

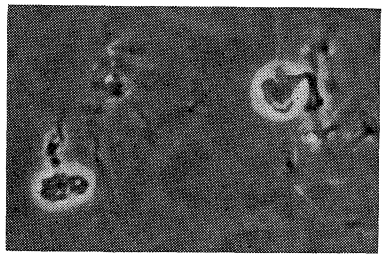


Plate 2. *Actinoplanes brasiliensis.* Sporangia developing on the surface of agar media. The highly irregular outer surface of the sporangia is evident (×600×1/1.5)

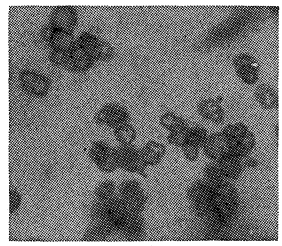


Plate 3. *Actinoplanes philippinensis.* Regular, smooth walled, sphaerical sporangia developing on the surface and also imbeded in the vegetative mycelium (×600×1/1.5)

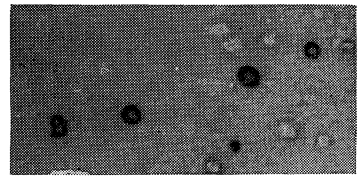


Plate 4. *Actinoplanes philippinensis.* Sporangia imbeded in the vegetative mycelium (×520×1/1.5)

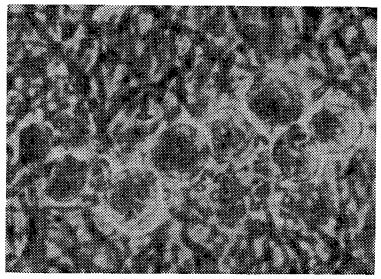


Plate 5. *Actinoplanes philippinensis.* Young, sphaerical and smooth walled sporangia forming on a vegetative hyphae (×1520×1/1.5)

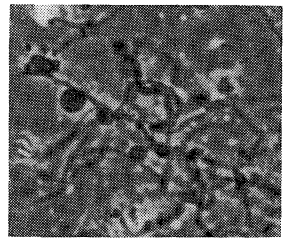


Plate 6. *Actinoplanes brasiliensis.* Flagellation of a motile sporangiospore (×1520×1/1.5)

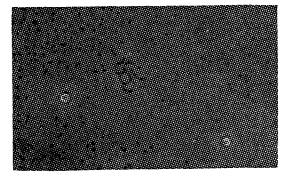
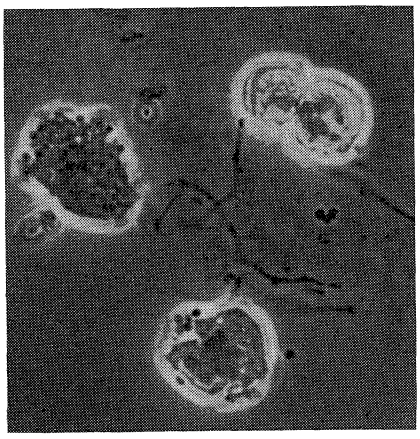


Plate 7. *Actinoplanes brasiliensis.* Spore liberation by means of the unoriented rupture of the sporangia (×1520×1/1.5)



characteristic of the genus *Actinoplanes*. An exception to this rule can be found in *Actinoplanes armeniacus*. In this species the concomitant formation of aerial mycelium containing conidiospores and of sporangia emerging from the substrate mycelium and containing motile spores was described by KALAKOUTSKI and KUZNETSOV[12]. The surface of culture A/672 is usually rough and its color ranging from light orange to intense orange. Soluble pigments are normally absent. Sporangium formation is very abundant on soil agar[13] and calcium malate agar. The sporangia of strain A/672 are irregular, club-shaped, but more frequently umbrella shaped (Plate 1) and very occasionally globose but always with a highly wrinkled surface (Plate 2). They were never observed to be imbeded in the vegetative mycelium as it frequently occurs with the sporangia of *Actinoplanes philippinensis* (Plate 3) in which the sporangia have also a characteristically smooth surface and are spherical to sub-globose (Plates 4, 5).

The size of the sporangia, measured at their widest side, varied from 3.5 to 11.5 μ. The sporangiophores are usually straight, however, occasionally wavy ones are found. They always grew into the air. They are 1.2 μ wide and 4.5~1.5 μ long, tending to enlarge versus the apex. The morphology of the spores varied from sub-spherical (1.2 μ) to rod shaped (2.3×1.2~1.7×1.2 μ). Due to the highly irregular sporangial morphology, an oriented Ampullariella-like pattern of spore disposition inside the sporangium is highly improbable. At least no clear-cut microscopic evidence could be obtained.

Spore dehiscence and motility initiates, in strain A/672, very vigorously a short time after the plates are flooded with water. Flagella staining[16] reveals the presence of short lophotrichous flagella (Plate 6). Spore liberation takes place by the unoriented rupture of the sporangium (Plate 7).

