A NEW BROAD-SPECTRUM AMINOGLYCOSIDE ANTIBIOTIC COMPLEX, SPORARICIN

II. TAXONOMIC STUDIES ON THE SPORARICIN PRODUCING STRAIN *SACCHAROPOLYSPORA HIRSUTA* SUBSP. *KOBENSIS* NOV. SUBSP.

AKIO IWASAKI, HISAKATSU ITOH and TOSHIHITO MORI

Tokyo Research Laboratories, Kowa Co., Ltd., Higashimurayama, Tokyo 189, Japan

(Received for publication December 28, 1978)

Morphological, cultural and physiological characteristics of a new nocardioform actinomycete are reported. The microorganism which produces the antibiotic complex sporaricin[1] has been selectively isolated from a sample of soil obtained from Kobe City, Hyogo Prefecture, Japan. By whole-cell analysis of the actinomycete, *meso*-diaminopimelic acid, arabinose and galactose were identified. But lipid LCN-A (lipid characteristic of *Nocardia*) and nocardomycolic acid were not detected. The taxonomic characteristics of this strain is closely related to the genus of *Saccharopolyspora*, described by LACEY and GOODFELLOW[2]. Based on the taxonomic comparison with *Saccharopolyspora hirsuta* ATCC 27875, the strain was considered to be a subspecies of *Saccharopolyspora hirsuta*. Therefore, the proposed subspecies is named *Saccharopolyspora hirsuta* subsp. *kobensis*.

An organism which produces sporaricin, a new aminoglycoside antibiotic complex, was isolated by selective isolation procedure from soil sample obtained from Kobe City, Hyogo Prefecture, Japan. The isolation and characterization of the antibiotic complex was reported in our previous paper[1].

In this paper, morphological, cultural and physiological characteristics of strain KC-6606 will be described and the name, *Saccharopolyspora hirsuta* subsp. *kobensis* will be proposed.

Taxonomic Studies

The isolation of strain KC-6606 from the soil sample was accomplished by plating soil suspension onto the following medium: arginine 1 g, casein 2 g, starch 10 g, K_2HPO_4 1 g, NaCl 50 g, $MgSO_4 \cdot 7H_2O$ 0.5 g, agar 12 g, distilled water 1,000 ml pH 7.0. Inoculated plates were incubated at 37°C for 7 days.

Stock slant cultures of strain KC-6606 and the reference strain, *Saccharopolyspora hirsuta* ATCC 27875, were maintained on yeast extract-malt extract agar. These slants were incubated at 37°C for 14 days.

The methods described by SHIRLING and GOTTLIEB[3] were employed for this taxonomic study in principle except for certain changes in procedures and additional tests.

Morphological observations were made on cultures which had been grown at 37°C for 14 days and 21 days on yeast extract-malt extract agar and inorganic salts-starch agar with a light microscope and an electron microscope.

Strain KC-6606 was a Gram-positive and non-acid fast actinomycete which produced both substrate hyphae and aerial hyphae on most of the media used in this study. The substrate hyphae (0.5~0.7 μ in diameter) spread out to form long branches and tangled complicatedly with each other (Plate 1). Occasionally typical nocardioform fragmentation was observed in the colony on most

Plate 1. Substrate hyphae of strain KC-6606 on inorganic salts-starch agar incubated at 37°C for 14 days. A mark equals 20 μ.

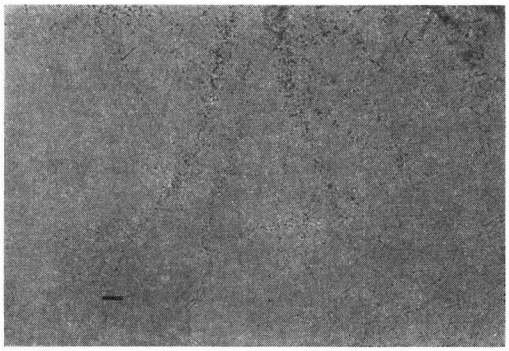


Plate 2. Substrate hyphae of strain KC-6606 on yeast extract-malt extract agar incubated at 37°C for 21 days. A mark equals 20 μ.

