FLUVIRUCINS A_1, A_2, B_1, B_2, B_3, B_4 AND B_5, NEW ANTIBIOTICS ACTIVE AGAINST INFLUENZA A VIRUS

IV. TAXONOMY ON THE PRODUCING ORGANISMS

KOJI TOMITA, NAHOMI ODA, YUTAKA HOSHINO, NORIYUKI OHKUSA and HIROTAKA CHIKAZAWA†

Bristol-Myers Squibb Research Institute, 2-9-3 Shimo-meguro, Meguro-ku, Tokyo 153, Japan

(Received for publication March 27, 1991)

The morphology, chemotaxonomy, and cultural and physiological characteristics were examined on the five strains of actinomycetes which produce antiviral antibiotics, fluvirucin congeners. All strains have *meso*-2,6-diaminopimelic acid in the cell wall. Four strains, Q464-31, L407-5, R359-5 and R516-16, belong to the maduromycetes since they have madurose in the whole cell. The remaining one strain, R869-90, has rhamnose but no madurose, and is a nocardioform actinomycete. These five strains were classified and designated as follows:
Strain Q464-31 (fluvirucin A_1 producer): *Microtetraspora tyrrhenii* sp. nov. (*Actinomadura pusilla* group).
Strain L407-5 (fluvirucin B_2 producer): A maduromycete.
Strain R359-5 (fluvirucin B_1 producer): *Microtetraspora pusilla* (*Actinomadura pusilla* group).
Strain R869-90 (fluvirucin A_2 producer): *Saccharothrix mutabilis*.
Strain R516-16 (fluvirucins B_2, B_3, B_4 and B_5 producer): A maduromycete.

In the course of searching for new antiviral antibiotics from the fermentation broth of soil microorganisms, five unusual actinomycetes were found to produce a family of antibiotics consisted of seven components, designated fluvirucins A_1, A_2, B_1, B_2, B_3, B_4 and B_5. The production, biological and chemical properties and structure of fluvirucins were described in the preceding papers[1~3].

This paper describes the characterization and taxonomic position of these five unusual actinomycete strains.

Materials and Methods

The cultural and physiological characteristics of the five strains were examined by the methods of SHIRLING and GOTTLIEB[4], and GORDON *et al.*[5]. Diagnostic components of amino acid and sugar in the whole cell hydrolysate were analyzed by the methods of LECHEVALIER[6]. The phospholipids were identified by the methods of LECHEVALIER *et al.*[7]. The menaquinone samples were prepared by the procedures of COLLINS *et al.*[8] and analyzed with a mass spectrometer. The glycolate test and the detection of mycolate were carried out by the methods of UCHIDA and AIDA[9], and MINNIKIN *et al.*[10], respectively. The composition of methyl esterified cellular fatty acids was analyzed by GC with SPB-1 fused silica capillary column (0.25 mm × 30 m), and determined by GC-MS.

Results

The morphology, cell chemistry, fatty acid composition, and cultural and physiological characteristics

† Preclinical Research Laboratories, 1 Futagoyama, Sakazaki, Kohda-cho, Nukata-gun, Aichi Prefecture 600, Japan.

of the five strains are shown in Tables 1, 2, 3, 4 and 5, respectively.

The supplemental characteristics and their taxonomic position are described below.

Table 1. Morphology of five strains of actinomycetes.

	Strain Q464-31	Strain L407-5	Strain R359-5	Strain R869-90	Strain R516-16
Aerial mycelium:					
Formation	Moderate (ISP No. 3)	None	Abundant (ISP No. 2)	None or sparse (ISP Nos. 2, 5, 7)	None
Spore chain	Short; hook or spiral (5~15 spores)	None	Short; hook or spiral (10~30 spores)	Long straight chains of spores of various lengths	None
Spores	Oval (1.2×1.6~2.5 μm); surface with vertical ridges	None	Oval (0.8×1.0×2.0 μm); smooth surface	Rod (0.6×0.8~3.0 μm); smooth surface	None
Special morphology	None	None	Pseudosporangium (coiled spore chain with pseudomembrane)	Fascicled thick hyphae, and sclerotic granules	None
Substrate mycelium:					
Fragmentation	None	None	None	Occurred (various lengths)	None
Special morphology	None	Dichotomously and repeatedly branched curled hyphae	None	None	Dichotomously and repeatedly branched curled hyphae

Table 2. Cell chemistry of five strains of actinomycetes.

