PHOLIPOMYCIN, A NEW MEMBER OF PHOSPHOGLYCOLIPID ANTIBIOTICS

I. TAXONOMY OF PRODUCING ORGANISM AND FERMENTATION AND ISOLATION OF PHOLIPOMYCIN

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Pholipomycin is a new member of the phosphoglycolipid family of antibiotics. Taxonomic studies of the producing organism revealed that it has morphologically characteristic aerial mycelia in which two to three spores are borne usually on short and clavate side branches. The species name, *Streptomyces lividoclavatus*, has been proposed. Pholipomycin is produced mainly in the solid residue of the fermentation culture broth and is isolated by methanol extraction of the mycelial cake followed by purification on ion-exchange resin and silica gel chromatography.

Since 1965, there have been isolated from various species of *Streptomyces* more than a dozen closely related antibiotics containing phosphorus, lipid and amino sugars. This family of antibiotics, the phosphoglycolipids, includes the moenomycins,[1,2,3] 8036 RP (quebemycin),[4] 11837 RP,[5] the prasinomycins,[6] 19402 RP,[7] the diumycins,[8,9] the macarbomycins,[10,11] prenomycin[12] and ensanchomycin.[13] In addition to their structural characteristics, these antibiotics are distinguished from other antibiotics by their unique biological activities, including activity against R factor-carrying gram-negative bacteria, inhibition of bacterial cell wall synthesis at a unique synthetic step and growth-promotion in animals.

This paper describes the producing organism, as well as the fermentation and isolation of pholipomycin, a newly discovered antibiotic of this family.

1. Taxonomy of Producing Organism

Strain No. 3176 was isolated from a soil sample collected at Obana, Tochigi Prefecture, Japan. Its morphological characteristics are as follows: When observed under microscope, aerial hyphae of strain No. 3176 were characterized by short and clavate side branches. It was sympodially branched, but neither spiral nor straight spore chains were detected. As shown in Plates 1 and 2 the usual oval to cylindrical spores were formed from the keystone area of the triangular-shaped spore. Spores were $0.5 \sim 0.8 \times 0.8 \sim 1.1\ \mu$ in size and with smooth surfaces. The cultural characteristics were determined by use of conventional media and methods described by SHIRLING and GOTTLIEB[14]. Observation of the culture was made after cultivation for 2 weeks at 28°C unless otherwise stated. The taxonomic keys of BERGEY's Manual of Determinative Bacteriology (8th ed.) and of WAKSMAN in The Actinomycetes, Vol. 2, were used to compare with recognized genera and species of the actinomycetes.

As shown in Table 1, good growth was observed on most of the media. In general, the mass

Plate 1. Aerial hyphae of *Streptomyces lividoclavatus* on colloidal chitin agar, scanning electron micrograph, 10 days (×5,000).
A mark equals 1 μ.

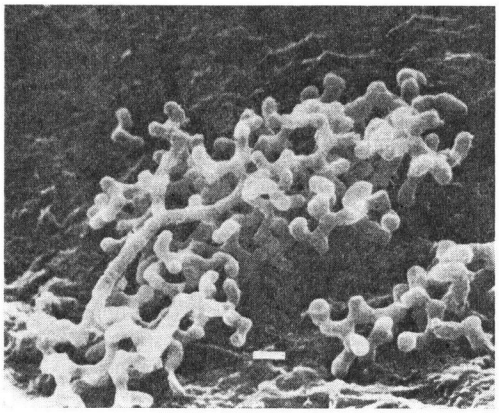
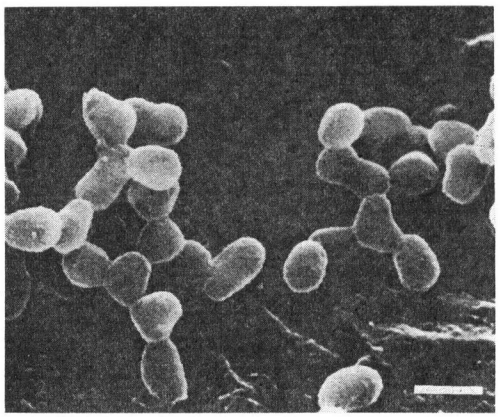