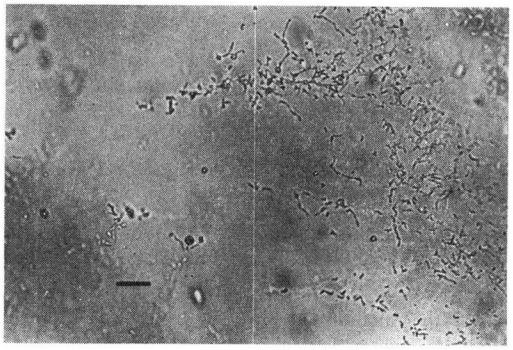


Plate 3. Aerial hyphae of strain KC-6606 on inorganic salts-starch agar incubated at 37°C for 21 days. A mark equals 10 μ.

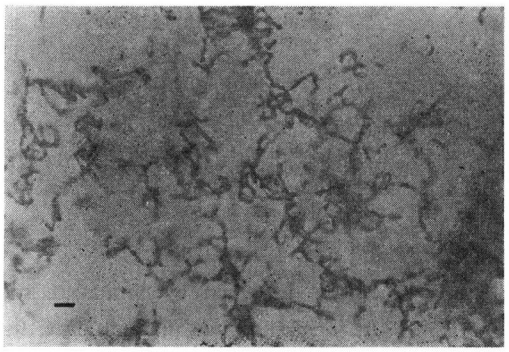


Plate 4. Aerial hyphae of strain KC-6606 on inorganic salts-starch agar incubated at 37°C for 14 days. A mark equals 20 μ.

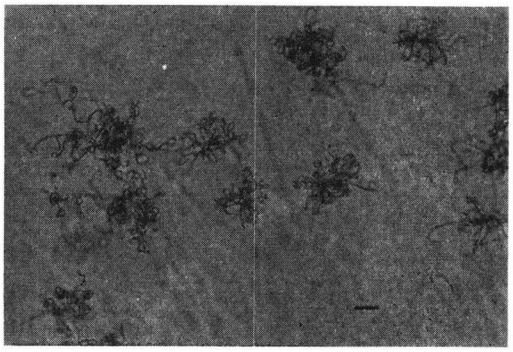


Plate 5. Electron micrograph of spores of strain KC-6606 on inorganic salts-starch agar incubated at 37°C for 14 days. A mark equals 0.3 μ.

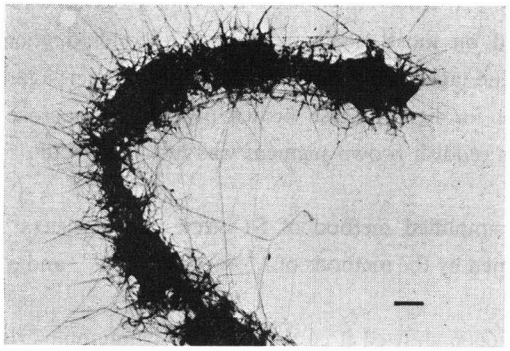
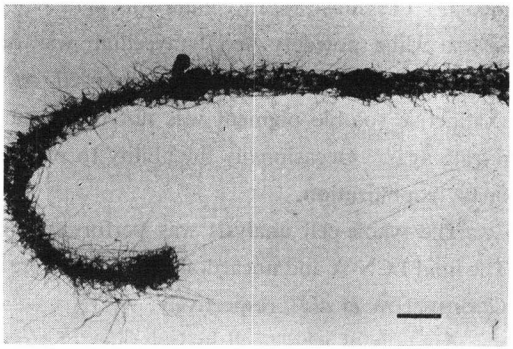