	Strain Q464-31	Strain L407-5	Strain R359-5	Strain R869-90	Strain R516-16
Whole cell hydrolysate:					
Diaminopimelic acid	*meso*	*meso*	*meso*	*meso*	*meso*
Sugar	Glucose, madurose	Galactose, glucose, mannose, madurose, ribose	Glucose, mannose, madurose, ribose	Galactose, glucose, mannose, ribose, rhamnose	Glucose, mannose madurose, ribose
Cell wall type	III_B	III_B	III_B	III_C	III_B
Phospholipids[a]	PIM, GluNU, PI, PE, OPE, DPG	PIM, GluNU, PI, PE, DPG	PIM, GluNU, PI, PE, OPE, DPG	PI, GluNU, PE, OPE	PI, PG, GluNU, DPG
Type	P-IV	P-IV	P-IV	P-IV	P-V (tentative)
Major menaquinone	MK-9 (H_4)	MK-9 (H_4)	MK-9 (H_4)	MK-9 (H_4)	MK-9 (H_4)
Mycolate	Absent	Absent	Absent	Absent	Absent
Fatty acid pattern[b]	3c	3c	3c	3f	3c
Glycolate test	Negative	Negative	Negative	Negative	Negative

[a] Abbreviations: PIM, Phosphatidylinositol mannoside; GluNU, unknown glucosamine-containing phospholipids; PI, phosphatidylinositol; PE, phosphatidylethanolamine; OPE, hydroxylated phosphatidylethanolamine; DGP, diphosphatidylglycerol; PG, phosphatidylglycerol.

[b] According to the classification of GRUND and KROPPENSTEDT[16].

Table 3. Fatty acid composition of five strains of actinomycetes.

Strain No.	Fatty acid composition (%)															
	Straight chain			Branched chain				Unsaturated					10-Methyl branched chain			Hydroxylated fatty acids
	15:0	16:0	17:0	*i*-15	*i*-16	*a*-15	*a*-17	*i*-16:1	*cis*9 16:1	*cis*9 17:1	*i*-18:1	*cis*9 18:1	10Me 16:0	10Me 17:0	10Me 18:0	
Q464-31	4	6	6	2	19			8	4	7	3	2	3	16	3	8
L407-5	4	3	8	2	10			8	5	11	3	3	1	17	5	10
R359-5	3	2	2	2	23			19	3	7	4	2	3	11	2	10
R869-90		5		10	9	12	10		15			1	11	2		16
R516-16	3	4	5	2	17			11	5	6	5	2	2	11	4	12

Abbreviations (examples): Straight chain saturated, 15:0, pentadecanoic acid; *iso*-branched chain, *i*-16, 14-methylpentadecanoic acid; *anteiso*-branched chain, *a*-17, 14-methylhexadecanoic acid; monounsaturated straight chain, *cis*9-18:1, 9-octadecenoic acid (oleic acid); 10-methylbranched chain, 10Me 18:0, 10-methyloctadecanoic acid (tuberculostearic acid); hydroxylated fatty acids, 2-hydroxy-14-methylpentadecanoic acid, 2-hydroxy-hexadecenoic acid, *etc.*

Table 4. Cultural characteristics of five strains of actinomycetes.

		Strain Q464-31	Strain L407-5	Strain R359-5	Strain R869-90	Strain R516-16
Yeast extract-malt extract agar (ISP No. 2)	G:	Good; deep yellowish brown (75)	Good; deep brown (56)	Good; strong brown (55)	Good; moderate orange yellow (71)	Good; deep brown (56)
	A:	Poor; white	None	Abundant; yellowish white (92)	Very scant; white	None
	D:	None	None	None	None	None
Oatmeal agar (ISP No. 3)	G:	Moderate; light yellow (86)	Good; light yellow (92)	Moderate; light brown (57)	Moderate; pale orange yellow (70)	Good; strong yellow (84)
	A:	Moderate; white	None	Moderate; yellowish white (92)	None	None
	D:	None	None	Pale yellow (89)	None	None
Inorganic salts-starch agar (ISP No. 4)	G:	Poor; light olive brown (94)	Poor; moderate yellow (87)	Poor; moderate yellow (87)	Moderate; light olive brown (94)	Poor; pale yellow (89)
	A:	Scant; white	None	None	None	None
	D:	None	None	None	None	None
Glycerol-asparagine agar (ISP No. 5)	G:	Moderate; dark yellow (88)	Poor; moderate yellow (87)	Scant; moderate yellow (87)	Moderate; deep yellow (88)	Poor; pale yellow (89)
	A:	None	None	None	Very scant; white	None
	D:	None	None	None	None	None
Peptone-yeast extract-iron agar (ISP No. 6)	G:	Moderate; deep yellowish brown (75)	Moderate; deep yellowish brown (75)	Moderate; light yellowish brown (76)	Good; colorless	Moderate; deep yellowish brown (75)
	A:	None	None	None	None	None
	D:	None	None	None	None	None
Tyrosine agar (ISP No. 7)	G:	Moderate; dark yellow (88)	Poor; grayish brown (61)	Scant; pale yellow (89)	Good; dark orange yellow (72)	Poor; grayish yellow (90)
	A:	None	None	None	Poor; yellowish white (92)	None
	D:	None	None	None	None	None

