STUDIES ON A NEW ANTIBIOTIC M-92 PRODUCED BY *MICROMONOSPORA*

I. TAXONOMY OF M-92 PRODUCING *MICROMONOSPORA* AND ANTIBIOTIC PRODUCTION THEREFROM

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An isolate (strain MCRL 0404) producing a new antibiotic, M-92, was identified as a new strain of *Micromonospora* for which the name *Micromonospora verruculosa* sp. nov. was proposed. A water-infusion of dried sea tangle and dried shiitake was utilized for the production of M-92. When this strain was fermented in the medium containing this infusion, M-92 accumulated in the mycelium at about 10 times that in the broth at the peak level.

Since sporaviridin[1,2] was found in 1966 in our laboratory as the first antibiotic from *Actinoplanaceae*, we have taken an interest in rare actinomycetes as antibiotic producers. As reported by NONOMURA *et al.*[3,4] the dry-heat treatment of a powdered soil sample eliminated most bacteria and aerial mycelium-forming actinomycetes, but not rare actinomycetes such as *Microbispora* and *Streptosporangium*.

Among the antibiotic-producing *Micromonospora* isolated from soil samples previously dry heated at 110°C for 6 minutes, a strain numbered MCRL 0404 produced an antibiotic in mycelium and in broth, which was active against some Gram-positive and Gram-negative bacteria. This antibiotic was found to be new and designated antibiotic M-92. Strain MCRL 0404 was identified as a new species of *Micromonospora*, based on its micromorphological, cultural, physiological and chemotaxonomical characteristics, and thus the name *Micromonospora verruculosa* sp. nov. is proposed. This paper deals with the taxonomy of the M-92 producing strain and production of the antibiotic. Isolation, properties and action mechanism of antibiotic M-92 and its components will be reported in succeeding papers.

Taxonomy of Strain MCRL 0404

Strain MCRL 0404 was isolated from a soil sample collected in Nago City, Okinawa, Japan. The taxonomic study was generally carried out by the methods adopted by the International Streptomyces Project (ISP)[5] using the media recommended by SHIRLING and GOTTLIEB[5], and WAKSMAN[6] and colors were described according to the color names and hue numbers of the Color Harmony Manual (4th edition)[7]. For cell wall composition determination, amino acid and sugar samples prepared from cells by the method of LECHEVALIER and LECHEVALIER[8] were qualitatively examined by TLC. Fatty acid samples prepared by the method of OKAMI *et al.*[9] were analyzed by gas chromatography.

Strain MCRL 0404 was aerobic and showed good growth at 37°C in a pH range of 7.0~8.0. The strain could grow at pH 5.0 or 9.0 at 18~37°C, but it could not grow at temperatures higher than 45°C in all pH ranges.

The cultural characteristics of strain MCRL 0404 is summarized in Table 1. Strain MCRL 0404 grew better on organic media than synthetic media. On organic agar media, vegetative mycelia puckered, thickened, raised and developed into the medium. Long straight or loosely curved vegetative

Table 1. Cultural characteristics of strain MCRL 0404 and reference *Micromonospora*.