Plate 6. Electron micrograph of 'empty' hairy sheath of strain KC-6606 on inorganic salts-starch agar incubated at 37°C for 14 days. A mark equals 1 μ.



media after 21 days (Plate 2). The aerial hyphae (0.6~0.8 μ in diameter) was short and segmented into bead-like chains of spores which often be connected by 'empty' hyphae (Plate 3). The chain of mature spores consisted of 20 or more spores and formed loops or spirals (Plate 4). The spores were oval to short cylindrical, $0.4 \sim 0.6 \times 0.7 \sim 1.3$ μ in size by electron microscopic observation. Spore surfaces

Table 1. Cultural characteristics of strain KC-6606.

Medium	Growth	Substrate mycelium	Aerial mycelium	Soluble pigment
Sucrose-nitrate agar	good	butyrous-gelatinous buff (2fb)	poor, white (a) powdery	reddish brown
Glucose-asparagine agar	moderate	butyrous-gelatinous pearl pink (3ca)-light melon yellow (3ea)	poor, white (a) powdery	faint yellow
Glycerol-asparagine agar (ISP No. 5 medium)	good	gelatinous light ivory (2ca)	poor, white (a) powdery	faint yellow
Inorganic salts-starch agar (ISP No. 4 medium)	good	cartilaginous light ivory (2ca)-buff (2fb)	poor, white (a) powdery	none
Tyrosine agar (ISP No. 7 medium)	good	butyrous-gelatinous buff (2fb)	good, white (a) powdery	light yellow
Nutrient agar	moderate	butyrous-gelatinous buff (2fb)	poor, white (a) powdery	none
Yeast extract-malt extract agar (ISP No. 2 medium)	abundant	gelatinous bamboo (2gc)	moderate, white (a) powdery	faint yellow
Oat meal agar (ISP No. 3 medium)	poor	almost colorless	poor, white (a) powdery	none
Peptone-yeast extract-iron agar (ISP No. 6 medium)	moderate	butyrous-gelatinous light amber (2ic)	poor, white (a) powdery	faint yellow
BENNETT's agar	good	gelatinous buff (2fb)	poor, white (a) powdery	none

were covered with a sheath carrying tufts of long straight or curved hairs (Plates 5, 6).

Cultural characteristics were observed on nine kinds of media described by SHIRLING and GOTTLIEB[3] and by WAKSMAN[4], as well as on BENNETT's agar. The incubation was made at 37°C for 14 days. The color names used in this study were based on Color Harmony Manual (Containers Corporation of America 1958)[5].

As shown in Table 1, colonies were thin, raised or convex and usually slightly wrinkled on most media. The substrate mycelium was light ivory to buff in color and usually butyrous or gelatinous. Sparse white powdery aerial mycelium was observed on most media, whereas moderate to good aerial mycelium formation was observed on tyrosine agar and yeast extract-malt extract agar. Distinctive soluble pigment was not produced except for light reddish brown pigment on sucrose-nitrate agar. Occasionally the ability to produce this reddish brown pigment was lost by subculture or by lyophilization.

The whole-cell analysis was performed by the simplified method of STANECK and ROBERTS[6]. The lipid LCN-A and nocardomycolic acid were examined by the methods of MORDARSKA *et al.*[7] and of GOODFELLOW *et al.*[8], respectively.

Analysis of whole-cell hydrolysates of strain KC-6606 showed it contained *meso*-diaminopimeric acid, and arabinose and galactose as sugars. Accordingly the cell wall of this strain is believed to be of type IV[9]. Analysis of the lipids in whole-cell showed that it did not contain the lipid LCN-A and nocardomycolic acid.

