LIPIARMYCIN, A NEW ANTIBIOTIC FROM *ACTINOPLANES*

I. DESCRIPTION OF THE PRODUCER STRAIN AND FERMENTATION STUDIES

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The producer strain of the new antibiotic, lipiarmycin, is described. The colony morphology, the presence of globose sporangia bearing motile spores, the absence of aerial mycelium and the presence of meso-DAP in cell wall, ascribe this strain to the genus *Actinoplanes*. The pigmentation and morphological characteristics together with the cultural and physiological features distinguish this strain from all the described *Actinoplanes* species. It is considered to be a new species for which the name *Actinoplanes deccanensis* nov. sp. is proposed. Lipiarmycin is produced in an organic complex medium containg NaCl. Production occurs at the end of trophophase and continues, though at decreasing rate, during idiophase.

During the course of screening for antibiotics produced by strains of *Actinoplanaceae*[1,2] a strain of *Actinoplanes* was found to produce a substance with a strong activity against gram-positive bacteria including strains resistant to several antibiotics. From its unique physico-chemical properties and its mechanism of action it was judged to be a new antibiotic and it was named lipiarmycin.*

In this paper, taxonomical studies of the producing strain, together with fermentation studies of lipiarmycin are reported.

Material and Methods

Growth of the organism: Lyophilized vials of the organism are streaked on oat meal agar slants and grown at 32°C for 1 week. The slant surface is scraped and used as inoculum for 3×500 ml indented shake flasks containing 100 ml of the medium V. The flasks are incubated at 32°C on rotary shaker at 200 rpm. The seed culture for the jar fermenter experiments is prepared by inoculating the content of two 72-hours-old flasks into a 4-liter jar and incubating it at 28°C for 24 hours in the medium V. The aeration rate, in jar fermenters, is 1 v/v min, and the propeller rate is 400 rpm. Foam is controlled by automatic addition of silicone. Sterilization is in autoclave at 121°C for 15 minutes.

Preparation of test organism: the bacteria are grown on a nutrient agar slant overnight at 37°; suspended in sterile H_2O to a density equivalent to 50 Klett units. One to two ml of such suspension is mixed with Difco Penassay, and 13 ml are poured into 9 cm diameter Petri dishes, Paper discs, 9 mm diameter, are obtained from Cartiere Galbani, Pordenone (Italy).

Media composition is as follows (g/liter): V medium: meat extract 3; tryptone 5; yeast extract 5; dextrose 1; starch 24; $CaCO_3$ 4, brought to 1,000 ml with distilled H_2O. E medium: meat extract 4; peptone 4; NaCl 2.5; yeast extract 1; soybean meal 10; destrose 50; $CaCO_3$ 5, brought to 1,000 ml with tap H_2O. The pH is titrated to 7.6 with NaOH before sterilization, and becomes 6.3 after sterilization.

Identification of diaminopimelic acid and sugars: washed mycelium was hydrolyzed in a

* From Leap year; the strain was isolated on February 29th, 1972.

sealed tube in 6 N HCl at 100°C for 18 hours for DAP and in 1 N H_2SO_4, at 100°C for 2 hours for sugars. Ascending paper chromatography was performed according to BECKER *et al.*[8]

Results and Discussion

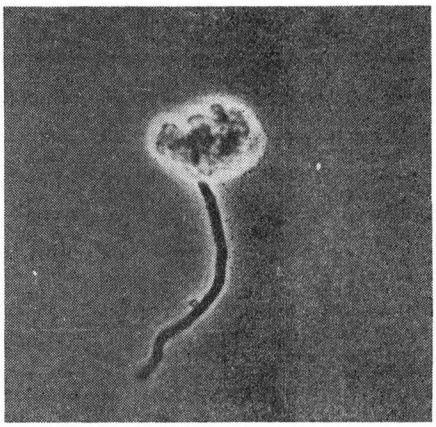
Taxonomical Studies of Strain A/10655

Strain A/10655 was isolated from a soil sample collected at locality Decca in India. According to the taxonomical studies described below it was identified as a member of the genus *Actinoplanes* similar but distinguishable from the known orange species[1].

Morphological properties: the morphology of the culture in agar-media is typical of the genus *Actinoplanes*. The surface is opaque and slightly rough to wrinkled. Its color is from light to deep orange depending on the medium. Aerial mycelium is always absent. The vegetative mycelium is branched with a diameter of about 1 μ.

Small sporangia (4~7 μ diameter) form abundantly on soil extract agar and are globose with irregular surface. They are distinctly smaller than those of other orange *Actinoplanes*[1]. After rupture of the wall of the sporangium the spore release is observed. The spores are sub-spherical and motile (size 1 $\mu \times 1.5$ μ) (Plate 1).

