TAXONOMY OF ACTINOMYCETES CAPABLE OF HYDROXYLATION OF ML-236B (COMPACTIN)

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Three actinomycetes having capability of 3β-hydroxylation of ML-236B were isolated from soil samples collected in Australia. Strain SANK 62781 was identified as *Nocardia autotrophica*. Strain SANK 62881 and strain SANK 62981 were identified as new subspecies of *N. autotrophica* for which the name *N. autotrophica* subsp. *canberrica* and *N. autotrophica* subsp. *amethystina* are proposed, respectively. The type strains of *N. autotrophica* subsp. *canberrica* and *N. autotrophica* subsp. *amethystina* are ATCC 35203 and ATCC 35204.

In the course of screening for microorganisms capable of 3β-hydroxylation of ML-236B[1], we isolated three actinomycetes with potent activity from soil samples collected in Australia. These strains were found to belong to the genus Nocardia. In this paper, the taxonomic characteristics of these strains are described.

Taxonomic Studies

Strains SANK 62781, SANK 62881 and SANK 62981 were isolated from eucalyptus forest soil, lake-side sand and lake-bottom mud, respectively, collected at Canberra, Australia. Water suspensions of each substrate sample were plated on agar medium, PY (glucose 0.2%, soluble starch 0.1%, pressed yeast 0.09%, peptone 0.05%, beef extract (Difco) 0.05%, $CaCO_3$ 0.03%, NaCl 0.05%, rifampicin 1 μg/ml, cycloheximide 25 μg/ml, nystatin 25 μg/ml, agar 2.0%, pH 7.4 before sterilization), GA (glycerol 1.0%, asparagine 0.1%, K_2HPO_4 0.05%, ISP-trace salts solution 0.1%, tetracycline 10 μg/ml, cycloheximide 25 μg/ml, nystatin 25 μg/ml, agar 2.0%, pH 7.0 before sterilization) and SC (soluble starch 1.0%, casein 0.1%, KH_2PO_4 0.05%, noboviocin 25 μg/ml, cycloheximide 25 μg/ml, nystatin 25 μg/ml, agar 2.0%, pH 7.2 before sterilization) and those plates were incubated at 28°C for 14 days.

The organisms were inoculated into ISP medium 1 in a Sakaguchi flask and grown for 3 days at 28°C on a reciprocal shaker.

The cultures were centrifuged, washed twice with sterile distilled water and then used as an inoculum for various studies.

Morphological, cultural and physiological properties of these strains were examined according to the methods described by SHIRLING and GOTTLIEB[2] and those recommended by WAKSMAN[3]. The characteristics of these strains were compared with those of the known species of actinomycetes described in "The Actinomycetes, Vol. 2" by WAKSMAN[3], "BERGEY's Manual of Determinative Bacteriology, 8th Edition"[4], the "ISP Reports" by SHIRLING and GOTTLIEB[5~8] and other recent literatures concerning taxonomy of nocardiae and streptomycetes.

Morphological Characteristics

The morphological characteristics of the hyphae on various agar media at 28°C for 7 to 21 days

Plate 1. Light micrograph of strain SANK 62781 (on potato extract - carrot extract agar, 28°C, 14 days). ×150.

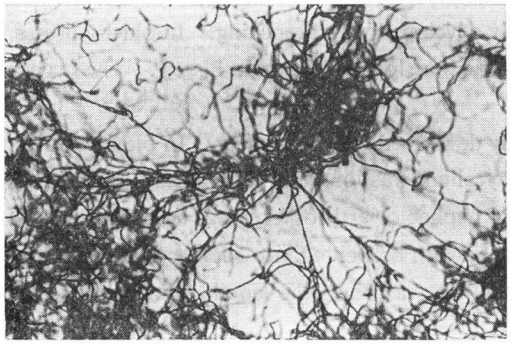


Plate 2. Scanning electron micrograph of strain SANK 62781 (on potato extract - carrot extract agar, 28°C, 14 days). A mark equals 5 μm.

Plate 3. Light micrograph of strain SANK 62881 (on potato extract - carrot extract agar, 28°C, 14 days). ×150.

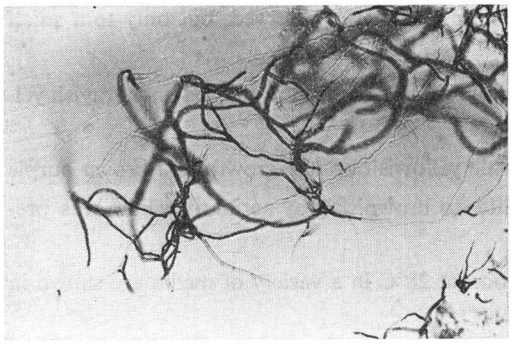


Plate 4. Scanning electron micrograph of strain SANK 62881 (on potato extract - carrot extract agar, 28°C, 14 days). A mark equals 5 μm.

