RAPAMYCIN (AY-22,989), A NEW ANTIFUNGAL ANTIBIOTIC

I. TAXONOMY OF THE PRODUCING STREPTOMYCETE AND ISOLATION OF THE ACTIVE PRINCIPLE

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A streptomycete was isolated from an Easter Island soil sample and found to inhibit *Candida albicans*, *Microsporum gypseum* and *Trichophyton granulosum*. The antibiotic-producing microorganism was characterized and identified as *Streptomyces hygroscopicus*. The antifungal principle was extracted with organic solvent from the mycelium, isolated in crystalline form and named rapamycin. Rapamycin is mainly active against *Candida albicans*; minimum inhibitory concentration against ten strains ranged from 0.02 to 0.2 μg/ml. Its apparent activity against *Microsporum gypseum* and *Trichophyton granulosum* is lower because of its instability in culture media on prolonged incubation required by these fungi. No activity was observed against gram-positive and gram-negative bacteria. Acute toxicity in mice is low.

Cultures of a streptomycete isolated from an Easter Island (Rapa Nui) soil sample were found to inhibit the yeast, *Candida albicans*, and the dermatophytes, *Microsporum gypseum* and *Trichophyton granulosum*; antibacterial activity was only marginal and limited to some gram-positive species, such as *Sarcina lutea* and *Staphylococcus aureus*; all gram-negative bacteria tested were resistant. The active principle was isolated from the mycelium of the streptomycete and the crystalline material obtained after purification was found to inhibit mainly *Candida albicans*; lesser activity was observed against the dermatophytes and no activity was demonstrated against any of the gram-positive and gram-negative bacteria tested. The antibiotic was named rapamycin [Etymol.: Rapa- (Rapa Nui=Easter Island), -mycin]. Its structure is still unknown.

This paper deals with the characterization of the producing streptomycete, the isolation and purification of the antibiotic and some of its biological properties.

Identification of the Rapamycin-Producing Streptomycete

Streptomycete strain AY B-994 was isolated from a soil sample collected in Easter Island (Rapa Nui): the soil was diluted in distilled water and the resulting suspensions were plated on yeast-starch agar according to the double-layer technique of PORTER *et al.*[1]. After one week of incubation at 28°C the streptomycete colonies were purified by repeated streaking and the pure strains grown separately on yeast-starch agar plates to yield confluent growth. After 4~10 days of incubation, discs (7 mm in diameter) were cut and transferred onto the surface of plates of Bacto-Blood Agar Base (Difco Laboratories, Detroit, Mich.) inoculated with test bacteria and SABOURAUD dextrose agar inoculated with test yeast and dermatophytes. The Blood Agar Base plates were inoculated with a standardized bacterial inoculum grown at 37°C for 5 hours in Bacto-Nutrient broth; the SABOURAUD dextrose agar plates were inoculated with a standardized inoculum consisting of cells of yeast or spores of dermatophytes. Plates were

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incubated at 37°C for bacteria and yeast and at 28°C for dermatophytes. Activity was read as diameter of inhibition zone after incubation of 18 hours, 48 hours and 5 days for bacteria, yeast and dermatophytes respectively. When tested under the above conditions strain AY B-994 showed the following antimicrobial activity (diameter of inhibition zone in mm): *Sarcina lutea*, 24; *Staphylococcus aureus*, 20; *Escherichia coli*, *Enterobacter aerogenes*, *Proteus mirabilis* and *Pseudomonas aeruginosa*, no zone of inhibition; *Candida albicans*, 24; *Microsporum gypseum*, 26; *Trichophyton granulosum*, 27. Strain AY B-994 was then maintained on tomato paste-oatmeal agar[3] and preserved by lyophilization[4].

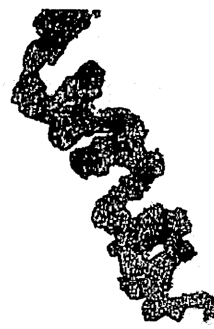
The methods used for characterization were those of the International *Streptomyces* Project published by SHIRLING & GOTTLIEB[5]. Streptomycete strain AY B-994 was found to belong to the species *Streptomyces hygroscopicus* in PRIDHAM & TRESNER's[2] key of classification; it was deposited in the ARS culture collection of USDA (Peoria, Ill.) and assigned the number NRRL 5491.

1. Morphological Characteristics

Streptomycete strain AY B-994 sporulates moderately to abundantly on most media studied. The morphology as revealed by light microscopy and electron microscopy is illustrated in Figs. 1 and 2. Aerial mycelium is monopodially branched; sporophores are terminated by spore chains in the form of short, narrow, compact and closed coils (*Spiral*) of three or more turns; ten or more spores are present in each spiral. Spore color *en masse* is predominantly gray (gray series) turning gray-brown on prolonged incubation. Aerial mycelium is hygroscopic: on absorption of water, spores crowd in masses and a black pigment is produced, giving the appearance of black, gelatinous spots on the surface of several solid media. Under the electron microscope spores are oval, smooth, $0.8 \times 1.6\ \mu$.