Medium	MCRL 0404	*M. olivoasterospora* ATCC 21819	*M. globosa* NRRL B-2673
Sucrose nitrate agar (Waksman medium No. 1, 30°C)	G : Poor, ivory (2db) Spore: None RC : Colorless SP : Faintly	Poor, biscuit (2ec) None or scant, olive gray (lig) Biscuit (2ec) None	Poor, bisque (3ec) None Colorless Faintly
Glucose asparagine agar (Waksman medium No. 2, 30°C)	G : Moderate, bright melon yellow (3ia) Spore: Beaver (31i) RC : Beige brown (3ig) SP : Dark olive (1 1/2 nl)	Moderate, bamboo (2gc) Lamp black (p) Dark olive (1 1/2 pn) Light citron gray (lec)	Poor, bright melon yellow (3ia) None Light tan (3gc) Light tan (3gc)
Glycerol asparagine agar (ISP medium No. 5, 30°C)	G : Moderate, light tan (3gc) Spore: Dark olive (1 1/2 pn) RC : Dark olive (1 1/2 pn) SP : Olive gray (1 1/2 ig)	Poor, olive gray (1 1/2 ig) Olive (1 1/2 ni) Olive gray (lig) None	Poor, light tan (3gc) Mustard tan (21q) Clove brown (3ni) Bamboo (2fb)
Inorganic salts starch agar (ISP medium No. 4, 30°C)	G : Good, camel (3ie) Spore: Lamp black (p) with oyster white (b), thin aerial mycelia RC : Dark brown (3nl) SP : Light olive drab (1 1/2 li)	Moderate, bisque (3ec) Lamp black (p) Mole (1) Biscuit (2ec)	Moderate, apricot (4ia) None Amber (31c) Light amber (3ic)
Tyrosine agar (ISP medium No. 7, 30°C)	G : Moderate, covert tan (2ge) Spore: Charcoal gray (n) RC : Dark olive (1 1/2 pn) SP : Light olive drab (1 1/2 li)	Moderate, bamboo (2gc) Dark brown (2pn) Chocolate (4nl) Oak brown (4pi)	Moderate, cinnamon (31e) Scant, adobe brown (31g) Light tan (3gc) Light tan (3gc)
Nutrient agar (Waksman medium No. 14, 30°C)	G : Moderate, camel (3ie) Spore: Lamp black (p) RC : Olive (1 1/2 pl) SP : Olive gray (1 1/2 ig)	Moderate, ivory (2db) Lamp black (p) Dark olive (1pn) Olive gray (1 1/2 ig)	Moderate, apricot (4ia) None Light amber (3ic) Bamboo (2fb)
Yeast extract-malt extract agar (ISP medium No. 2, 30°C)	G : Good, mustard tan (21g) Spore: Lamp black (p) with oyster white (b), thin aerial mycelia RC : Dark olive (1 1/2 pn) SP : Dark olive (1 1/2 pn)	Good, bamboo (2gc) Lamp black (p) with oyster white (b), thin aerial mycelia Dark green (24pn) Olive (1 1/2 ni)	Good, camel (3ie) Sepia brown (3pn) Sepia brown (3pn) Dark luggage tan (4pg)
Oatmeal agar (ISP medium No. 3, 30°C)	G : Good, camel (3ie) Spore: Lamp black (p) with oyster white (b), thin aerial mycelia RC : Dark olive (1 1/2 pn) SP : Covert brown (21i)	Good, bamboo (2gc) Lamp black (p) Dark olive (1 1/2 nl) Dark olive (1 1/2 pn)	Good, light tan (3gc) Camel (3ie) Adobe brown (31g) Adobe brown (31g)
Calcium malate agar (Waksman medium No. 7, 30°C)	G : Poor, melon yellow (3ga) Spore: Scant, charcoal gray (n) RC : Bamboo (2fb) SP : None	Poor, whitish Charcoal gray (n) None None	No growth
Bennett agar (Waksman medium No. 30, 30°C)	G : Good, clove brown (3ni) Spore: Sepia brown (3pn) RC : Sepia brown (3pn) SP : Oak brown (4pi)	Good, bamboo (2gc) Lamp black (p) Light olive drab (11i) Slate tan (2ig)	Good, adobe brown (31g) Sepia brown (3pn) Dark brown (2pn) Yellow maple (3ng)
Peptone iron agar (Difco 30°C)	G : Moderate, folded, mustard tan (21g) Spore: Scant, charcoal gray (n) RC : Topaz (3ne) SP : Mustard brown (2ni)	Moderate, ivory (2db) None Bamboo (2gc) None	Good, folded, orange rust (4pe) None Orange rust (4pe) Topaz (3ne)

G; Growth, RC; reverse color, SP; soluble pigment.

mycelia were initially almost orange in color, but later turned to olive and then to dark olive on some agar media. At the tip of a short sporophore, MCRL 0404 bore a single greenish black to black spore. At maturity the spores almost covered the growth surface. Under an electron microscopic observation,

Plate 1. Spores of *Micromonospora verruculosa* MCRL 0404. (Bennett agar, ×5,300).