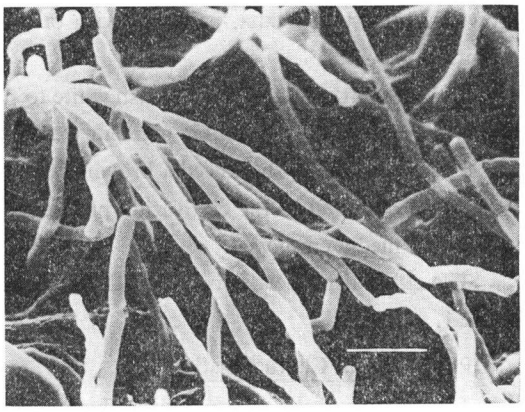


Plate 5. Light micrograph of strain SANK 62981 (on potato extract - carrot extract agar, 28°C, 14 days). ×150.

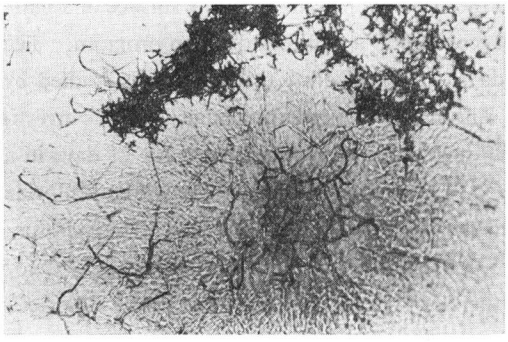
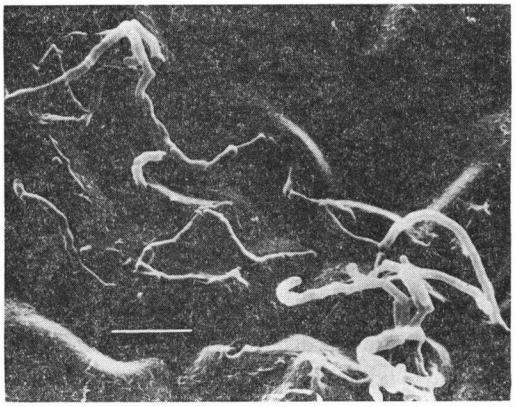


Plate 6. Scanning electron micrograph of strain SANK 62981 (on potato extract - carrot extract agar, 28°C, 14 days). A mark equals 5 μm.



were examined under a light microscope. The aerial hyphae prepared with a critical point dryer (HCP-1, Hitachi Co., Ltd.) were observed under a MSM-6 scanning electron microscope (Akashi Seisakusho Co., Ltd.).

Vegetative hyphae of strain SANK 62781 were fully developed with branching. In older cultures, they fragmented into bacillary elements. The aerial hyphae were long, as shown in Plate 1. The surface of hyphae or arthrospores was smooth (Plate 2).

Vegetative hyphae of strain SANK 62881 were fully developed with branching. In older cultures, they fragmented into bacillary elements. The aerial mycelium was long and sometimes formed knots or nest-like tangles as shown in Plate 3. The surface of hyphae or arthrospores was smooth (Plate 4).

Vegetative hyphae of strain SANK 62981 developed with branching into bacillary elements, and sometimes with a "nocardoid" zig-zag. The aerial mycelium was poor, the length was short, and the surface was smooth (Plates 5 and 6).

Cultural Characteristics

Observations of cultures on various agar media were made after cultivation at 28°C for 7 to 21 days. The mass colors of the mycelium are described in common terminology, but the exact color was determined by comparing the mycelial color with color-tips from the Japan Color Standard[9].

Strain SANK 62781 possessed white aerial mycelium on a yellowish gray to pale yellowish orange growth. In certain media, pale yellowish brown soluble pigment was observed, but only to a small extent.

Strain SANK 62881 had brownish white to pale yellowish orange aerial mycelium on a grayish yellow brown growth. No soluble pigment was observed.

Strain SANK 62981 showed a brownish white to pale yellowish orange growth and brown purple spots were observed as the cultivation proceeded. White to brownish gray aerial mycelium was present on all media employed.

The cultural properties on the 14th day of cultivation at 28°C in a variety of media are shown in Table 1.

