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SELECTION AND CHARACTERIZATION OF HIGH PRODUCING RIFAMYCINS COLONY FROM *AMYCOLATOPSIS MEDITERRANEI*

MOHAMED A. FARID*, AHMED I. EL-DIWANY and HESHAM A. EL ENSHASY

National Research Center, Natural and Microbial Products Dept., Dokki, Cairo, Egypt

A study on different colonies of rifamycins producing strain of Amycolatopsis mediterranei CBS 42575 revealed 6 colonies. The isolated colonies differed in their cultural and morphological properties and antibiotic productivity. The most promising colony which produced the highest amount of rifamycins of 784 and 2844 µg/ml for rifamycin B and SV respectively was characterized by 2-3 mm in diameter, red in color and with a star shape.

Keywords: *Rifamycins; Amycolatopsis mediterranei*

Introduction

It has previously been reported that, spontaneous variants making up parallel series of heredity variability inside the population of antibiotics producing *Actinomycetes* differ in the level of their antibiotic activities (Kuznetsov et al., 1992). They reported also that spontaneous variants of the basic type possess the highest antibiotic activity and the other variants representing parallel series have a lower activity level. Kuznetsov and Filippova (1974) studied the variability of *Actinomyces olivobrunneus* and isolated 9 stable natural variants differing in their cultural and morphological properties, antibiotic spectra and the degree of melaninogenesis. On the other hand, the storage of a lyophilized *Streptomyces griseus* for several years leads to an increase in the percentage of some variants in the population of this strain (Kuznetsov et al., 1975).

Amycolatopsis mediterranei, formerly assigned to the *Nocardia mediterranei*, the producer of rifamycins antibiotics (Sensi et al., 1959; Sensi and Thiemann, 1967; Ghisalba et al., 1983) usually differs in its cultural and morphological characteristics from one strain to the other. The strain produces five antibiotic substances which have been named as rifamycins A, B, C, D and E. In 1964 Sugawara et al. described a new strain which produced rifamycin O. Rifamycin S has been isolated from the acid hydrolsis of rifamycin O (Gallo et al., 1962). After reduction of rifamycin S with ascorbic acid it gives another active substance named rifamycin SV (Korzybski et al., 1967; Sensi et al., 1962). Rifamycin SV can also be obtained directly from a mutant strain of *Nocardia mediterranei* (Birner et al., 1972; Lancini and Hengeller, 1969)) and from *Micromonospora ellipsospora* which was fermented under submerged aerobic conditions in the presence of assimilable sources of carbon and nitrogen (Weinstein et al., 1975). Birner et al. (1972) isolated two different colonies from an Australian isolate of *Nocardia mediterranei*, differing in their morphological and cultural characteristics.

The present work relates to the direct selection of active colonies from population of *Amycolatopsis mediterranei* CBS 42575 on the basis of their easily morphological properties. The description of the cultural characteristics of the active colony on different media was also studied.

Materials and Methods

Microorganism and media

Amycolatopsis mediterranei CBS 42575 was obtained from the Centraalbureau voor Schimmel culture, Baarn, The Netherlands. It was maintained on a slant culture for 7 days on Bennett's agar medium. The medium had the following composition (g/l): 10.0 glucose, 1.0 yeast extract, 1.0 beef extract, 2.0 N-Z amine, 20.0 agar and 1000 ml distilled water. The pH of the medium

* Correspondence

was adjusted to 6.8 before sterilization. The fermentation medium had the following composition (g/l):30.0 glucose, 1.0 $MgSO_4.7H_2O$, 0.016 $FeSO_4.7H_2O$, 0.001 zinc acetate, 5.0 yeast extract and 1000 ml distilled water. The pH of the medium was adjusted to 7.0 with 0.1 M NaOH. Inoculum was carried out by transferring a selected colony under study obtained from 7 days cultures on Bennett's agar slant to 50 ml seed medium in 250 ml erlenmayer flasks, followed by incubation at 28°C on a rotary shaker 200 rpm for 96 h.