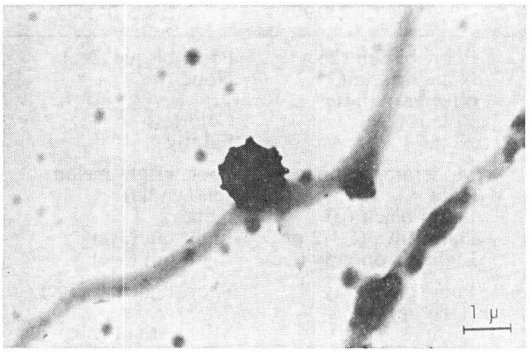
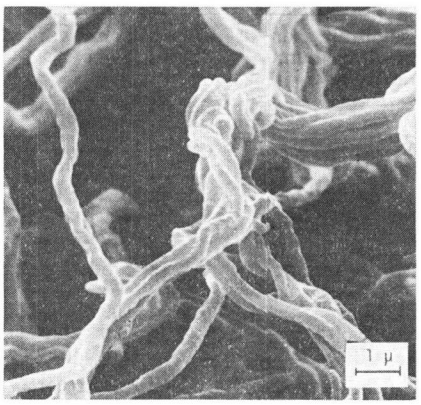


Plate 2. Rudimentary and retarded aerial mycelia of *Micromonospora verruculosa* MCRL 0404. (Inorganic salts starch agar, ×6,000).



the spore was oval (1.0~1.3 μm). The spore-surface had a warty-like apperance (Plate 1). On inorganic or organic agar plates, true aerial mycelium was not formed, but white to gray rudimentary and retarded aerial mycelia were formed (Plate 2). Soluble pigments were light or dark olive.

Physiological properties, utilization of carbon sources and cell wall components of strain MCRL 0404 are shown in Tables 2, 3 and 4, respectively. According to LECHEVALIER and LECHEVALIER[8], the cell wall type of strain MCRL 0404 is classified as type II, as DL-diaminopimelic acid and glycine were found as constitutional amino acids, and neither arabinose nor galactose were detected as constitutional sugars. The fatty acid spectrum showed a branched type.

Strain MCRL 0404 which is mesophilic, bears a single spore at the tip of a sporophore, forms no

Table 2. Physiological properties of strain MCRL 0404 and reference *Micromonospora*.

	MCRL 0404	*M. olivoasterospora* ATCC 21819	*M. globosa* NRRL B-2673
Calcium malate hydrolysis (Waksman medium No. 7)	−	−	−
Starch hydrolysis (ISP medium No. 4)	+	+	+
Gelatin liquefaction (Waksman medium No. 19)	+	+	+
H_2S production (Difco peptone iron agar)	+	+	+
Nitrate reduction (Difco nitrate broth)	+	+	+
Tyrosinase reaction (ISP medium No. 7)	−	+	−
Milk coagulation (Difco 10% skim milk)	+	−	+
Milk peptonization (Difco 10% skim milk)	+	−	+
Melanin formation (ISP medium No. 7 or Difco peptone iron agar)	−	−	−
Cellulose decomposition (Waksman medium No. 39)	+	−	−

+: Positive. −: Negative.

Table 3. Utilization of various carbon sources by strain MCRL 0404 and reference *Micromonospora*.

Carbon source	MCRL 0404	*M. olivoasterospora* ATCC 21819	*M. globosa* NRRL B-2673
D(+)-Melibiose	++	−	++
Dextrin	++	+	++
D-Glucose	++	++	++
Sucrose	++	+	++
D-Xylose	++	++	++
D(+)-Galactose	++	+	++
D(+)-Raffinose	++	−	++
Maltose	++	+	++
L(+)-Arabinose	++	−	++
Inulin	++	−	++
Starch	++	++	++
D-Mannose	++	++	++
Fructose	+	−	+
Lactose	+	−	+
D(−)-Sorbitol	−	−	−
Inositol	−	−	−
D(+)-Melezitose	−	−	−
Dulcitol	−	−	−
Mannitol	−	−	−
D(−)-Arabinose	−	−	+
D(−)-Ribose	−	+	−
L(+)-Rhamnose	−	−	−
Salicin	−	−	−
Glycerol	−	−	−

Basal medium: ISP medium No. 9.
++: Positive utilization, +: poorly positive utilization, −: negative utilization.

true aerial mycelium, and has a type II cell wall is rationally classified in the genus *Micromonospora*.