Based on the above taxonomical studies, strain KC-6606 was compared with known genera and species of actinomycetes having cell wall type IV described in BERGEY's Manual of Determinative

Bacteriology (8th edition)[10]. There were eight genera having cell wall type IV (or IV + glycine) in the above BERGEY's edition, namely *Corynebacterium* (*diphteriae*), *Mycobacterium, Bacterionema, Frankia, Nocardia, Thermomonospora* (*Saccharomonospora*), *Pseudonocardia* and *Micropolyspora*. Strain KC-6606 differed from these eight genera in characteristics described below. *Corynebacterium, Mycobacterium* and *Bacterionema* do not form true mycelium. *Frankia* does not produce aerial mycelium and does not grow on artificial media. *Nocardia* does not produce aerial spores generally and contains lipid LCN-A and nocardomycolic acid in the cells. *Thermomonospora* does not form chains of spores on aerial mycelium. *Pseudonocardia* has distinctive morphological features of spore chains and spores especially the large size (*ca.* 2.5 μ long) of the spores. On the other hand, *Micropolyspora* exhibits relatively many common features with strain KC-6606 but differs from the latter in respect to short chain of spores having smooth to short spiny surfaces and prominently thickened end walls.

In 1975, LACEY and GOODFELLOW reported a new genus, *Saccharopolyspora*[2] which was isolated from sugar cane baggase. Taxonomic characteristics of this genus had a close relation to those of strain KC-6606 in morphology, cultural characteristics and fine structure. Therefore, strain KC-6606 was compared with *Saccharopolyspora hirsuta* ATCC 27875.

Physiological properties of strain KC-6606 and *Saccharopolyspora hirsuta* ATCC 27875 were as follows: The range of growth temperatre and optimum growth temperature were determined on yeast extract-malt extract agar using a temperature gradient incubator (Toyo Kagaku Sangyo Co., Ltd.). Gelatin liquefaction was examined at 20°C and 27°C for 14 days on a medium composed of gelatin 200 g, glucose 20 g, peptone 5 g and distilled water 1,000 ml. The medium was placed in an ice-box after incubation to detect liquefaction. Starch hydrolysis was observed by the starch-iodine reaction after incubation on inorganic salts starch agar plate at 37°C for 14 days. Milk peptonization and coagulation were observed in skim-milk medium at 37°C for 14 days. Melanoid pigment production was observed on tyrosine agar and peptone-yeast extract-iron agar, as well as in tryptone-yeast extract broth. Nitrate reduction was examined in a medium composed of peptone 10 g, NaCl 5 g, $NaNO_3$ 10 g and distilled water 1,000 ml. Formation of nitrite was detected with reagents consisted of 0.8% sulfanilic acid in 5 N acetic acid and 0.5% α-naphthylamine in the same solvent. Resistance to lysozyme was examined by the method of GORDON[11]. Salt tolerance was tested on yeast extract-malt extract agar containing NaCl, constituted 0%, 3%, 6%, 9%, 12% and 15%, respectively.

As shown in Table 2, growth of the strains were observed over a wide range of temperature from

Table 2. Physiological properties of strain KC-6606 and *Saccharopolyspora hirsuta* ATCC 27875.

Physiological properties	KC-6606	ATCC 27875
Temperature requirement	Growth from 18°~45°C	Growth from 25°~50°C
Optimum temperature	37°~42°C	37°~40°C
Gelatin liquefaction	Very slow at 20°C, positive at 27°C	No liquefaction at 20°C, positive at 27°C
Starch hydrolysis	Positive	Positive
Action on milk	No coagulation, positive for peptonization	No coagulation, positive for peptonization
Melanoid pigment production	Negative	Negative
Nitrate reduction	Positive	Negative
Resistance to lysozyme	Sensitive	Sensitive
NaCl tolerance	>12%, but <15%	≦15%

about 20° to 50°C with the optimum temperature about 40°C when yeast extract-malt extract agar was employed. Gelatin liquefaction, starch hydrolysis and milk peptonization were positive, whereas melanoid pigment production and milk coagulation were negative in both strains. Nitrate reduction was positive in strain KC-6606 but negative in *Saccharopolyspora hirsuta* ATCC 27875. Both strains were sensitive to lysozyme and showed higher tolerance to NaCl.