Observation after incubation at 28°C for 3 weeks.
Color name used: ISCC-NBS Color-name Charts.
Abbreviations: G, Growth and reverse color; A, aerial mycelium; D, diffusible pigment.

Table 5. Physiological characteristics of five strains of actinomycetes.

Strain	Q464-31	L407-5	R359-5	R869-90	R516-16
Decomposition of:					
Adenine	+	−	−	−	−
Casein	+	+	+	+	+
Hippuric acid	+	+	+	+	+
Hypoxanthine	+	+	+	+	+
Tyrosine	+	−	−	+	−
Xanthine	−	−	−	−	−
Decarboxylation of:					
Benzoate	−	−	−	−	−
Citrate	+	−	+	+	−
Mucate	−	−	−	−	−
Succinate	+	+	+	+	+
Tartrate	−	+	−	−	+w
Production of:					
Amylase	+	+	+	+	+
Esculinase	+	+	+	+	+
Gelatinase	−	+	+	+	+
Nitrate reductase[a]	−/+	−/+	+/+	+/−	+/+
Tyrosinase	−	−	−	−	−
Urease	+	+w	−	−	+w
Growth in/at:					
Lysozyme, 0.01%	−	−	−	+	−
NaCl, 3%	+	+	+	+	+
5%	−	−	−	+	−
Temperature, 43°C	−	+	−	+	+
46°C	−	+	−	−	−
Carbohydrate utilization:[b]					
Adonitol	+	+	+	+w	+
D-Arabinose	+	+	+	+	+
L-Arabinose	+	+	+	+	+
Cellobiose	+	+	+	+	+
Dulcitol	−	−	−	−	−
Erythritol	−	+	−	−	−
D-Fructose	+	+	+	+	+
D-Galactose	+	−	+	+	+
D-Glucose	+	+	+	+	+
Glycerol	+	+	+	+	+
Inositol	+	+	+	+	+
Lactose	+	+	+	+	+
D-Mannitol	+	+	+	+	+
D-Mannose	+	+	+	+	+
D-Melezitose	−	−	−	−	+w
Melibiose	+	+	+	+	+
Methyl-α-glucoside	+w	+	+	+w	+
Raffinose	+	+	−	+	+
L-Rhamnose	+	+	+	+	+
D-Ribose	+	−	+	+	+w
Salicin	+	−	+	+	+
D-Sorbitol	−	−	−	−	−
D-Sorbose	−	−	−	−	−
Sucrose	+	+	+	+	+
Trehalose	+	+	+	+	+
D-Xylose	+	+	+	+	+

[a] Sucrose-nitrate broth/peptone-nitrate broth.

[b] Basal medium: PRIDHAM-GOTTLIEB's inorganic salts medium (ISP No. 9).

+w: Weakly positive.

Strain Q464-31 (Fluvirucin A_1 Producer)

Hook or spiral spore-chains were formed monopodially on the tip of aerial hyphae. The spores were big and had vertical folds on the surface (Fig. 1). White aerial mycelium was formed on ISP media Nos. 2, 3 and 4. The colony on ISP medium No. 2 was rigid, convex or crateriform and brownish. Melanin and other distinct pigments were not produced. Growth occurred between 17°C and 40°C, or in NaCl at 3% or less.