According to the criteria of *Micromonospora* taxonomy by LUEDEMANN *et al.*[10], strain MCRL 0404, which shows monopodial branching, belongs morphologically to the types of *Micromonospora chalcea, Micromonospora echinospora* and *Micromonospora purpurea*. Among these, *M. echinospora* and its subspecies show a spore-surface of a spiny or a warty-like structure as does strain MCRL 0404. At present, many *Micromonospora* are known to form spiny or warty spores. Most of these strains, however, form vegetative mycelium, characteristic in their violet color or produce reddish-brown to brown-violet soluble pigments. These strains, therefore, are readily differentiated from strain MCRL 0404 which, in some media, forms vegetative mycelium of green to olive color and produces a soluble olive-colored pigment. Because of the mycelial color and warty surface of spores, strain 0404 could be compared with the fortimicin-producing *Micromonospora olivoasterospora*[11]. In addition to some differences in cultural characteristics shown in Table 1, *M. olivoasterospora* did not utilize D-melibiose, L-arabinose and D-raffinose, and was positive in the tyrosinase reaction and negative on milk coagulation, milk peptonization and cellulose decomposition as shown in Tables 2 and 3. Moreover, as shown in Table 4, cell wall components of the strain are quite different from those of MCRL 0404.

According to the observation of KAWAMOTO *et al.*[12] on the 19 representative strains of *Micromono-*

Table 4. Components in cells of strain MCRL 0404 and reference *Micromonospora*.

	Component		MCRL 0404	*M. olivoasterospora* ATCC 21819		*M. globosa* NRRL B-2673	
Amino acid*	Glycine		++++	++++		++++	
	LL-Diaminopimelic acid		−	−	(−)**	−	(+)
	DL-Diaminopimelic acid		++++	++	(−)	++++	(++++)
	3-Hydroxydiaminopimelic acid		−	+++	(++++)	−	(−)
Sugar*	Glucose		++++	++++	(++++)	++++	(++++)
	Xylose		+	+++	(++++)	+	(+)
	Arabinose		±	++	(++)	±	(+)
	Galactose		−	+++	(+)	−	(+)
Fatty acid***	Straight	C_{16}	1	2			
		C_{17}	5	3		12	
		C_{18}	8	3			
	Iso branched	C_{16}	34	31		39	
		C_{17}	4	5		8	
		C_{18}	1	3		9	
	Anteiso branched	C_{15}	19	26		10	
		C_{17}	21	18		15	
		C_{19}	4	3		1	
	Unknown		3	6		6	

* Amino acid and sugar are expressed in relative amounts according to the sizes and intensities of their spots on the TLC.

** Symbols in parenthesis are cited from the reference 12.

*** Fatty acid is expressed in per cent of relative amounts according to each peak level of the gas chromatogram pattern.

spora species, and as re-confirmed by the present authors, cell wall compositions of *Micromonospora globosa* were quite unique compared to other strains, in that the cell wall was composed of DL-diaminopimelic acid and glucose. Strain MCRL 0404 was quite similar to *M. globosa* in its cell wall components. Not only the cell wall compositions, but also the physiological properties (except for cellulose decomposition) and carbon source utilization patterns of *M. globosa* are quite similar to those of strain MCRL 0404. However, *M. globosa* forms spores with a smooth surface. As shown in Table 1, its cultural characteristics are also definitely differ from those of strain MCRL 0404.

As a result, strain MCRL 0404 is considered to be a new species of *Micromonospora*, for which the name *Micromonospora verruculosa* Matsuzawa and Tani sp. nov. is proposed. "*Verruculosa*" (Latin, adj, warty) was derived from the warty structure of the spore-surface. The type strain (MCRL 0404) has been deposited in the culture collection of the Fermentation Research Institute, Tsukuba, Ibaraki-Prefecture, Japan, under the accession number FERM-P 738.

Strain MCRL 0404 was mutated by artificial techniques or spontaneously. These mutants bore few spores, but formed white to gray rudimental aerial mycelia on orange vegetative mycelia and selectively produced the component called VA-2, the most active component of the antibiotic M-92 complex.

Production of Antibiotic M-92

In an attempt to find a suitable medium for antibiotic production by *M. verruculosa*, the effectiveness of water-infusions of dried sea tangle and shiitake (the air-dried fruit bodies of an edible mushroom, *Lentinus edodes*) were found as nutrients. The seed culture was prepared by cultivating at 37°C for 72 hours under rotary shaking in the medium[13] composed of 0.1% glucose, 2.4% soluble starch, 0.3% beef extract, 0.5% Tryptone, 0.5% yeast extract and 0.4% $CaCO_3$ (pH 7.0). Five ml of the seed culture were inoculated to 500-ml Sakaguchi flasks provided with 100 ml of the test medium shown in Table 5

and cultivated under reciprocal shaking at 37°C. Antibiotic production in broth was examined. As shown in Table 5, production of antibiotic M-92 was significantly increased in the presence of dried sea tangle and dried shiitake.