Colony selection

Sterile glass petri dishes were prepared in a sterile laminar flow area. The Bennett's agar medium was melted in a boiling water bath for about 10 min. After cooling at about 60°C the medium was distributed 20 ml per petri dish and after 10 min the petri dishes were stored in a refrigerator. The strain of 10-15 days old was transferred into a sterile Hand-Homogenizer (Eppendorf type-B.Braun, Melsungen, Germany) containing 5 ml of sterile saline solution and homogenized. A serial dilution was prepared using a series of test tubes containing 9 ml of sterile saline solution. 0.25 ml of diluted suspension was transferred into the petri dishes containing Bennett's agar medium and platted with sterile glass spreader sticks. After platting, the petri dishes were kept 20 min in sterile area and incubated at 28-30°C for 10-15 days in reverse position. The growing colonies were selected, characterized and photographed using Stereo microscope series SM 33 (Hund, Wetzlar, Germany) and propagated on Bennett's agar slant.

Cultural characteristics

For the investigation of growth characteristics, the active colony of *A. mediterranei* CBS 42575 was grown in a variety of standard media according to Waksman (1961), Gottlieb and Shirling (1966) and in addition, some media recommended by Sugawara et al. (1964) and Birner et al. (1972) were also used.

Determination of rifamycins in the fermentative broth

Rifamycins B and SV were measured spectrophotometrically according to the method of Pasqualacci et al. (1970) as follows:
Reagent solutions: Solution A: Acetate buffer (pH 4.6) Solution B: Solution A containing 0.1% w/v of $NaNO_2$.
Procedure: Two 0.1 ml portions of the fermentative broth in two 10 ml volumetric flasks were made to volume one with the pH 4.6 acetate buffer solution (solution A) and the other one with the oxidized pH 4.6 acetate buffer solution containing 0.1% w/v of $NaNO_2$ (solution B). The absorbance of the solution diluted with solution A was determined against the solution diluted with solution B using a double beam spectrophotometer (CE 595, Cecil Co., UK) at 425 nm for rifamycin B and 447 nm for rifamycin SV. The amounts of rifamycins were calculated from the measured absorbances.

Results and Discussion

Amycolatopsis mediterranei CBS 42575 cells were platted into petri dishes containing Bennett's agar medium. After 10 days incubation at 28-30°C, the growing colonies were picked up and the cultural and morphological characteristics had been determined. The results in Table 1 and Figs 1a-f show that a considerable morphological variation could be observed between the isolated colonies. The selected colonies had the following morphological characteristics: The size of the isolated colonies in cross section varied from 1-10 mm. The selected colonies grew well on Bennett's agar medium and the cultures were variously colored: orange, orange-red, red, reddish-brown and buff depending on the colony type. The shape of such colonies were regular, mucoid with hollow center and irregular in shape folding toward the center. The aerial mycelium was either altogether absent or weakly developed.

Table 2 shows the results of antibiotic productivity of the isolated colonies at the end of the fermentation time (96 h). These data indicated that the different colonies posses different levels of rifamycins B and SV when they grow under the same condition of cultivation. The highest yield of rifamycins was obtained by using the red-brown colored colony, star shaped, devoid of a hollow center and not exceeding 3 mm in diameter. Thus, a clear correlation was found between the level of rifamycins produced by different kinds of colonies and their cultural and morphological properties. These results are in accordance with that reported by Kuznetsov et al. (1992), when they studied the antibiotic activities of spontaneous variants of seven *Streptomyces* producing seven different antibiotics.

Cultural characteristics of the most active colony

The selected colony No.1 grows well on the usual media, synthetic and complex-organic one. The results in Table 3 were based on observation after 1, 2 and 3 weeks of incubation at 28°C unless otherwise noted. The selected colony (No.1) forms orange-red-brown pigments, changing of color with change in media

composition. The pigment is not formed on nutrient agar, Ca-malate glucose or glycerol agar media. The culture of selected colony liquefy gelatin slowly, hydrolyze starch, assimilate many sugars and does not grow on cellulose.

Finally for fermentative production purpose, the selection should be carried out from time to time to keep the amount of antibiotics rifamycins B and SV constant.