The morphology and cell chemistry of strain Q464-31 indicated that the strain should be placed in the *Actinomadura pusilla* group[11] which was proposed to be separated from the genus *Actinomadura* and reclassified in the genus *Microtetraspora*[12]. Based on the direct or descriptive comparisons of the strain to the known species of the *A. pusilla* group[13,14], strain Q464-31 was differentiated from the known species by its spores with vertical ridges and some physiological characteristics including the decomposition of adenine, the presence of urease and the absence of gelatinase. Thus, strain Q464-31 is classified as a new species of the genus *Microtetraspora*. The proposed designation is *Microtetraspora tyrrhenii* sp. nov. (*tyrrhenii*, tyr-re'-ni-i, L. adj. referring to the Tyrrhenian Sea in Mediterranean Sea, which the soil source site of this organism faces). The type strain is No. Q464-31 (ATCC 53931), which is single isolate.

The source of strain Q464-31 was a soil sample collected in the river side of Tevere (Tiber) River, Rome, Italy.

Strain L407-5 (Fluvirucin B_2 Producer)

None of the aerial mycelium or spores was formed (Fig. 2). Formation of aerial mycelium was neither seen on the additional media such as starch-casein agar and soil extract agar and nor induced by the supplement of vitamin B complex to chemically defined ISP media or CZAPEK's sucrose-nitrate agar. The vegetative mycelium was non-fragmentary. The colony on ISP medium No. 2 was rigid, convex or crateriform and brownish. Melanin and other distinct pigments were not formed. Growth occurred between 20°C and 46°C or in NaCl at 4% or less.

The taxonomic position (genus) of strain L407-5 is unknown due to its non-sporulating property. Based on the chemotaxonomy of strain L407-5, the strain was designated tentatively as a maduromycete[15].

The source of strain L407-5 was a soil sample collected in Madhya Pradesh State, India.

Strain R359-5 (Fluvirucin B_1 Producer)

Hook or spiral short spore-chains were formed monopodially on the tip of aerial hyphae. Some coiled

Fig. 1. Strain Q464-31.

A chain of spores with vertical folds (cultivation on ISP medium No. 2 for 3 weeks).

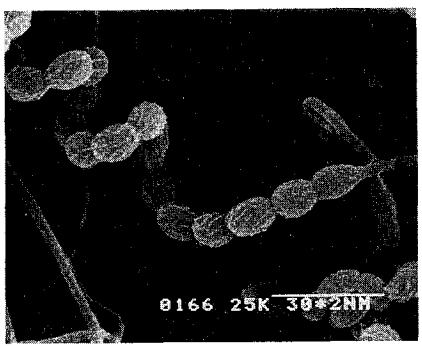


Fig. 2. Strain L407-5.

Non-sporogenic hyphae.

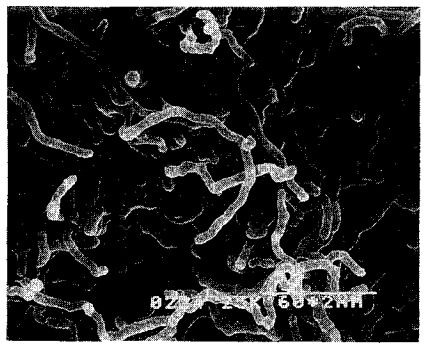
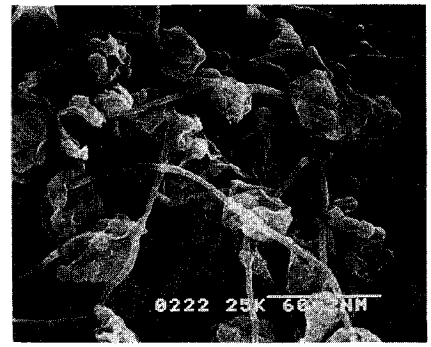


Fig. 3. Strain R359-5.

A chain of spores with smooth surface (cultivation on ISP medium No. 2 for 3 weeks).

Fig. 4. Strain R359-5.

Pseudosporangia on the aerial mycelium (cultivation on ISP medium No. 2 for 3 weeks).



spore-chains were observed as a pseudosporangium with pseudomembrane by scanning electron microscopy. The spores were oval and had a smooth surface (Figs. 3 and 4). Aerial mycelium was formed only on ISP media Nos. 2 and 3, and the color of mycelium was white or yellowish white. On chemically defined media the growth was scant, and aerial mycelium was not formed. The substrate mycelium was non-fragmentary. The colony on ISP medium No. 2 was rigid, convex or crateriform and brownish. Melanin and other distinct pigments were not formed. Growth occurred between 20°C and 41°C or in NaCl at 3% or less.