Plate 1. Sporangium obtained on soil extract-agar. Magnification × 800



Cultural properties: the cultural properties on different media suggested by SHIRLING and GOTTLIEB[3] are shown in Table 1. The strain grows well in all media with the exception of Ca-malate agar. The cultural characteristic were determined after 6~14 days of incubation at 30°C.

Carbon utilization: the carbon utilization was examined according the PRIDHAM and GOTTLIEB[3] (Table 2).

Strain A/10655 utilizes readily the carbohydrates metabolized via pentose-phosphate cycle (xylose-arabinose) and those feeding at the level of glucose-6-phosphate (glucose,

Plate 2. Group of sporangia immediately prior to rupture. Magnification × 800

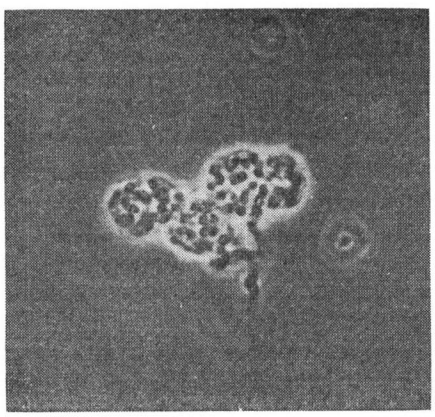


Plate 3. Spores, immediately after release from sporangium. Magnification × 800

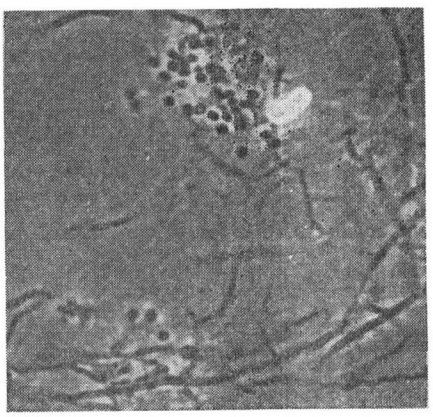


Table 1. Cultural characteristics of strain A/10655. The number of some of the culture media refers to those given by SHIRLING and GOTTLIEB[3].

Culture media	Cultural characteristics
Medium No. 2 (yeast extract-malt agar)	Abundant growth, very wrinkled, light orange
Medium No. 3 (oatmeal agar)	Moderate growth, crusty, light amber
Medium No. 4 (inorganic salts-starch agar)	Abundant growth, crusty, orange
Medium No. 5 (glycerol-asparagine agar)	Moderate growth, rough surface, orange
Medium No. 6 (peptone-yeast extract-iron agar)	Moderate growth, wrinkled, light orange
Medium No. 7 (tyrosine agar)	Abundant growth, wrinkled, amber to light brown, diffusible brown pigment
Oatmeal agar according to WAKSMAN	Abundant growth, crusty, opaque, light orange
HICKEY and TRESNER's agar	Abundant growth, wrinkled, light orange-pinkish
CZAPEK glucose agar	Moderate growth, crusty, cream to light orange
Glucose asparagine agar	Moderate growth, slightly crusty, opaque, cream to light orange
Nutrient agar	Moderate growth, crusty, opaque, light orange
Potato agar	Abundant growth, very wrinkled, pale orange
BENNETT's agar	Abundant growth, wrinkled, light orange
Calcium malate agar	Scanty growth, wrinkled, opaque, light orange
Skim milk agar	Abundant growth, wrinkled, opaqe orange
CZAPEK agar	Moderate growth, crusty, light orange
Egg agar	Scanty growth, thin, opaque, white-waxy
Peptone glucose agar	Moderate growth, crusty, orange
Agar	Very scant growth, thin, hyaline
LOEFFLER serum	Moderate growth, rough surface, orange
Potato	Moderate growth, wrinkled, light orange
Gelatin	Scanty growth, light orange
Cellulose agar	Very scanty growth, thin, hyaline

Table 2. Carbon utilization by strain A/10655.

Carbon sources	Utilization*
Inositol	−
Fructose	−
Rhamnose	+
Mannitol	−
Xylose	+
Raffinose	−
Arabinose	+
Cellulose	−
Sucrose	+
Glucose	+
Mannose	+
Lactose	+
Salicin	−

* +=Positive utilization. −=No growth.