Utilization of carbon sources was examined according to the method of PRIDHAM and GOTTLIEB[12]. The results of growth on 21 carbon sources were recorded after 21 days of incubation at 37°C.

As shown in Table 3, almost all carbon sources were utilized by both strains except that strain KC-6606 did not utilize L-arabinose, D-xylose, L-rhamnose, D-sorbitol, dulcitol and inositol, whereas *Saccharopolyspora hirsuta* ATCC 27875 lacked ability to utilize only two carbon sources, namely L-arabinose and dulcitol.

Degradation tests were examined by using the methods of GORDON[13], GOODFELLOW[14] and LACEY and GOODFELLOW[2]. The substrates used in this study were adenine, aesculin, casein, elastin, hypoxanthine, keratin, L-tyrosine, urea, xanthine and xylan. The test was run at 37°C for 14 days.

Except for xylan, all substrates were degraded by both strains. Degradation of aesculin by strain KC-6606 was weaker than by *Saccharopolyspora hirsuta* ATCC 27875.

Antibiotic resistance tests against eight aminoglycoside antibiotics were conducted on yeast extract-malt extract agar. Agar plates containing two fold dilutions of each antibiotics were prepared and mycelium suspensions were subsequently inoculated on the plates. The plates were incubated at 37°C for 7 days. Antibiotics used were as follows: gentamicin, streptomycin, neomycin, tobramycin, paromomycin, kanamycin, lividomycin and sporaricin A. As shown in Table 5, strain KC-6606 and *Saccharopolyspora hirsuta* ATCC 27875 showed remarkable resistance to the aminoglycoside antibiotics tested, except lividomycin. Strain KC-6606 was slightly susceptible

Table 3. Carbon source utilization of strain KC-6606 and *Saccharopolyspora hirsuta* ATCC 27875.

Carbon sources	KC-6606	ATCC 27875
L-Arabinose	−	−
D-Xylose	−	+
D-Ribose	+	+
D-Mannose	+	+
L-Rhamnose	−	+
D-Galactose	+	+
D-Glucose	+	+
D-Fructose	+	+
Maltose	+	+
Cellobiose	+	+
Trehalose	+	+
Sucrose	+	+
Raffinose	+	+
Inulin	+	+
D-Sorbitol	−	+
Dulcitol	−	−
Inositol	−	+
D-Mannitol	+	+
Sodium acetate	+	+
Sodium citrate	+	+
Sodium succinate	+	+

(+: utilized, −: not utilized)

Table 4. Abilities of strain KC-6606 and *Saccharopolyspora hirsuta* ATCC 27875 to degrade different substrates.

Substrates	KC-6606	ATCC 27875
Adenine	+	±
Aesculin	±	+
Casein	+	+
Elastin	+	+
Hypoxanthine	+	+
Keratin	+	+
L-Tyrosine	+	+
Urea	+	+
Xanthine	+	+
Xylan	−	−

(+: strongly degraded, ±: weakly degraded, −: not degraded)

to the antibiotics than *Saccharopolyspora hirsuta* ATCC 27875.

Summarizing the above results, strain KC-6606 and *Saccharopolyspora hirsuta* ATCC 27875 correspond in common with each other in basal properties such as morphology, cultural characteristics, cell wall composition, almost all physiological properties including hydrolytic activities on various organic compounds and susceptibility to antibiotics, although they differ in nitrate reduction and in the utilization of D-xylose, L-rhamnose, D-sorbitol and inositol, as shown in Tables 2 and 3. Accordingly, it would be reasonable to consider that these strains belong to the same species and can be a new subspecies designated as *Saccharopolyspora hirsuta* subsp. *kobensis* IWASAKI *et* MORI subsp. nov. The strain KC-6606 was deposited as type at the American Type Culture Collection, Rockville, Md., and was assigned ATCC 20501. The strain was also deposited as FERM-P No. 3912 at Fermentation Research Institute, Chiba, Japan.