Immediately after mounting the sporangia in water between cover slides, the sporangial wall of strain A/672 is not clearly visible. Prior to the spore enclosure, however, the sporangial wall as well as the spores inside the sporangium become clearly differentiated. Approaching the sporangiospore liberation, the sporangia loose their irregular shape and become more uniform and sphaerical, very probably because

Table 2. Physiological characteristics of *Actinoplanes* A/672 and related microorganisms

Test	*Actinoplanes* A/672	*A. missouriensis*	*A. utahensis*	*A. philippinensis*
Starch hydrolysis	+++	+	+	+++
H_2S formation	−	−	+	±
Melanin	−	−	+	−
Tyrosin hydrolysis	−	+++	±	+
Xanthine hydrolysis	−	−	−	−
Casein hydrolysis (Skim milk)	+	+	+	+
Calcium malate hydrolysis	+	±	+	+
Nitrate reduction	reduced	reduced	reduced	reduced
Litmus milk	no coagulation no peptonization	no coagulation no peptonization	no coagulation no peptonization	no coagulation no peptonization
Gelatine liquefaction	+	−	±	+

Table 3. Utilization of carbon compounds by strain A/672 and related species of *Actinoplanes*

Carbon source	Strain A/672	*Actinoplanes philippinensis*	*Actinoplanes utahensis*	*Actinoplanes missouriensis*
Inositol	+	++	−	−
Fructose	++	++	++	++
Rhamose	++	++	++	++
Mannitol	++	++	++	++
Xylose	++	++	++	++
Raffinose	−	++	−	−
Arabinose	++	++	++	++
Cellulose	+	+	−	−
Sucrose	++	++	++	++
Glucose (positive control)	++	++	++	++
No carbon (negative control)	−	−	−	−

++ : Strongly positive utilization : growth is similar to or greater than growth on positive control.
+ : Positive utilization : growth is significantly greater than "no carbon" although somewhat less than on glucose.
− : Utilization negative : growth is similar to "no carbon" and much less than on positive control.

of the swelling of the intrasporangial substance. Similar observations have also been made for *A. utahensis* (COUCH, 1963).

Formation of palisade hyphae has not been observed. The vegetative mycelium is formed of long, wavy and twisted filaments, occasionally highly branched in the form of short lateral ramifications. The diameter of the hyphae varies from 0.6 μ to 1.2 μ.

Appearance on various media.

The cultural and physiological characteristics of strain A/672 are listed respectively in Tables 1 and 2. The optimum temperature for development was found to be from 28° to 37°C; no growth occurred at 45°C.

The results of the carbon utilization test, performed according to SHIRLING and GOTTLIEB[8] are listed in Table 3.

Isolation of Antibiotic A/672

The product is of an acid nature and can be extracted with butanol from the fermentation broth acidified to pH 3.0. To achieve a preliminary purification the broth culture is first brought to pH 8.0 and extracted with butanol. At this pH the antibiotic is completely soluble in water and the butanol containing some of the impurities is eliminated. The broth is then acidified (pH 3.0), re-extracted with butanol, washed with water to eliminate the excess of acid and concentrated to a small volume. The antibiotic is precipitated from the concentrated solution with an excess of petroleum ether. The product thus obtained can be further purified by re-extracting into phenol from an aqueous solution, followed by a precipitation with an excess of acetone.

The antibiotic obtained is a whitish amorphous powder, acidic, highly soluble in water, soluble in methanol and dimethylformamide, poorly soluble in butanol, and insoluble in the higher alcohols, esters, acetone and chloroform.

Antibiotic A/672 shows UV absorption with a maximum at 265 mμ in methanol, 0.1 N HCl, 0.1 N NaOH and 0.15 M phosphate buffer at pH 7.38 ($E^{1\%}_{1cm}$ 103 in methanol).