Plate 2. Spore-chain morphology of *Streptomyces lividoclavatus* on colloidal chitin agar, scanning electron micrograph, 10 days (×10,000).
A mark equals 1 μ.



color of aerial hyphae was greenish gray with some exceptions, such as white on sucrose-nitrate and nutrient agar and orange on oatmeal agar. Soluble pigment was absent or almost absent. Physiological properties and utilization of carbon sources are summarized in Tables 2 and 3. Most of the carbon sources tested were utilized but not pentoses, such as arabinose and xylose.

From the results of the taxonomic studies mentioned above, strain No. 3176 was assigned to the genus *Streptomyces*. Among known species of *Streptomyces*, *S. clavuligerus*[15] and *S. triangulata*[16] were selected as closely related species, especially in view of spore formation. Both species

Table 1. Cultural characteristics of *S. lividoclavatus*

Medium	Characteristics	
Sucrose-nitrate agar	G: Moderate, SM: White, scant	AM: White, scant SP: None
Glucose-asparagine agar	G: Good SM: White, scant	AM: Bluish gray, scant SP: None
Glycerol-asparagine agar (ISP 5)	G: Good SM: Dull orange	AM: Bluish gray, scant SP: None
Inorganic salts-starch agar (ISP 4)	G: Good SM: Pale yellowish brown, moderate	AM: Bluish gray, moderate SP: None
Tyrosine agar (ISP 7)	G: Good SM: Light brownish gray, moderate	AM: Bluish gray, scant SP: Brownish gray, scant
Nutrient agar (Difco)	G: Good SM: Pale yellowish brown, scant	AM: White, scant SP: None
Yeast extract-malt extract agar (ISP 2)	G: Abundant SM: Light brownish gray, abundant	AM: Bluish gray, abundant SP: None
Oatmeal agar (ISP 3)	G: Moderate SM: Pale yellowish orange, abundant	AM: Pale yellowish orange, moderate SP: None

G: Growth, AM: Aerial mycelium, SM: Substrate mycelium, SP: Soluble pigment.
Color names were assigned according to 'Guide to Color Standard', a manual published by Nippon Shikisai Kenkyusho, Tokyo, Japan.
Medium numbers in parenthesis are those used by the International Streptomyces Project (ISP).

Table 2. Physiological characteristics of *S. lividoclavatus*

Temperature requirement	Growth from 10 to 37°C
Gelatin liquefaction (18°C)	Weak
Starch hydrolysis	Weak
Action on milk (25°C)	No coagulation, positive for peptonization
Melanin production	Positive on tyrosine agar Negative on peptone-yeast extract iron agar
Nitrate reduction	Positive

Table 3. Carbon utilization

D-Glucose	++
L-Arabinose	−
Sucrose	++
D-Xylose	−
i-Inositol	++
D-Mannitol	−
D-Fructose	++
Rhamnose	++
Raffinose	++
Cellulose	−

++ Strongly positive utilization.
− Negative utilization.

were also reported to have short and clavate side branches on the triangular-shaped spores. However, the aerial mass color of *S. clavuligerus* on most media is pale yellowish-green. Further, many carbon sources utilized by strain No. 3176 are not utilized by *S. clavuligerus*, *e.g.* D-fructose, sucrose, L-rhamnose and raffinose. *S. triangulata* exhibits aerial mass color of grayish blue on most media. Formation of T-shaped spore chains are characteristic of *S. triangulata* only. D-Glucose and *i*-inositol are utilized by *S. triangulata* but many other carbon sources including those described above are not (Table 4).