Table 3. Physiological characteristics of strain A/10655

Test	Results
Hydrolysis of starch	positive
H_2O formation	negative
Tyrosinase reaction	positive
Casein hydrolysis	negative
Solubilization of calcium malate	positive
Nitrate reduction	positive
Liquefaction of gelatine	positive
Litmus milk coagulation	negative
Peptonization	negative
Cellulose decomposition	negative

sucrose, lactose, starch); it grows poorly on those feeding at the level of fructose-6-phosphate (fructose, mannitol, but grows on mannose); it does not grow on higher polymers such as raffinose, cellulose nor grows on the salicyl alcohol-β-glucoside (salicin). The inability to split the galactose-α(1, 6)-glucoside bond of raffinose is shared with all the other described species of *Actinoplanes* except *A. filip-*

pinensis[1,4]. The very poor and erratic growth on fructose and mannitol is a peculiarity of this isolate.

Physiological properties: the physiological properties of strain A/10655 are summarized in Table 3. A/10655 like *A. brasiliensis* and *A. missouriensis*[1,5,6] but unlike all the other described species[1,4] is unable to produce H_2S. It can be distinguished however from the former two species by its ability to produce melanin and to hydrolyze tyrosine. Furthermore unlike all *Actinoplanes* species, it is unable to hydrolyze casein.

Temperature of growth: Strain A/10655 grows on agar surface at temperature between 26° and 42°C. The surface growth at 42°C is unusual among *Actinoplanes*.

Cell wall composition and sugar pattern: Strain A/10655 contains meso-diaminopimelic acid in its cell wall like all the *Actinoplanes* species so far analyzed. The sugar pattern of whole cell hydrolyzate is a combination of type D (presence of xylose and arabinose) and type A (predominance of galactose) described by LECHEVALIER *et al.*[7]

From the above reported taxonomical studies it can be concluded that strain A/10655:

1. Belongs to the genus *Actinoplanes* (globose sporangium, motile spores, lack of aerial mycelium, presence of meso-DAP).
2. Is similar to the group of the orange species, but does not superimpose completely with any such species (small sporangia, scanty growth on fructose and mannitol, inability to hydrolyze casein).

For these reasons it is considered to be a new species for which the name *Actinoplanes deccanensis* nov. sp. proposed. The type strain A/10655 has been deposited in the American Type Culture Collection (ATCC) under the number 21983.

Production of Lipiarmycin

Microbiological Assay of Lipiarmycin

To determine the antibiotic potency of the fermentation broth a microbiological assay for lipiarmycin was developed. Figure 1 reports the graphs of inhibition zone sizes *vs* lipiarmycin concentration with several bacteria sensitive to the antibiotic. All the bacteria tested yielded a linear relationship between the size of the inhibition zone and the log of the concentration of lipiarmycin over a 100-times concentration range. *Sarcina lutea* and *Flavobacterium deidrogenans* yielded the most sensitive curves giving readable inhibition zones at concentrations as low as 0.1~0.5 μg/ml and 1~2 μg/ml respectively. A less sensitive but more precise assay could be developed growing *S. lutea* at pH 8.5 instead of pH 7. *B. subtilis*, *B. cereus* and *B. stearothermophylus* yielded good, reproducible curves in the concentration range 30~60 to 250~500 μg/ml. This is the range of concentrations often found in fermentation broths so that the assay with these test organisms does not require dilution of broth.

Fermentation Studies

The organism grows well in shake flasks on a large variety of nutrient media. Medium E was found to be the best for growth and antibiotic production.

As the lipiarmycin molecule contains chlorine, the effect of NaCl on growth and antibiotic yield was studied. The Cl-containing component of medium E was found to be the meat extract, beside NaCl. Omission of these two components from medium E did not influence growth levels but strongly reduced the antibiotic yield which was restored by NaCl addition

Fig. 1. Paper-disc assay of lipiarmycin. Graphs of inhibition zone *vs* lipiarmycin concentration with several bacteria. Each value is the average of three replicates.
Inhibition zones are read with an optical reader affording a 3 time magnification.
1. *Bacillus subtilis* (spores) ATCC 6633 in PS medium pH 6
2. *Bacillus cereus* var. *mycoides* ATCC 9634 in PS medium pH 7
3. *Bacillus stearothermophylus* ATCC 12980 in S3 medium pH 7, temperature 50°C
4. *Sarcina lutea* ATCC 9341 in PS medium pH 8.5
5. *Flavobacterium deidrogenans* Schering 1111 in PS medium pH 7
6. *Sarcina lutea* ATCC 9341 in PS medium pH 7
Ten mg lipiarmycin were dissolved in 0.6 ml dimethylformamide; 4 ml sterile 0.15 M phosphate buffer pH 7.2 was then added and the mixture was brought to 10 ml with sterile water.
The solution was serially diluted with H_2O. Curve (6) refers the ordinate values on the right side of the graph. PS: Difco Penassay medium S3 (g/liter): Trypticase 10, dextrose 10, NaCl 5, phenol red 0.018, agar 15.