Of the temperature tested (27°C, 32°C and 37°C), cultivation at 37°C was found to give the highest antibiotic production.

Large scale production of antibiotic M-92 was carried out in a 2,000-liter tank fermentor. The media used for seed preparation and production were composed of 2% glucose, 2% soluble starch, 0.5% beef extract, 0.01% yeast extract (0.5% for seed medium), 0.05% $MgSO_4 \cdot 7H_2O$, 0.05% K_2HPO_4, 0.2% NaCl, 0.4% $CaCO_3$, 0.01% silicon KM-75, 0.5% dried sea tangle and 0.5% dried shiitake (the latter two were infused with water for 20 minutes at 100°C, before addition) at pH 7.0. To prepare a seed culture, spores were first cultivated on a rotary shaker for 72 hours at 37°C in a 1-liter Erlenmeyer flask provided with 180 ml of the sterilized seed medium and transferred to a 30-liter jar fermentor provided with 16 liters of the sterilized seed medium and cultivated for 72 hours at 37°C. The seed culture thus obtained was inoculated to a 2,000-liter tank fermentor provided with 1,100 liters of the sterilized production medium and cultivated under the following conditions: temperature, 37°C±1°C; aeration, 550~650 liters/minute; agitation, 125 r.p.m.; internal pressure, 0.5~0.7 kg/cm².

Table 5. Effect of some nitrogen sources and others on antibiotic production.

Nitrogen source	%(w/v)	Antibiotic production in broth (Diameter of inhibitory zone, mm**) 3 days	5 days
Soy bean meal	1.5	13	10
Pharmamedia	1.5	13	10
Yeast extract	1.0	10	10
Beef extract	1.0	13	10
Peptone	1.0	13	15
Casamino acids	1.0	14	15
Amber BYF50X	1.0	10	13
Dried shiitake*	0.5	14	20
Dried sea tangle*	0.5	21	19
Beef extract Dried sea tangle*	0.5 0.5	24	22
Beef extract Dried sea tangle* Dried shiitake*	0.5 0.5 0.5	25	22

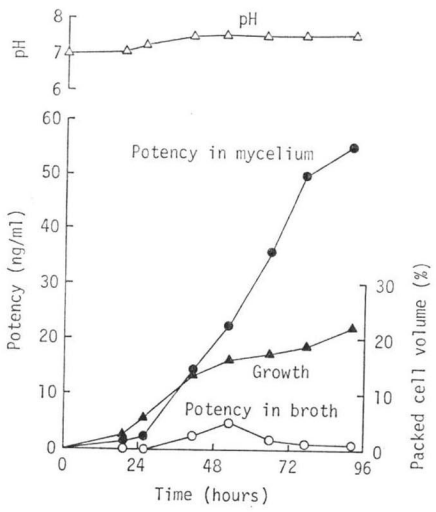
Basal medium: Soluble starch 2%, glucose 2%, $MgSO_4 \cdot 7H_2O$ 0.1%, K_2HPO_4 0.1%, pH 7.0 (after addition of nitrogen sources).

* Dried sea tangle and dried shiitake were infused with water for 20 minutes at 100°C, before addition.

** Diameters were measured by disk-plate method using *S. aureus* Terajima as test organism.

Antibiotic potency in the broth or mycelium was measured as follows. An aliquot of broth was sampled into a centrifuging tube, centrifuged and separated from the supernatant. To determine the potency in the aqueous portion of the broth, the supernatant was assayed by a disc-plate method using *Staphylococcus aureus* Terajima as a test organism and expressed as VA-2, the most active component of M-92 complex, used as a

Fig. 1. Time-course of M-92 production by *Micromonospora verruculosa* MCRL 0404.



standard material for the bioassay. The precipitate was suspended in dimethylformamide to a level equal to the volume of the broth and M-92 in the mycelium was extracted by shaking for 10 minutes. The potency in dimethylformamide solution was assayed as above. A typical time course of fermentation is shown in Fig. 1. M-92 production in the culture filtrate reached a maximum at about 50 hours after incubation, while the antibiotic accumulated in the mycelia reached a maximum at 77 to 91 hours. In general, the total amount accumulated in the mycelia was about 10 times that produced in the broth.

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