Table 5. Susceptibility of strain KC-6606 and *Saccharopolyspora hirsuta* ATCC 27875 to aminoglycoside antibiotics.

Aminoglycoside antibiotics	MIC in mcg/ml	
	KC-6606	ATCC 27875
Gentamicin	50	>100
Streptomycin	50	100
Neomycin	100	>100
Tobramycin	>100	>100
Paromomycin	50	100
Kanamycin	>100	>100
Lividomycin	12	50
Sporaricin A	>100	>100

Acknowledgement

The authors wish to express their thanks to Drs. H. UMEZAWA, Y. OKAMI and M. HAMADA of the Institute of Microbial Chemistry for their helpful advice and to Dr. A. SEINO of Kaken Chemical Company Limited for making *Saccharopolyspora hirsuta* ATCC 27875 available to them.

We are indebted to Miss K. UCHINO for technical assistance with some of the experiments.

References

1) DEUSHI, T.; A. IWASAKI, K. KAMIYA, T. KUNIEDA, T. MIZOGUCHI, M. NAKAYAMA, H. ITOH, T. MORI & T. ODA: A new broad-spectrum aminoglycoside antibiotic complex, sporaricin. I. Fermentation, isolation and characterization. J. Antibiotics 32: 173~179, 1979
2) LACEY, J. & M. GOODFELLOW: A novel actinomycete from sugar-cane bagasse: *Saccharopolyspora hirsuta* gen. *et* sp. nov. J. Gen. Microbiol. 88: 75~85, 1975
3) SHIRLING, E. B. & D. GOTTLIEB: Methods for characterization of *Streptomyces* species. Internat. J. Syst. Bacteriol. 16: 313~340, 1966
4) WAKSMAN, S. A.: The actinomycetes. Vol. II. Classification, identification and description of genera and species. The Williams and Wilkins Co., Baltimore, 1961
5) Color Harmony Manual, 4th Edition: Color Standards Department, Containers Corporation of America, 38 South Dearborn Street, Chicago 3, Illinois, 1958
6) STANECK, J. L. & G. D. ROBERTS: Simplified approach to identification of aerobic actinomycetes by thin-layer chromatography. Appl. Microbiol. 28: 226~231, 1974
7) MORDARSKA, H.; M. MORDARSKI & M. GOODFELLOW: Chemotaxonomic characters and classification of some nocardioform bacteria. J. Gen. Microbiol. 71: 77~86, 1972
8) GOODFELLOW, M.; D. E. MINNIKIN & P. V. PATEL: Free nocardomycolic acid in the classification of Nocardias and strains of the 'rhodochrous' complex. J. Gen. Microbiol. 74: 185~188, 1973
9) BECKER, B.; M. P. LECHEVALIER & H. A. LECHEVALIER: Chemical composition of cell-wall preparations from strains of various form-genera of aerobic actinomycetes. Appl. Microbiol. 13: 236~243, 1965
10) BUCHANAN, R. E. & N. E. GIBBONS: BERGEY'S Manual of Determinative Bacteriology. 8th edition, The Williams and Wilkins Co., Baltimore, 1974
11) GORDON, R. E.: Some criteria for the recognition of *Nocardia madurae* (VINCENT) BLANCHARD. J. Gen. Microbiol. 45: 355~364, 1966

12) PRIDHAM, T. G. & D. GOTTLIEB: The utilization of carbon compounds by some *Actinomycetales* as an aid for species determination. J. Bacteriol. 56: 107~114, 1948
13) GORDON, R. E.: The taxonomy of soil bacteria. Ecology of soil bacteria. pp. 293~321, edited by T. R. G. GRAY & D. PARKINSON, Liverpool University Press, Liverpool, 1967
14) GOODFELLOW, M.: Numerical taxonomy of some nocardioform bacteria. J. Gen. Microbiol. 69: 33~80, 1971