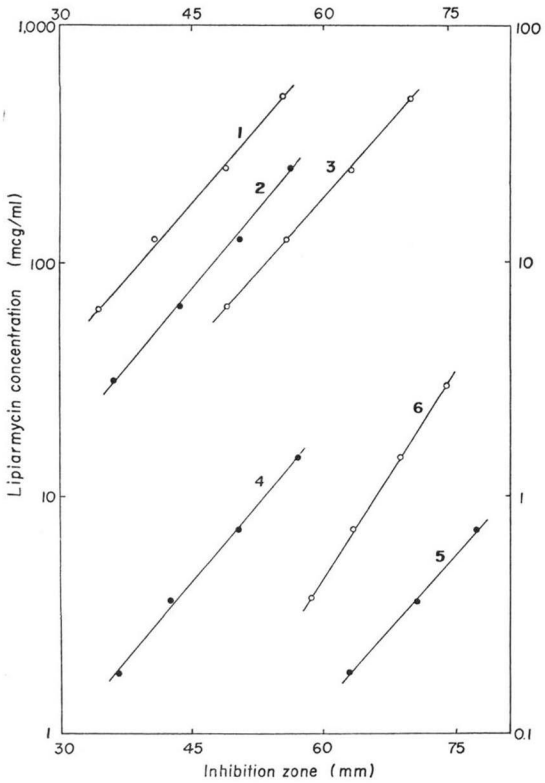


Fig. 2. Effect of Cl^- and Br^- on lipiarmycin production
(A) Medium E depleted of Cl (less than 5 μg/ml)
(B) Medium E depleted of Cl supplemented with 2.5 % (w/v) NaCl
(C) Medium E depleted of Cl supplemented with 2.5 % (w/v) NaBr.

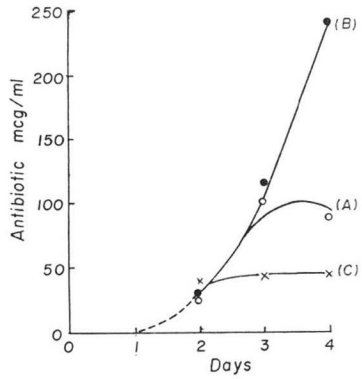
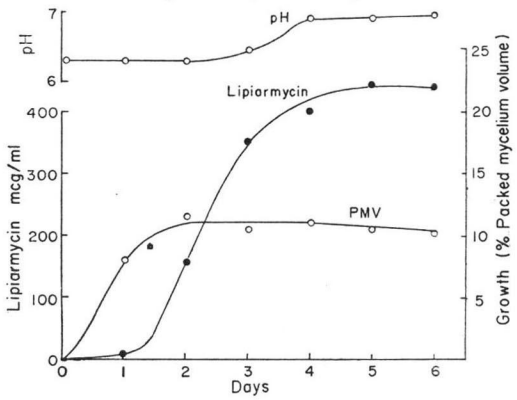


Fig. 3. Time-course of fermentation of lipiarmycin in 4-liter jar fermenter. PMV: Growth measured as percent packed mycelium volume



to the medium. NaBr, on the other hand, further reduced antibiotic yield with little effect on growth (Fig. 2).

One peculiarity of this organism is its ability to growth at 42°C, a non-permissible temperature for most *Actinoplanes*. Growth in medium E in a 4-liter jar fermenter is somewhat slower at 42°C than at 28°C, but it reaches the same maximum level. However, no antibiotic production is observed at the higher temperature. The organism grows in several synthetic media containing 1 % (w/v) of one of the following carbon sources: glu-

cose, sucrose, lactose, glycerol, glutamate, with either $NaNO_3$ or $(NH_4)_2SO_4$, at 1 % (w/v) concentration and trace metal supplement. Yeast extract (0.5 %), tryptone (1 %) or both together stimulate growth. No antibiotic production was observed in any synthetic medium even with yeast or tryptone supplementation.

The time-course of the fermentation in a 4-liter jar fermenter in medium E at 28°C is shown in Fig. 3. Growth was measured as percent solid of a centrifuged aliquot of fermentation broth.

The trophophase, *i.e.* the phase of active biomass synthesis, is completed within 48 hours. Antibiotic production begins toward the end of the trophophase and continues, though at diminishing rate during idiophase. The small increase in antibiotic concentration observed after the 4th day probably reflects the evaporation of the medium.

The pH of the medium keeps constant at the zero-time value (pH 6.3), during trophophase and increases rapidly in the first 48 hours after the onset of the idiophase.

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