The morphology and cell chemistry of strain R359-5 indicated that the strain is placed in the *A. pusilla* group[11] which was proposed to be reclassified in the genus *Microtetraspora*[12]. Among the known species of the *A. pusilla* group, strain R359-5 shares many common characteristics with *A. pusilla*[13,14]. The two organisms form the short spiral spore-chain pseudosporangium, the spores with smooth surface, the yellowish white aerial mycelium, the brownish substrate mycelium, and none of diffusible pigments. They show a similar sugar utilization profile. Strain R359-5 is differentiated from *A. pusilla* in its predominant formation of spiral spore-chains, limited formation of pseudosporangium, growth in 3%-NaCl, and hydrolysis of casein and starch. Since the differences between the two organisms are limited, strain R359-5 was identified as *Microtetraspora pusilla* (Nonomura and Ohara) KROPPENSTEDT *et al.*[12].

The source of strain R359-5 was a soil sample collected in La Carlota, Negros Island, Philippines.

Strain R869-90 (Fluvirucin A_2 Producer)

Aerial mycelium was sparsely formed on ISP media Nos. 2, 5 and 7. Long and straight chains of segments were born in the total parts of the aerial mycelium. These segments consisted of rods of varying lengths and had a smooth surface. Mature spores occurred discontinuously in the segmented hyphae (Fig. 5). Fascicled thick hyphae and sclerotic granules were formed on the aerial mycelium. Zigzag hyphae were not observed. The substrate mycelium was fragmentary. The colony on ISP medium No. 2 was soft, smooth, drop-like and colorless to yellowish. Melanin and other pigments were not formed. Growth occurred between 17°C and 44°C or in NaCl at 6% or less.

The morphology, cell chemistry, and cultural and physiological characteristics of strain R869-90 indicated that the strain is placed in *Saccharothrix mutabilis*, (Shearer, Colman and Nash III) GRUND and KROPPENSTEDT[16], a nocardioform actinomycete[17,18], which was proposed to be reclassified from the genus *Nocardiopsis*[16].

The source of strain R869-90 was a soil sample collected in Maharashtra State, India.

Fig. 5. Strain R869-90.

Segmented aerial hyphae with mature spores (cultivation on ISP medium No. 7 for 3 weeks).

Fig. 6. Strain R516-16.

Non-sporogenic hyphae.

Strain R516-16 (Fluvirucins B_2, B_3, B_4, and B_5 Producer)

None of the aerial mycelium and spores was formed (Fig. 6). Formation of the aerial mycelium was neither seen in additional media such as starch-casein agar and soil extract agar, and nor induced by the supplement of vitamin B complex to chemically defined ISP media or CZAPEK's sucrose-nitrate agar. The vegetative mycelium was non-fragmentary. The colony on ISP medium No. 2 was rigid, convex or crateriform and brownish. Melanin and other distinct pigments were not formed. Growth occurred between 22°C and 43°C or in NaCl at 3% or less.

Like strain L407-5, strain R516-16 was a non-sporulating maduromycete. Strain R516-16 was similar to strain L407-5 in the cultural characteristics, but differed in the phospholipid pattern (its absence of phosphatidylethanolamine and the presence of phosphatidylglycerol). Physiologically, strain R516-16 was different from the latter in the absence of growth at 46°C, utilization of D-galactose, and lack of utilization of erythritol.

Thus, strain R516-16 was designated tentatively as a maduromycete[15], which has distinct diferences from another non-sporogenic maduromycete, strain L407-5. The source of strain R516-16 was a soil sample collected in Maharashtra State, India.

Discussion

The final taxonomic position of the two strains, L407-5 and R516-16, was not determined due to their absence of sporulation. The taxonomic relationships among the two-non-sporogenic strains, strain Q464-31 (*M. tyrrhenii*), strain R359-5 (*M. pusilla*) and the type strains of *M. pusilla* and related species are of interest, and will be followed up using additional chemotaxonomical and/or molecular genetic methods.

Acknowledgments

The authors are grateful to Dr. T. OKI, the director of their research institute, for his encouragement through this work, and to Dr. T. TSUNO and Miss M. BABA for the menaquinone analysis by mass spectrometry and the quantitative fatty acid analysis by gas chromatography.