Table 1. The morphological characteristics of the different colonies of *Amycolatopsis mediterranei* on Bennet's agar medium

Colony No.	Average diameter (mm)	Morphological characteristics
1	2-3	Red, rosate, irregular in shape
2	7-8	Orange, irregular
3	3-5	Orange, mucoid, hollow centered
4	8-10	Orange-red, irregular with folding toward the centre
5	1-2	Reddish-brown, mucoid, rounded, regular
6	5-7	Buff, hollow centered, irregular

Table 2. Rifamycins production by different colonies of *Amycolatopsis mediterranei*

Isolated colonies	Growth parameters		Antibiotic parameters			
	pH	Cell dry weight (mg/50 ml)	Rifamycin B (µg/ml)	Rifamycin SV (µg/ml)	R. Y. (rifa B) (µg/mg cells)	R. Y. (rifa SV) (µg/mg cells)
1	6.60	540	784	2844	72.5	263.0
2	6.55	680	630	2380	46.3	175.0
3	6.63	543	504	1955	46.3	180.0
4	6.54	493	581	2181	58.9	221.2
5	6.44	520	350	1091	33.6	104.9
6	6.32	430	245	907	28.5	105.5
Parent strain (mixed colonies)	6.58	566	497	1771	43.9	156.4

Table 3. Growth characteristics of selected colony of *Amycolatopsis mediterranei.* on different media.

Media used	Age (days)	Growth	Aerial Mycelium		Substrate mycelium		Sporulation	Exopigment
			Presence	Color	Presence	Color		
Bennett's Agar.	7	+	+	Red	+	Reddish-Brown	-	Orange
	14	++	+	Brown	+	Brown	-	Reddish-Brown
	21	++	+	Brwon	+	Brown	+	Brown
Tryptone-Yeast extract Agar.	7	+	+	faint beige	+	beige	-	-
	14	+	+	beige	+	beige	+	-
	21	+	+	beige	+	beige	+	-
Yeast extract-Malt extract Agar	7	+	+	Reddish-Brown	+	Reddiah-Brown	-	Orange
	14	++	+	Reddish-Brown	+	Brown	-	Red
	21	++	+	Brown	+	Brown	+	Red

Media used	Age (days)	Growth	Aerial Mycelium		Substrate mycelia		Sporulation	Exopigment
			Presence	Color	Presence	Color		
Ca-Malate glucose Agar	7	+	-	-	+	beige	-	-
	14	+	+	beige	+	beige	-	-
	21	+	+	beige	+	beige	-	-
Glucose casein digested yeast beef agar.	7	+	+	Orange	+	Orange	-	Orange
	14	++	+	Brown	+	Brown	-	Brown
	21	++	+	Brown	+	Brown	-	Brown
Nutrient Agar	7	+	-	-	+	beige	-	-
	14	+	+	beige	+	beige	-	-
	21	+	+	beige	+	beige	-	-
Czapek's Agar	7	+	+	Orange	+	Orange	-	Yellowish-Orange
	14	+	+	Orange	+	Red	-	Orange
	21	++	+	Orange	+	Brown	-	Orange

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Table 3. cont.

Media used	Age (days)	Growth	Aerial Mycelium		Substrate mycelium		Sporulation	Exopigment
			Presence	Color	Presence	Color		
Inorganic salt-								
Starch Agar.	7	+	+	Orange	+	Orange	+	-
	14	+	+	Orange	+	Orange	+	-
	21	++	+	Red	+	Red	+	-
Glycerol-Asparagine								
Agar.	7	+	+	Orange	+	Orange	-	-
	14	+	+	Orange	+	Orange	-	Orange
	21	++	+	Red	+	Red	-	Orange
Tyrosine-Agar.	7	+	+	Red	+	Red	-	-
	14	++	+	Red	+	Red	+	Orange
	21	++	+	Brown	+	Brown	+	Red
Ca-Malate								
glycerol Agar	7	-	-	-	-	-	-	-
	14	+	+	beige	+	beige	-	-
	21	+	+	beige	+	beige	-	-

