NOTE

AN AUSTRALIAN ISOLATE OF *NOCARDIA MEDITERRANEA* PRODUCING RIFAMYCIN SV

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Since its first announcement by P. SENSI *et al.* in 1959[1], the rifamycin complex has been extensively studied by a number of workers[2]. The type culture *Nocardia mediterranea* (MARGALITH *et* BERETTA) THIEMANN *et al.* 1969*, then known as *Streptomyces mediterranei*[3] was isolated from a soil sample collected in France. SUGAWARA *et al.*[4] culturing an isolate (4107 A2) from a Japanese soil sample, reported that this organism was a variant strain of the same streptomycete which differed in certain cultural characteristics and produced in culture rifamycin O as the main active principle. LANCINI and HENGELLER[5] treated *S. mediterranei* with N′ methyl-N′-nitro-N-nitrosoguanidine and isolated a mutant (ATCC 21271) which produced mainly rifamycin SV in culture.

An antibiotic-producing organism, later identified as a *Nocardia* isolated by one of us (W.R.L.) from a soil sample from the arid Northern Territory of Australia, produced in culture mainly rifamycin SV without deliberate genetic manipulation. As SUGAWARA and co-workers found with their strain 4107 A2, our strain, indexed as NT19 in our collection, differed from *Nocardia mediterranea* in some respects, and it further differs from strain 4107 A2.

Comparison of the cultural characteristics of all three strains is made and our procedures for isolation of rifamycin SV are described in this note.

Taxonomy

The strain, NT19, was obtained from a soil sample collected near Alice Springs, Northern Territory, Australia.

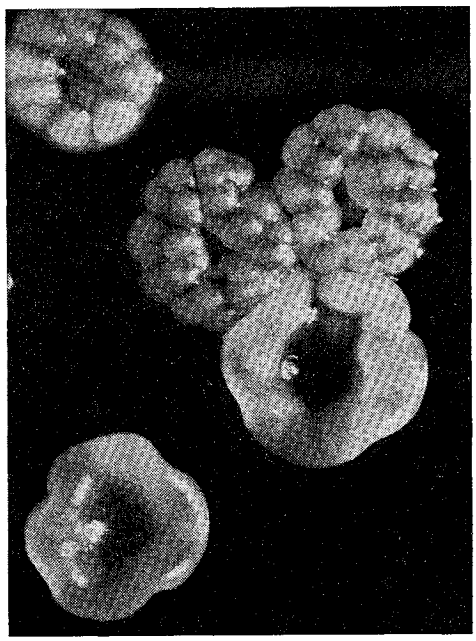
The colour of the vegetative mycelium is orange pink and that of the aerial mycelium is white to very pale pink. The strain produces light brown to yellow pigment on some media. Electron microscopy of the spores shows that they are oval in shape, and of size 0.5~0.85 $\mu \times 1.7$~2.7 μ. When whole cell hydrolysate was examined by paper chromatography according to the procedure of BECKER *et al.*[8] it was established that the hydrolysate contains only the *meso*-isomer of diaminopimelic acid. On these bases the isolate was identified as belonging to the genus *Nocardia*[9].

Cultural characteristics of strain NT19 on various media are given in Table 1.

Comparing these characteristics with those of strain 4107 A2 and *Nocardia mediterranea* as summarized by SUGAWARA *et al.*[4] in their Table 1, several differences are evident.

1. Two types of colony are formed on glucose asparaginase agar. A photograph

Plate 1. NT19-Colony forms (×6).



* *Streptomyces mediterranei* MARGALITH and BERETTA 1960 has been transferred to the genus *Nocardia* as *Nocardia mediterranea* (MARGALITH and BERETTA) THIEMANN *et al.* 1969, on the basis of cell wall composition[9].

Table 1. NT 19-Cultural and physiological characteristics

Medium	Cultural characteristics
Glucose-asparagine agar	Growth : Good with two colony forms present. Colony I : Orange colour, raspberry shape, with pitted mucoid-like surface. Colony II : Very pale orange colour, concave shape, with a smooth mucoid-like surface. No aerial mycelium or soluble pigment on either colony. Each colony form was dominant among the colonies produced when sub-cultured twice on the same medium. White fluffy aerial mycelium increased on subculture. No soluble pigment. Both colony forms when transferred to the other media listed in this table showed similar characteristics. No difference in antibiotic forming ability could be detected.
Oatmeal agar	G : Good, white circular concave colonies with rough surface. AM : Very pale pink, fluffy. SP : Light brown to yellowish.
Yeast extract glucose agar	G : Good, colonies similar to Colony I on G. A. agar but with a dry looking surface. AM : White, fluffy, on the more dense areas of growth. SP : Light brown to yellowish.
BENNETT's agar	G : Good, colonies similar to Colony I on G. A. agar but with a drier looking surface. AM : White, fibrous, on the more dense areas of growth. SP : Amber.
Tyrosine agar	G : Good, colonies the same as Colony I on G. A. agar. AM : None SP : Very pale yellow.
Yeast extract molasses agar	G : Good, single colonies very similar to Colony I on G. A. agar. AM : Very pale pink, fibrous or wool-like on dense areas of growth. SP : None
CZAPEK's agar	G : Good, white circular concave colonies. AM : Fine white, fibrous. SP : None
Nutrient agar	G : Poor, round very pale orange raised colonies with a mucoid-like but pitted surface. AM : None SP : None
Starch agar	G : Good, colonies orange in colour, similar shape to Colony I on G. A. agar but only slightly raised. AM : None SP : None
Ca-malate agar	G : Good, circular white convex colonies. AM : White, fluffy. SP : Yellow.
Calcium citrate glycerol agar	G : Good, circular white concave colonies. AM : Fine white, wool-like. SP : Yellow.
Potato wedge	G : Fair, pale orange colonies, shapeless indistinct forms.
Gelatin	G : Fair. Liquefaction after 30 days at 25°C.