References

1) NARUSE, N.; O. TENMYO, K. KAWANO, K. TOMITA, N. OHGUSA, T. MIYAKI, M. KONISHI & T. OKI: Fluvirucins

A_1, A_2, B_1, B_2, B_3, B_4 and B_5, new antibiotics active against influenza A virus. I. Production, isolation, chemical properties and biological activities. J. Antibiotics 44: 733~740, 1991

2) NARUSE, N.; T. TSUNO, Y. SAWADA, M. KONISHI & T. OKI: Fluvirucins A_1, A_2, B_1, B_2, B_3, B_4 and B_5, new antibiotics active against influenza A virus. II. Structure determination. J. Antibiotics 44: 741~755, 1991
3) NARUSE, N.; M. KONISHI, T. OKI, Y. INOUYE & H. KAKISAWA: Fluvirucins A_1, A_2, B_1, B_2, B_3, B_4 and B_5, new antibiotics active against influenza A virus. III. The stereochemistry and absolute configuration of fluvirucin A_1. J. Antibiotics 44: 756~761, 1991
4) SHIRLING, E. B. & D. GOTTLIEB: Methods for characterization of *Streptomyces* species. Int. J. Syst. Bacteriol. 16: 313~340, 1966
5) GORDON, R. E.; S. K. MISHRA & D. A. BARNETT: Some bits and pieces of the genus *Nocardia*: *N. carnea*, *N. vaccinii*, *N. transvalensis*, *N. orientalis* and *N. aerocolonigenes*. J. Gen. Miocrobiol. 109: 69~78, 1978
6) LECHEVALIER, M. P.: Identification of aerobic actinomycetes of clinical importance. J. Lab. Clin. Med. 71: 934~944, 1968
7) LECHEVALIER, M. P.; C. D. BIEVRE & H. LECHEVALIER: Chemotaxonomy of aerobic actinomycetes: Phospholipid composition. Biochem. Syst. Ecol. 5: 249~260, 1977
8) COLLINS, M. D.; T. PIROUZ, M. GOODFELLOW & D. E. MINNIKIN: Distribution of menaquinones in actinomycetes and corynebacteria. J. Gen. Microbiol. 100: 221~230, 1977
9) UCHIDA, K. & K. AIDA: Taxonomic significance of cell-wall acyl type in *Corynebacterium-Mycobacterium-Nocardia* group by a glycolate test. J. Gen. Appl. Microbiol. 25: 169~183, 1979
10) MINNIKIN, D. E.; L. ALSHAMAONY & M. GOODFELLOW: Differentiation of *Mycobacterium*, *Nocardia*, and related taxa by thin-layer chromatographic analysis of whole-organism methanolysates. J. Gen. Microbiol. 88: 200~204, 1975
11) GOODFELLOW, M.; E. STACKEBRANDT & R. M. KROPPENSTEDT: Chemotaxonomy and actinomycete systematics. *In* Biology of Actinomycetes '88. *Ed.*, Y. OKAMI *et al.*, pp. 233~238, Japan Scientific Societies Press, 1988
12) KROPPENSTEDT, R. M.; E. STACKEBRANDT & M. GOODFELLOW: Taxonomic revision of the actinomycete genera *Actinomadura* and *Microtetraspora*. System. Appl. Microbiol. 13: 148~160, 1990
13) ATHALYE, M.; M. GOODFELLOW, J. LACEY & R. P. WHITE: Numerical classification of *Actinomadura* and *Nocardiopsis*. Int. J. Syst. Bacteriol. 35: 86~98, 1985
14) MEYER, J.: *Genus Actinomadura* Lechevalier and Lechevalier 1970. *In* BERGEY's Manual of Systematic Bacteriology. Volume 4. *Ed.*, S. T. WILLIAMS *et al.*, pp. 2511~2526, Williams & Wilkins, 1989
15) GOODFELLOW, M.: Section 30. Maduromycetes. *In* BERGEY's Manual of Systematic Bacteriology. Volume 4. *Ed.*, S. T. WILLIAMS *et al.*, pp. 2509~2510, Williams & Wilkins, 1989
16) GRUND, E. & R. M. KROPPENSTEDT: Chemotaxonomy and numerical taxonomy of the genus *Nocardiopsis* Meyer 1976. Int. J. Syst. Bacteriol. 40: 5~11, 1990
17) SHEARER, M. C.; P. M. COLMAN & C. H. NASH, III: *Nocardiopsis mutabilis*, a new species of nocardioform bacteria isolated from soil. Int. J. Syst. Bacteriol. 33: 369~374, 1983
18) TAKAHASHI, A.; K. HOTTA, N. SAITO, M. MORIOKA, Y. OKAMI & H. UMEZAWA: Production of novel antibiotic, dopsisamine, by a new subspecies of *Nocardiopsis mutabilis* with multiple antibiotic resistance. J. Antibiotics 39: 175~183, 1986