Fig. 1(a). Colony No. 1

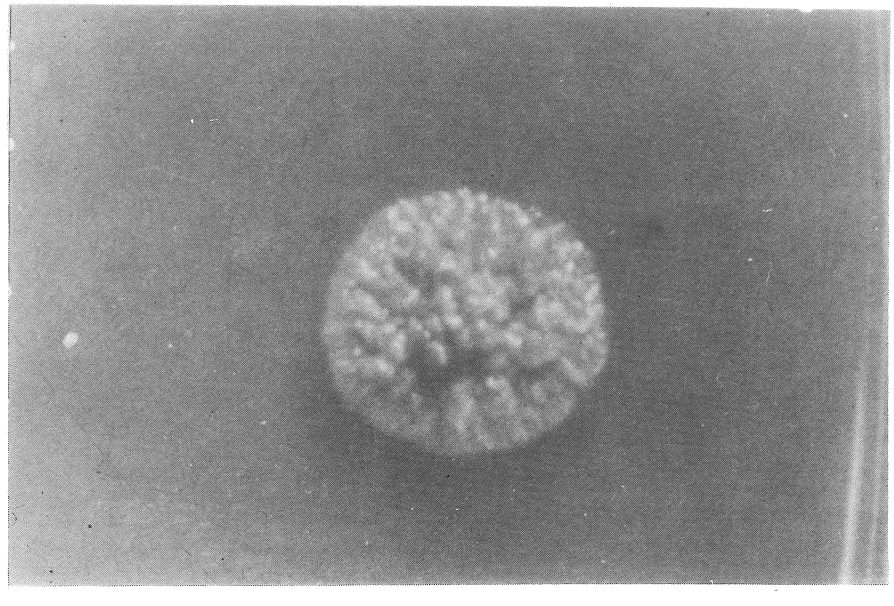

Fig. 1(b). Colony No. 2

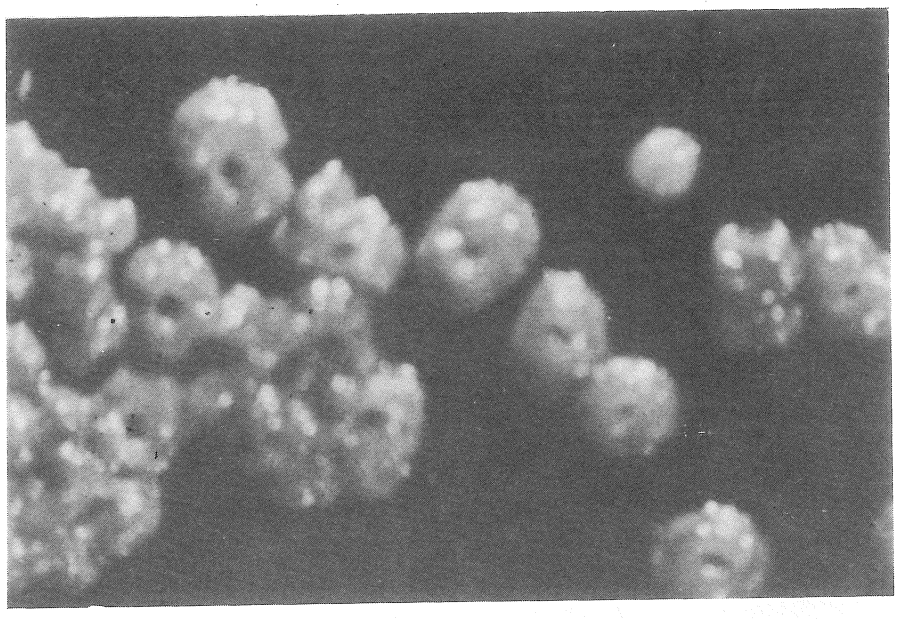

Fig. 1(c). Colony No. 3

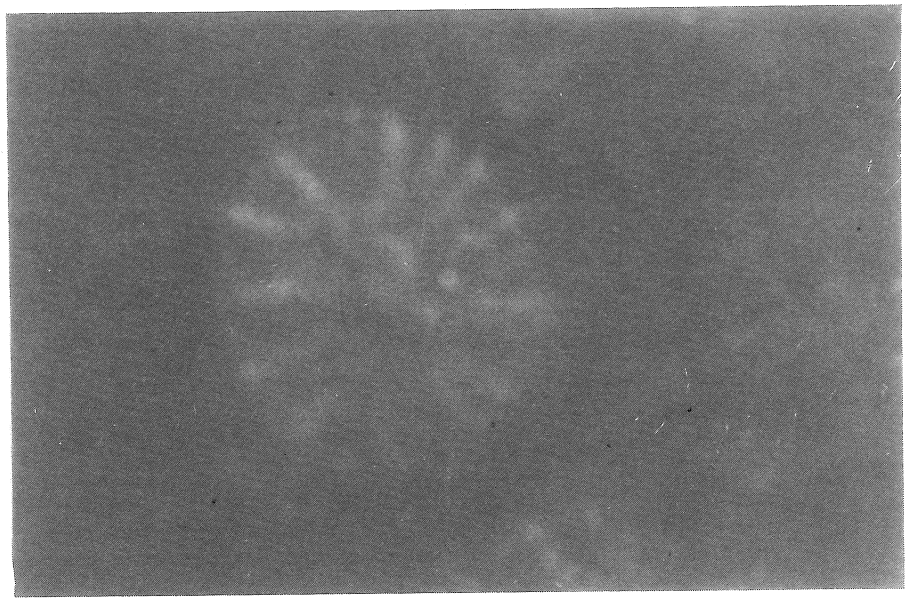

Fig. 1(d). Colony No.4

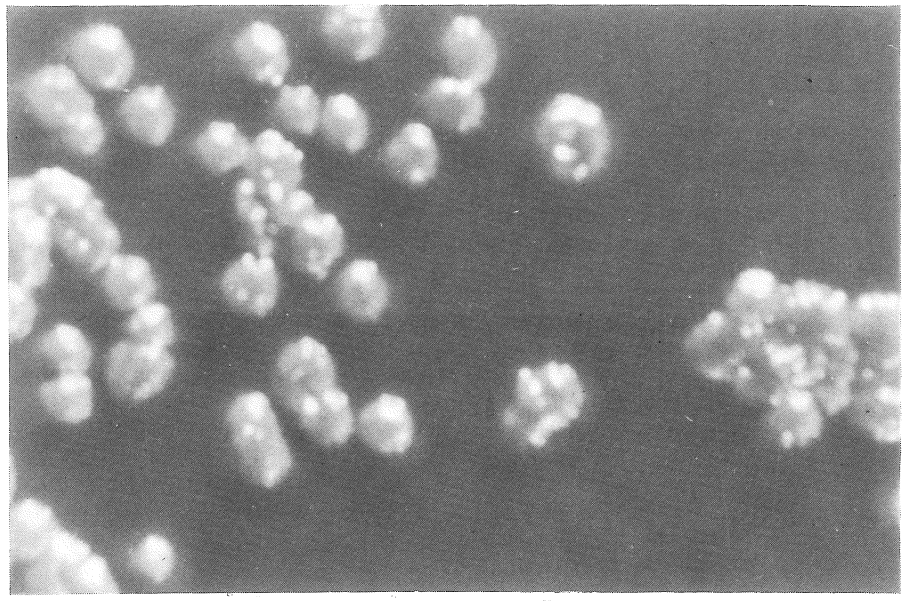