Fig. 1. Photomicrograph of *Streptomyces hygroscopicus* AY B-994
(ISP Medium 2, 14 days at 25°C, ×455)

Fig. 2. Electron micrograph of *Streptomyces hygroscopicus* AY B-994
(ISP Medium 2, 14 days at 25°C, ×2,500)



2. Cultural Characteristics

The cultural characteristics of strain AY B-994 are reported in Table 1 for International *Streptomyces* Project (ISP) media and various other media. Tomato paste-oatmeal agar[3] is by far the best medium for growth, sporulation and development of black spots. After two weeks of incubation at 28°C followed by one week of storage at 4°C the surface of this medium appears carbonaceous black; the preparation of homogenous spore suspensions from these black gelatinous masses is difficult. On this medium as well as on ISP Medium 1 (solidified) well

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Table 1. Cultural characteristics of streptomycete strain AY B-994

Culture media*	Vegetative mycelium (growth and color)	Aerial mycelium		Pigment
		Development and color	Spores/spiral; shape of sporophores**	
Tomato paste-oatmeal agar[3]	Rapid and abundant; colorless to yellowish to grayish	Very abundant; grayish with many black spots	≫10 spores/spiral; SCL >90%	
Tryptone-yeast extract agar (ISP Medium 1 solidified)	Rapid and abundant; yellowish	Moderate to abundant; yellowish, with black spots	>10 spores/spiral	Yellow
Yeast extract-malt extract agar (ISP Medium 2)	Rapid and abundant; yellow to dark yellowish	Moderate to abundant; white to grayish to gray, with black spots	>10 spores/spiral; SCL 90%	Yellow
Oatmeal agar (ISP Medium 3)	Very abundant; pale yellowish to brownish	Abundant; whitish to gray with black spots	>10 spores/spiral; SCL 90%	Pale yellow
Inorganic salts-starch agar (ISP Medium 4)	Slow, but eventually abundant; colorless to whitish; starch slowly hydrolyzed	Moderate; whitish to gray, with some black spots	>10 spores/spiral; SCL 90%	None
Glycerol-asparagine agar (ISP Medium 5)	Slow, moderate after 14 days; colorless to pale yellowish to pinkish	Very poor; grayish beige; no black spots	—	Pale pink
Peptone-yeast extract-iron agar (ISP Medium 6)	Poor; colorless to pale yellowish; no H_2S produced	No sporulation (or extremely poor)	—	Yellow
CZAPEK's solution agar (WAKSMAN's Medium 1)	Slow, moderate after 14 days; colorless to pale yellowish	Poor, whitish to grayish	*ca* 10 spores/spiral; SCL 70~80%	None
Glucose-asparagine agar (WAKSMAN's Medium 2)	Rapid and abundant; pale yellowish to whitish, gray later	Moderate; whitish to yellowish, gray later, with black spots; surface brownish black after 28 days	>10 spores/spiral; SCL 70~90%	Pale yellow
BENNETT's agar	Very abundant; pale to medium yellow to greenish	Moderate; whitish to gray to yellowish, with black spots	>10 spores/spiral; SCL 90%	Yellow
Potato sucrose agar	Moderate; colorless to pale yellowish brown, later yellow-gray	Moderate; whitish to gray, with black spots	Hyphae without spores; spirals irregular	Yellow
SABOURAUD dextrose agar	Rapid and abundant	Moderate to abundant		
SABOURAUD maltose agar	Rapid and abundant	Moderate		

* For composition of ISP media refer to SHIRLING & GOTTLIEB;[5] in this study Bacto dehydrated media were used.

** SCL=spirals, closed loops.

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isolated colonies are of two types. One type is powdery white, elevated and well sporulated; the other type is brownish, flat and much less sporulated. The white type is more productive.

3. Physiological Characteristics

The physiological characteristics of strain AY B-994 are summarized in Table 2. pH and temperature ranges for growth and sporulation are much wider than for production of rapamycin.

From the observations reported above strain AY B-994 appears to belong to the species *Streptomyces hygroscopicus*. Its characteristics fit well within the range of characteristics observed by TRESNER & BACKUS[6] for several strains which are recognized to belong to this species. Strain AY B-994 also compares well to *Streptomyces hygroscopicus*, as recently determined by PRIDHAM & TRESNER[2]; it differs from the type strain listed by these authors in that it can also use raffinose and *i*-inositol. We feel these discrepancies do not justify the description of a new species for strain AY B-994.