Differences in antibiotic production are noted also among these three species. β-Lactam antibiotics are produced by *S. clavuligerus*, while antifungal and antiactinomycete antibiotics are produced by *S. triangulata*. Phosphoglycolipid antibiotics are not produced by either these two species. From the above, strain No. 3176 was identified as a new species and designated as

Table 4. Comparison of *Streptomyces* with triangular-shaped spores

	S. clavuligerus	*S. triangulata*	*S. lividoclavatus*
Spore, Surface	Smooth	Smooth	Smooth
Shape (Normal)	Oblong to cylindrical	Oval to cylindrical	Oval to cylindrical
(Triangular)	+	+	+
(T-shaped)	−	+	−
Culture on glycerol asparagine agar	AM: White SP: None	White Pink	Bluish gray None
Yeast extract-malt extract agar	AM: Light grayish olive	Grayish blue	Bluish gray
Oatmeal agar	AM: White SP: None	Grayish blue Faint light brown	Pale yellowish orange None
Action on milk			
Coagulation	−	+	−
Peptonization	+	+	+
Carbon utilization			
D-Glucose	−	++	++
Sucrose	−	−	++
D-Fructose	−	−	++
Rhamnose	−	−	++
Raffinose	−	−	++

AM: Aerial mycelium, SP: Soluble pigment

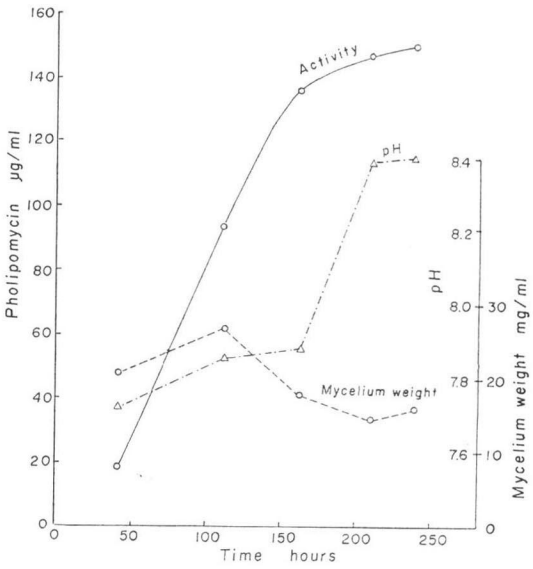
Streptomyces lividoclavatus ENOKITA *et* ARAI sp. nov. (livido = bluish, clavatus = club shape).

2. Fermentation

Pholipomycin was produced in a medium composed of glucose 4.0%, meat extract 0.2%, soybean meal 1.5%, cotton seed oil 0.1%, NaH_2PO_4 0.05%, and a mixture of small amounts of inorganic salts, including 0.01% $MgSO_4 \cdot 7H_2O$, 0.001% $MnSO_4 \cdot 7H_2O$ and 0.002% each of $ZnSO_4 \cdot 7H_2O$, $FeSO_4 \cdot 7H_2O$, $CuSO_4 \cdot 7H_2O$, $AlCl_3 \cdot 6H_2O$, $Na_2MoO_4 \cdot 2H_2O$ and $CoSO_4 \cdot 7H_2O$. The pH of the medium was 7.0~7.2 before sterilization.

Fermentation was carried out in a 30-liter jar fermentor containing 15 liters of the above medium. After inoculation of 50 ml of the seed culture, shaken-cultured in the medium described above in an Erlenmeyer flask on a rotary shaker at 28°C for 72 hours, the fermentation was conducted at 28°C, with aeration of 20 liter/minute, agitation of 135 rpm and inner pressure of 1.1 kg/cm^2. The potency of the culture broth was estimated by cylinder-plate method using *Pseudomonas aeruginosa* SANK 73860 as the test organism. Pholipomycin was produced predominantly in the mycelial portion of the broth. Samples were prepared for bioassay by centrifugation of the broth at 3,000 rpm for 10 minutes followed by extraction of the centrifugate with 80% aqueous methanol. An example of the time course of the fermentation is presented in Fig. 1. The maximal potency of pholipomycin, 150 μg/ml, was obtained after 240 hours of cultivation. The changes observed in mycelial weight, analyzed after drying at 105°C for 5 hours, were gradual, but sudden changes in pH from 7.9 to 8.4 within 160~210 hours may be related to discontinuation of antibiotic production and to the exhaustion of a key nutrient.