Fig. 1(e). Colony No. 5

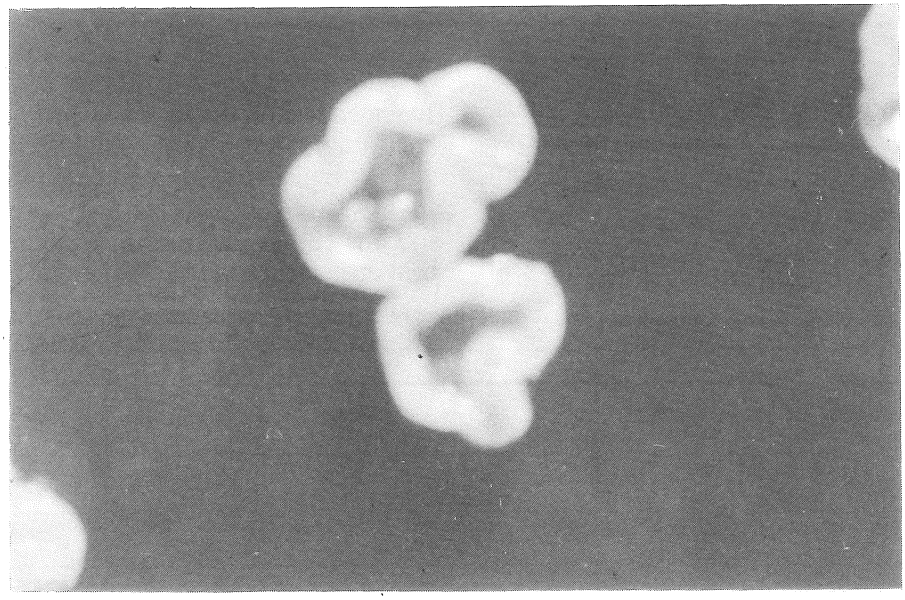

Fig. 1(f). Colony No. 6

Fig. 1. Different morphological structures of *Amycolatopsis mediterranei* colonies on Bennett's agar medium. Magnification X 5

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