G : Growth. AM : Aerial mycelium. SP : Soluble pigment.

of these appears as Plate 1.

2. In distinction from the other strains, no soluble pigment is produced on yeast extract molasses agar.

3. A pale yellow soluble pigment is produced on tyrosine agar.

4. Strain NT19 utilises only four (xylose, arabinose, glucose and fructose) of the common sugars tested as carbon sources. A comparison of this utilization with that of the other two strains is given in Table 2.

Table 2. NT19-Utilisation of carbohydrates

Carbon source	NT19	4107 A2[4]	*Nocardia mediterranea*[8,9]
Xylose	+	—	++
Arabinose	+	+	+++
Rhamnose	—	+	++
Glucose	+	++	++
Fructose	+	++	+++
Galactose	—	++	+++
Mannose	—	++	++
Sucrose	—	+	++
Maltose	—	+	+
Lactose	—	±	++
Raffinose	—	—	—
Inulin	—	±	—
Mannitol	—	—	++
Sorbitol	—	—	—
Dulcitol	—	—	—
Inositol	—	++	++
Salicin	—	—	+
Starch	—	±	not described

Basal medium was 1% proteose peptone (Difco), 0.5% NaCl, with bromocresol purple indicator dye. Sugars were of analytical reagent grade, for biochemical tests. Cultures were incubated at 37°C for 18 days.

Culture of the Strain

The strain was maintained by culture on Minimal Medium: 15 g agar, 6 g $NaNO_3$, 0.5 g KCl, 0.5 g $MgSO_4 \cdot 7H_2O$, 1.5 g KH_2PO_4, 10 g glucose, 0.001 g $FeSO_4 \cdot 7H_2O$, 0.0005 g $(CH_3COO)_2$ Zn per litre, pH 6.5, and freeze dried. Lyophile preparations were reconstituted on Complete Medium: Minimal Medium to each litre of which was added 2 g Evans peptone, 1 g Bacto yeast extract, and amino-acids and vitamins in the concentrations recommended by EAGLE[6] (if liquid media were required, agar was omitted), and used as inoculum for the cultural studies. Growth was prolific at temperatures between 25°C and 37°C.

Production and Identification of Rifamycin SV

Preliminary work established that much more reproducible results were obtained in surface culture than in submerged fermentations, although shaking flask culture was useful for preparation of seed cultures. Bottles 20 cm×30 cm×3 cm containing 0.5 litre (1 cm depth) of Fermentation Medium: 6 g HY Case-amino acids, 10 g glucose, 6 g $NaNO_3$, 3.75 g KH_2PO_4, 1.5 g K_2HPO_4, 1 g $MgSO_4 \cdot 7H_2O$, 16 mg Fe (as $FeSO_4 \cdot 7H_2O$), and 1 mg Zn (as $[CH_3COO]_2Zn$), water to 1 litre, pH 7, inoculated with 5% by volume of 48-hour shaking flask culture, were incubated at 25°C for 48~72 hours. Culture fluid when assayed by the cylinder plate method using a penicillin-resistant strain of *Staphylococcus aureus*, and a methicillin standard had an activity in the range equivalent to methicillin at 50~80 μg/ml, and later when the antibiotic identity was established was found to have an activity in the range equivalent to rifamycin SV at 15~35 μg/ml. Filtered culture fluid concentrated tenfold under vacuum was treated with 20 volumes of acetone, filtered from the inactive precipitate and freed from acetone and a little more water by a further vacuum distillation. The concentrate, acidified with H_2SO_4 to pH 2.7 was extracted twice with 5 volumes of chloroform, the separated chloroform extract dried with Na_2SO_4 and the chloroform removed by distillation. Extraction several times of the viscous semisolid oil with cold acetone removed a waxy solid, biologically inactive. The final oily concentrate represents about 9% of the total solids content of the crude culture fluid filtrate and 70~85% of the total culture fluid activity. We were unable to separate it in pure crystalline form for more detailed chemico-physical examination. Chromatography was performed on phosphate treated paper pH 8.6, developed by amyl alcohol: *n*-butanol 9:1 in normal and reducing (0.1% ascorbic acid) systems[7], and by the thin layer technique using (a) Silica (Merck) developed with acetone, and (b) Silica-Avicel (microcrystalline cellulose - technical grade, 1:1), developed with acetone - chloroform (4:1). In all cases the mobility

corresponded to a rifamycin SV standard. Eluted samples examined spectrophotometrically showed maximum absorption at 445 nm. If ascorbic acid was not present, the characteristic yellow spot of rifamycin SV changed to the red colour of rifamycin S when allowed to stand in the air. Further confirmation of the presence of rifamycin SV was obtained by assaying samples biologically using the cylinder plate assay in the presence and absence of sodium ascorbate. In the former reducing situation its biological activity was over 50 % higher than when oxidised. Crude concentrated material was active *in vitro* against *Mycobacterium tuberculosis*.

Cultivation of the strain NT19 under various conditions of stationary and submerged fermentation with and without sodium barbitone showed that 0.2 % sodium barbitone inhibited formation of rifamycin SV, but no rifamycin B was detected.

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