Physiological Characteristics

The young mycelia grown under submerged culture were used to determine acid fastness as described in the Manual of Clinical Microbiology[10]. Decomposition of adenine, guanine, casein, xanthine, hypoxanthine, tyrosine and urea, acid production from carbohydrate, resistance to lysozyme and survival at 50°C were studied by the procedures described by GORDON *et al.*[11]. The minimal inhibitory concentration (MIC) for each antibiotic was determined by streaking inocula onto the surface of a plate of ISP medium 2. The antibiotics tested were diluted according to a two-fold dilution program. The growth was observed after incubation at 28°C for 21 days. Carbohydrate utilization was studied by the procedures described by SHIRLING and GOTTLIEB[2]. The effect of temperature on growth was investigated by streaking the inoculum over the surface of ISP medium 2 and incubating it for 21 days in a temperature gradient incubator (Toyo Kagaku Sangyo Co., Ltd., Japan). The test for sodium chloride tolerance was examined by streaking the inoculum on to the same medium as used for the temperature study, but containing sodium chloride at 1.0, 2.0, 3.0, 5.0, 7.0 and 10.0%, and incubating at 28°C for 21 days. For growth tests under anaerobic condition, the Gas Pak system (BBL) was used. The activity for DNase was determined by the method by JEFFERIES *et al.*[12]. The media used for other tests were as follows; ISP media 1, 6 and 7 for melanoid pigment formation, nitrate broth (Difco) for nitrate reduc-

Table 1. Cultural characteristics of strains SANK 62781, SANK 62881 and SANK 62981.

Medium		SANK 62781	SANK 62881	SANK 62981
Yeast - malt agar (ISP 2)	G:	Very good, pale yellowish brown (6–7–9)	Very good, brown (6–4–1)	Very good, brownish white (2–9–8) to grayish red brown (4–3–5)
	AM:	Abundant, white	Abundant, brownish white (2–9–7)	Trace, white
	R:	Dull yellowish orange (8–8–8)	Brown (4–4–7)	Brownish white (2–9–8) to grayish red brown (4–3–5)
	SP:	Yellowish brown (8–7–9)	None	None
Oatmeal agar (ISP 3)	G:	Good, pale brown (2–8–9)	Very good, pale yellowish orange (2–9–9)	Very good, dark reddish brown (4–3–4)
	AM:	Fair, white	Abundant, pale yellowish brown (2–9–9)	Fair, pale pink (2–8–4)
	R:	Yellowish brown (4–7–9)	Pale yellowish brown (4–8–9)	Brown purple (3–3–2)
	SP:	Pale yellowish brown (4–8–9)	None	None
Inorganic salts - starch agar (ISP 4)	G:	Good, yellowish gray (2–9–10)	Poor, yellowish gray (1–9–10)	Very good, brown purple (3–3–2)
	AM:	Fair, white	Abundant, pale yellowish orange (2–9–9)	Good, bright brownish gray (2–8–2)
	R:	Pale yellow (3–9–10)	Pale yellowish orange (2–9–9)	Dark reddish brown (4–3–4)
	SP:	None	None	None
Glycerol - asparagine agar (ISP 5)	G:	Good, pale brown (2–8–9)	Good, grayish yellow brown (4–5–7)	Very good, pale brown (2–9–9) to brown purple (3–3–2)
	AM:	Abundant, white	Abundant, brownish white (1–8–6)	Abundant, white
	R:	Pale yellowish brown (6–8–9)	Brown (4–4–6)	Pale yellowish orange (2–9–9) to grayish red brown (4–3–6)
	SP:	Pale yellowish brown (6–9–11)	None	None
Tyrosine agar (ISP 7)	G:	Very good, pale yellowish orange (3–8–8)	Very good, grayish yellow brown (4–5–7)	Good, grayish brown (4–6–6)
	AM:	Abundant, white	Abundant, brownish white (2–9–7)	Trace, white
	R:	Yellowish gray (1–9–10)	Light brown (6–5–7)	Pale yellowish orange (2–9–9) to brown purple (3–3–2)
	SP:	Pale yellowish brown (6–7–9)	None	None
Sucrose - nitrate agar	G:	Good, pale yellowish brown (2–9–9)	Poor, pale yellowish brown (2–9–9)	Poor, pale yellowish orange (2–9–9)
	AM:	Fair, white	Abundant, brownish white (2–9–7)	Fair, white
	R:	Yellowish gray (1–9–10)	Brownish white (1–9–6)	Pale yellowish orange (2–9–9)
	SP:	None	None	None
Glucose - asparagine agar	G:	Very good, pale yellowish orange (2–9–9)	Good, grayish yellow brown (4–5–7)	Very good, pale yellowish orange (2–9–9) to brown purple (3–3–2)
	AM:	Fair, white	Abundant, light brownish white (1–7–6)	Fair, white