Fig. 1. Fermentation of pholipomycin.



3. Isolation

Forty liters of culture broth, combined from 3 jar fermentors, was filtered with diatomaceous earth and the mycelial cake washed with an equal volume of water. The mycelial cake (8.5 kg) was extracted three times with 75% aqueous methanol, yielding 36 liters of extract. The extract was passed through a column packed with 1.9 liters of Dowex 50W-X4 (H^+ form), which was then washed with 70% aqueous methanol to obtain a combined effluent of 40 liters.

Pholipomycin was adsorbed from the effluent on a column containing 2 liters of Duolite A-2 (CH_3COO^- form). The column was washed with 2 liters of 70% aqueous methanol and subsequently with 2 liters of water, and then eluted with 0.5 N aqueous ammonia.

The active eluate, monitored by the characteristic UV absorption maximum at 257 nm, was concentrated to dryness under reduced pressure and the concentrate dissolved in methanol to

remove insoluble impurities.

The methanol solution (84 ml) was then added dropwise to 2 liters of acetone to precipitate pholipomycin, which was collected and dried to give 12.7 g of crude powder (purity 27.1%, yield 56.0%).

Further purification of pholipomycin was performed by column chromatography on silica gel. The crude pholipomycin in methanol was adsorbed on a column packed with 160 ml of Wakogel C-200 in chloroform - methanol (6: 4 in volume ratio). After removal of impurities with 3 liters of the same solvent as above, pholipomycin was recovered in the eluate with chloroform - methanol (5: 5). The antibiotic in the eluates was monitored on silica gel TLC (Silica gel Chromagram Sheet 6060, Kodak) using *n*-PrOH - 2 N NH_4OH (7: 3) as developing solvent. The active eluate (4 liters) was concentrated under reduced pressure to dryness to give 2.07 g of pholipomycin (purity 75.8%, yield 25.6%). The crude powder thus obtained was dissolved in 9 ml of water and adsorbed on a column of 200 ml of DEAE-cellulose (OH^- form). After washing with one liter of aqueous ammonia at pH 9, elution was effected with 0.01 N aqueous ammonia to give 1.06 liters of the active fractions showing a single spot of pholipomycin on TLC. The combined fractions were concentrated to dryness under reduced pressure to give 955 mg of pholipomycin with a purity of 97.0% (yield 15.1%). The product was dissolved in 5 ml of methanol on heating and the resulting precipitate was then dried to give 475 mg of pure pholipomycin in 7.7% recovery.

Discussion

Pholipomycin, a new member of an antibiotic family of phosphoglycolipid was found to be produced by a new species of a streptomycete designated as *Streptomyces lividoclavatus*.

Taxonomic studies on the organism revealed it to have sporophore morphology distinctly different from that of the previously described species of *Streptomyces* producing members of this family of antibiotics. Although *S. triangulata* and *S. clavuligerus* have been reported to have similar morphological characteristics, they are also apparently different from the present pholipomycin-producing organism by their aerial mass color as well as their physiological properties.

Among known antibiotics of this family, diumycin A'[9] is most closely related one having similar physico-chemical properties to pholipomycin. Acid hydrolysis of these two antibiotics alone yields glucosamine and chromophore, but not 6-deoxyglucosamine, glucose and glycine. However, pholipomycin was easily distinguished from diumycin A' by its C_{25} lipid components. As will be reported in a subsequent paper, moenocinol-type lipid components were found in pholipomycin in contrast to diumycinol-type components in diumycin A'.

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