Fig. 1. Infrared spectrum of antibiotic A/672

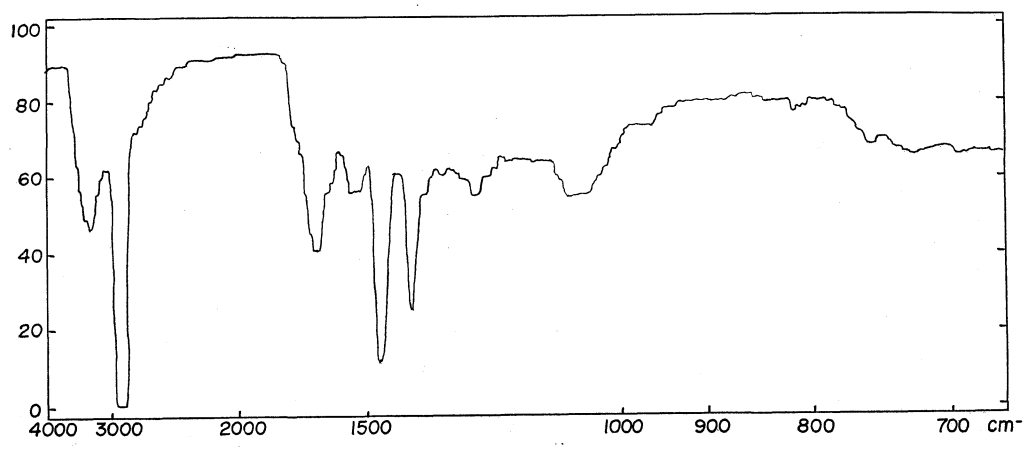


Table 4. Chromatographic pattern of antibiotic A/672 in various solvent systems

Solvent system	Rf *
Water-saturated *n*-butanol	0.0
Water-saturated *n*-butaonl containing 2 % *p*-toluensulfonic acid	0.0
Water-saturated butanol containing 2 % concentrated ammonia	0.05
n-Butanol-saturated water	0.85
Ammonium chloride (20% solution in water)	0.20
Phenol - water (75 : 25)	0.90
n-Butanol - methanol - water (40 : 10 : 20) containing 0.75 g methyl orange	0.20
n-Butanol - methanol - water (40 : 20 : 20)	0.20
Water - acetone (1 : 1)	0.0
Water-saturated ethyl acetate	0.0

* Antibiotic visualized on agar plates seeded with a suspension of *B. subtilis*.

Table 5. Antimicrobial activity of antibiotic A/672

Microorganisms	MIC (mcg/ml)
Staphylococcus aureus 209 P, ATCC 6538	20
Staphylococcus aureus Tour*	50
Streptococcus pyogenes C. 203	50
Streptococcus faecalis ATCC 10541	>100
Diplococcus pneumoniae UC. 41	20
Proteus vulgaris X 19 ATCC 881	>100
Escherichia coli ATCC 10536	>100
Pseudomonas aeruginosa ATCC 10145	>100
Candida albicans ATCC 10231	>100
Trichophyton mentagrophites ATCC 8757	>100
Mycobacterium tuberculosis $H_{37}Rv$ ATCC 9360	>100

* Clinical isolate.

The infrared spectrum is given in Fig. 1. Paper chromatography using different solvent systems followed by microbiological assay with *Sarcina lutea* gave only a single inhibition zone. The behavior of antibiotic A/672 in the different solvent systems is given in Table 4.

The antibiotic is active against Gram-positive bacteria but inactive against Gram-negative bacteria and fungi. Antibiotic A/672 showed no cross-resistance with the following antibiotics: cycloserine, gentamicin, oleandomycin, polymyxin, kanamycin, streptothricin, penicillin, rifamycin, bacitracin, tetracycline, lincomycin, erythromycin, cloramphenicol, streptomycin and neomycin. Its antibacterial spectrum is shown in Table 5. Using the intraperitoneal route an LD_{50} of approximately 300 mg/kg was obtained. No delayed toxicity was observed.

The *in vivo* activity was tested in mice against experimental infections using *Streptococcus hemolyticus* and *Diplococcus pneumoniae*. The product was administered subcutaneously for three consecutive days as a suspension in carboxymethyl-cellulose. A 100 per cent protection was obtained against *D. pneumoniae* at 100 mg/kg.

Discussion

From the taxonomic studies performed it is evident that strain A/672 should be classified in the genus *Actinoplanes*. Comparative studies with three known species of this genus, *Actinoplanes philippinensis*, *A. missouriensis* and *A. utahensis*, showed that the color of the vegetative mycelium of strain A/672 did not differ very significantly from that of any of the known species.

The pigmentation of the vegetative mycelium in various shades of yellow seems to be a general characteristic of the genus *Actinoplanes* since this color is found in all the species so far described. Absorption spectra performed on the intra-mycelial pigments of three different genera of the Actinoplanaceae showed them to be identical[15]. Strain A/672 can be distinguished, however, from the known species by its morphological and biochemical characteristics.

Strain A/672 has large and highly irregular sporangia distinctly different from the sphaerical ones found in *A. philippinensis* and *A. missouriensis*. From these two species it can be further separated on the basis of its biochemical characteristics as can be seen in Tables 2 and 3.

With *A. utahensis*, strain A/672 has in common the highly irregular shape of the sporangia but differs from that culture by the inability to form hydrogen sulfide, melanoid pigments and tyrosine hydrolysis, three important characters for which *A. utahensis* gives positive results (Tables 2 and 3). The characteristically moist and glistening colony surface of *A. utahensis* on some culture media further differentiate these two cultures (Table 1).

In view of the above characteristics strain A/672 was considered to be a new species of the genus *Actinoplanes* for which the name *Actinoplanes brasiliensis* is proposed.

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