Table 1. (Continued)

Medium		SANK 62781	SANK 62881	SANK 62981
Glucose - asparagine agar	R:	Pale yellowish brown (4–8–9)	Grayish red brown (4–3–6)	Pale yellowish orange (2–9–9) to grayish red brown (4–3–6)
	SP:	Pale yellowish brown (4–8–9)	None	None
Nutrient agar	G:	Good, yellowish gray (2–9–10)	Very good, pale yellowish brown (6–8–9)	Good, pale yellowish orange (2–9–9)
	AM:	Fair, white	Pale yellowish orange (2–9–9)	Trace, white
	R:	Yellowish gray (4–9–10)	Pale yellowish brown (6–8–9)	Pale yellowish orange (2–9–9)
	SP:	None	None	None
Water agar	G:	Poor, yellowish gray (1–9–10)	Poor, colorless	Poor, pale yellowish orange (2–9–9)
	AM:	Fair, white	Abundant, white	Fair, white
	R:	Yellowish gray (1–9–10)	Pale yellowish orange (2–9–9)	Pale yellowish orange (2–9–9)
	SP:	None	None	None
Potato extract - carrot extract agar	G:	Poor, yellowish gray (1–9–10)	Poor, colorless	Poor, pale yellowish orange (2–9–9)
	AM:	Fair, white	Fair, white	Fair, white
	R:	Yellowish gray (1–9–10)	Pale yellowish orange (2–9–9)	Pale yellowish orange (2–9–9)
	SP:	None	None	None

G: growth, AM: aerial mycelium, R: reverse, SP: soluble pigment.

tase, ISP medium 4 for starch hydrolysis, gelatin stab for gelatin liquefaction, and dehydrated skim milk for coagulation and peptonization. The cultures on all of the media tested were incubated at 28°C for 14 days except for those on milk (37°C, 10 days) and gelatin (25°C, 21 days) media. These physiological characteristics of the strains are shown in Table 2.

Cell Wall and Whole Cell Analyses

Cell walls and whole cells were analyzed by the procedure of BECKER *et al.*[13] and by the method of LECHEVALIER[14,15], respectively. The presence of mycolic acid and the acyl type in peptidoglycan were determined by the method of HECHT *et al.*[16] and the micro-scale column procedure of UCHIDA *et al.*[17], respectively. Phospholipid analysis was performed by the procedure described by LECHEVALIER[15]. The three strains had cellular components of the type IV-A, acetyl type and PII type of phospholipid. Mycolic acid was not detected as shown in Table 3.

The results of these taxonomic studies demonstrated that the strains belonged to the genus Nocardia. Among the known species of Nocardia, the characteristics of the strains are most closely related to those of *Nocardia autotrophica* (Takamiya and Tubaki 1956) Hirsch 1961[5,18~20], except for the differences shown in Table 4.

Strain SANK 62781 and *N. autotrophica* resembled each other in their morphological, cultural and physiological characteristics and it was thus concluded that this strain belonged to the species *N. autotrophica*.

Strain SANK 62881 differed from *N. autotrophica* in its decomposition of urea, resistance to lysozyme, acid production from carbohydrates and utilization of carbohydrates. However, these differences are not sufficient to classify strain SANK 62881 as a new species. Therefore, it is regarded as a new sub-

Table 2. Physiological properties of strains SANK 62781, SANK 62881 and SANK 62981.