Table 2. Physiological characteristics of streptomycete strain AY B-994

Parameters	Reactions and other observations
Hydrolysis of starch (ISP Medium 4)	Slow, but eventually extensive
Decomposition of cellulose	Negative
Production of hydrogen sulfide (H_2S) (ISP Medium 6)	Negative
Production of tyrosinase (ISP Medium 7)	Negative (melanin-negative)
Nitrate reduction (ISP Medium 8)	Very weakly positive or negative
Carbohydrate utilization (ISP Medium 9*)	Good growth on: D-glucose, D-fructose, D-mannitol, *i*-inositol, starch, glycerol
	Moderate growth on: D-xylose, L-arabinose, L-rhamnose, raffinose, lactose, D-maltose
	No growth on: sucrose, cellulose
Reaction to pH	Growth at pH 5~>8 (optimum pH 6~8)
Reaction to temperature	Growth at 20~37°C (optimum at 25~27°C) no growth below 18°C or above 47°C

* Bacto-Carbon Utilization agar was used as the basal medium.

Fermentation of Rapamycin in Shake Flasks

Strain AY B-994 was grown in several media at various pH and temperature values. Several sources of carbon and nitrogen were found to be suitable; optimal temperature and initial pH were 25°C and 6.0. Optimal composition of medium and agitation conditions have not been determined, but the following medium and conditions were found suitable for production of sufficient quantities of rapamycin for isolation and purification.

A spore suspension was prepared by suspending the spores from a 14-day culture on tomato paste-oatmeal agar in distilled water; the suspension was diluted to read 70 % transmittance (1-cm cuvette) in a Junior 6A Coleman spectrophotometer, and 1 % of the dilution used to inoculate 500-ml Erlenmeyer flasks each containing 100 ml of the following medium (g/liter): oatmeal, 50; "cerelose" (a pharmaceutical grade of glucose, Corn Products Corporation, New York, N.Y.), 20; N-Z Case (Sheffield Chemical, Norwich, N.Y.), 1; "blackstrap" molasses, 5; NaCl, 5 (in tap water; sterilization at 121°C for 20 minutes; pH after sterilization, 6.1). Flasks were incubated on a gyrotory shaker at 240 rev/min (2″-stroke), 25°C and 70 % R.H. Samples were taken daily for 7 days and assayed microbiologically, using a standard solution of nystatin as a reference and *Candida albicans* as the test organism. When pure rapamycin became available, a standard curve could be drawn and unknowns determined by interpolation.

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A typical fermentation yielded the following results:

Incubation	0 day	3 days	4 days	5 days	6 days
pH	6.0	6.6	7.1	5.6	5.6
Rapamycin (μg/ml)	—	40	45	100	100

A two-stage fermentation could also be run by inoculating flasks as above for 24 hours and using 2 % of this seed to inoculate production flasks prepared as above described; yields obtained were essentially the same as in the single-stage fermentation.

Isolation and Purification of Rapamycin

After five days of incubation the fermentation broth was filtered through Celite and the mycelium, which contained most of the antibiotic, was harvested and washed with 1~2 volumes of water. The washed mycelium was extracted three times with 5 volumes of methanol per weight of wet mycelium each time. The methanolic extracts were pooled and concentrated to the aqueous phase under reduced pressure. The concentrate was extracted three times with 1 volume of methylene dichloride each time; the methylene dichloride extracts were combined, dehydrated with anhydrous sodium sulfate and evaporated to yield an oily residue.

The oily residue was dissolved in 15 % acetone in hexane and charged on a column of silica gel G (50 : 1, w/v). The column was eluted with a solvent mixture of acetone and hexane containing gradually increasing concentrations of acetone. The elution of rapamycin was followed on TLC: an aliquot of each fraction was spotted on TLC silica gel G plates which were developed in acetone - hexane (40 : 60). Rapamycin was eluted from the column when the developing mixture consisted of acetone and hexane (25 : 75). The fractions containing the antibiotic were combined and evaporated to dryness under vacuum. The product was crystallized from the residue with ether. Ten liters of fermentation broth generally yielded 300 mg of pure, crstalline rapamycin.

Biological Activity of Rapamycin

The minimum inhibitory concentration of rapamycin for selected gram-positive and gram-negative bacteria, yeast and dermatophytes is reported in Table 3. Rapamycin is very active against all strains of *Candida albicans* tested. It shows no activity against bacteria. The MIC's against *Microsporum gypseum* and *Trichophyton granulosum* are much higher than anticipated from the inhibition zones observed

Table 3. Antimicrobial spectrum of rapamycin (AY-22,989)

Test organisms*	Minimum inhibitory concentration (μg/ml)
Staphylococcus aureus	> 100
Staphylococcus aureus pen[R]	> 100
Streptococcus faecalis	> 100
Escherichia coli	> 100
Enterobacter aerogenes	> 100
Klebsiella pneumoniae	> 100
Proteus mirabilis	> 100
Proteus vulgaris	> 100
Salmonella pullorum	> 100
Serratia marcescens	> 100
Pseudomonas aeruginosa	> 100
Candida albicans (10 strains)	<0.02~0.2
Microsporum gypseum	12.5
Trichophyton granulosum	> 1,000

* Medium for bacteria: Nutrient broth; medium for yeast and fungi: SABOURAUD dextrose broth. Incubation: 37°C for bacteria and yeast, 28°C for dermatophytes. Results read at 24 hours for bacteria, 48 hours for yeast, and 7 days for dermatophytes.

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