Determination	SANK 62781	SANK 62881	SANK 62981
Acid fastness:	−	−	−
Decomposition of:			
Adenine	−	−	−
Guanine	−	−	−
Casein	+	+	+
Xanthine	−	−	−
Hypoxanthine	+	+	+
Tyrosine	+	+	+
Urea	+	−	+
Hydrolysis of:			
Starch	−	−	−
Liquefaction of:			
Gelatin	+	+	+
Tolerance to:			
Lysozyme	−	+	−
MIC to:			
Chloramphenicol	50*	>100	>100
Tetracycline	>100	>100	>100
Streptomycin	3.1	6.2	6.2
Kanamycin	6.2	3.1	>100
Gentamicin	0.8	0.8	50
Erythromycin	25	>100	>100
Benzylpenicillin	25	>100	>100
Carbenicillin	>100	>100	>100
Ampicillin	50	>100	>100
Cephaloridin	50	>100	>100
Novobiocin	>100	>100	>100
Rifampicin	50	>100	>100
Nystatin	>100	>100	>100
Cycloheximide	>100	>100	>100
Amphotericin B	>100	>100	>100
Acid from:			
Acetate	−	−	−
Succinate	−	−	−
Citrate	−	−	−
Benzoate	NG**	NG	NG
Lactate	−	−	−
Pyruvate	−	−	−
Tartarate	−	−	NG
Glucose	+	+	+
Arabinose	+	−	+
Xylose	+	−	+
Inositol	+	+	+
Mannitol	+	+	+
Fructose	+	+	+
Rhamnose	+	−	+
Sucrose	+	±	NG
Raffinose	+	−	NG
Lactose	−	−	+
Maltose	+	−	+
Galactose	+	±	±

Determination	SANK 62781	SANK 62881	SANK 62981
Utilization of:			
Glucose	+	+	+
Arabinose	+	−	+
Xylose	+	−	+
Inositol	+	+	+
Mannitol	+	+	+
Fructose	+	+	+
Rhamnose	+	−	±
Sucrose	+	−	−
Raffinose	+	−	−
Glycerol	+	+	+
Trehalose	+	+	+
Dulcitol	+	−	−
Inulin	+	−	−
Salicin	+	−	−
Cellobiose	+	−	+
Melibiose	+	+	+
Cellulose	+	±	±
Acetate	+	+	+
Succinate	+	+	+
Citrate	+	+	+
Malate	+	+	+
Benzoate	−	−	−
Lactate	+	+	−
Pyruvate	+	+	+
Tartarate	+	+	−
DNase activity:	+	+	+
Production of:			
Nitrate reductase	−	±	+
Growth at/in/on:			
Anaerobic	−	−	−
1% NaCl	+	+	+
2% NaCl	+	+	+
3% NaCl	+	+	+
5% NaCl	+	+	+
7% NaCl	±	+	+
10% NaCl	−	±	−
50°C	−	−	−
45°C	−	−	−
37°C	+	+	+
28°C	+	+	+
20°C	+	+	+
10°C	+	+	±
4°C	−	−	−
Survival:			
50°C/8 hours	−	+	+
Action on milk:			
Peptonization	−	−	+
Coagulation	−	−	+
Melanoid pigment formation on:			
ISP 1	−	−	−
ISP 6	−	−	−
ISP 7	−	−	−

* μg/ml, ** NG: no growth.

species of *N. autotrophica* and named as *N. autotrophica* subsp. *canberrica* subsp. nov. The type strain is SANK 62881 (=ATCC 35203).

Strain SANK 62981 differed from *N. autotrophica* in its growth color, utilization of sucrose and susceptibility to antibiotics, as shown in Table 4. These differences were also not sufficient to classify this strain as a new species and it was therefore regarded as a subspecies of *N. autotrophica*, named as *N. autotrophica* subsp. *amethystina* subsp. nov. The type strain is SANK 62981 (=ATCC 35204).

Table 3. Chemical components of strains SANK 62781, SANK 62881 and SANK 62981.

	SANK 62781	SANK 62881	SANK 62981
Cell wall type	IV	IV	IV
Whole cell sugar pattern	A	A	A
Mycolic acid	—	—	—
Acyl type in peptidoglycan	Acetyl	Acetyl	Acetyl
Phospholipid type	PII	PII	PII

Table 4. Major differences of taxonomical properties between strains SANK 62781, SANK 62881 and SANK 62981.

	SANK 62781	SANK 62881	SANK 62981	*N. autotrophica* IFO 12743
Aerial mycelium color:	White to pale yellow	White to pale yellowish orange	White to brownish gray	White to pale yellow
Growth color:	Yellowish gray to pale yellowish brown	Grayish yellow brown	Brown purple	Pale yellow to yellowish gray
Decomposition of:				
Urea	+	−	+	+
Tolerance to:				
Lysozyme	−	+	−	−
Acid from:				
Arabinose	+	−	+	+
Xylose	+	−	+	+
Raffinose	+	−	NG*	−
Utilization of:				
Arabinose	+	−	+	+
Xylose	+	−	+	+
Rhamnose	+	−	+	+
Sucrose	+	−	−	+
MIC to:				
Kanamycin	6.2**	3.1	>100	1.5
Gentamicin	0.8	<0.8	50	<0.8

* NG: no growth, **: μg/ml.

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