STUDIES ON JUVENIMICIN, A NEW ANTIBIOTIC. I

TAXONOMY, FERMENTATION AND ANTIMICROBIAL PROPERTIES

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An actinomycete, strain No. T-1124, was found to produce new macrolide antibiotics, juvenimicins. Based on the results of taxonomic studies, the strain was considered to be a new variety of *Micromonospora chalcea* and the name *Micromonospora chalcea* var. *izumensis* is proposed. This strain also produced everninomicin. The production of juvenimicins was stimulated by addition of ferrous sulfate and magnesium sulfate in the fermentation medium. Among juvenimicins, juvenimicin A_3 exhibited the most potent antimicrobial activities against gram-positive bacteria and furthermore was active against gram-negative bacteria.

In the course of our screening program for a new macrolide antibiotic which is active against gram-positive and gram-negative bacteria and has curative *in vivo* effect, an actinomycete, strain No. T-1124 isolated from a soil sample collected in Izumi City, Osaka, was found to produce an antibiotic showing cross resistance against macrolide antibiotics. Based on the morphological properties and its antibacterial spectrum, the antibiotic produced by the strain was suggested to be new to science. The fermentation studies for the production of the antibiotic were carried out. The antibiotic was found to represent two groups of antibiotics, juvenimicins,[1] new antibiotics, and everninomicins.[2]

This paper deals with the taxonomic and fermentation studies on strain No. T-1124 and antimicrobial activities of juvenimicins.

Materials and Methods

I. Taxonomic Studies of Juvenimicin-producing Organism

(1) Strain No. T-1124: The strain was isolated from a soil sample collected in Izumi City, Osaka Prefecture, Japan.

(2) Morphology: The morphology of the strain was studied on glucose asparagine agar, yeast malt extract agar and BENNETT's agar. For studies on spore formation, the strain was incubated at 28°C for 14 days.

(3) Cultural characteristics: Spores of the strain collected from the 21-day-old culture on glucose asparagine agar were suspended in sterile water. A loopful of the suspension was inoculated on the various media according to the description of WAKSMAN[3] and SHIRLING and GOTTLIEB,[4] and was incubated at 24, 28 or 37°C. The cultural characteristics were observed in 14, 21 and 30 days. Determinations of spore-mass color and reverse color were carried out according to the "Color Harmony Manual."[5] The effects of temperature and pH range on growth of this strain were studied with glucose asparagine agar or BENNETT's agar.

(4) Utilization of carbon sources: Utilization of carbon sources was investigated by the method of PRIDHAM and GOTTLIEB[6] and that of LUEDEMANN and BRODSKY.[7]

II. Fermentation Procedure

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A loopful of spore suspension of strain No. T-1124 from agar slant culture was inoculated into 30 ml of a seed culture medium consisting of 5% soluble starch, 0.5% glycerol, 1% soybean flour, 0.5% meat extract and 0.5% $CaCO_3$ (pH 7.0) in a 200-ml Erlenmeyer flask. The inoculated flask was shaken on a rotary shaker (200 rpm, 5-cm radius) at 37°C for 40 hours, and was transferred into 500 ml of the sterilized seed culture medium of the same composition in a 2-liter SAKAGUCHI flask. Incubation was continued on a reciprocal shaker at 28°C for 40 hours. The seed culture totaling of 1.5 liters was transferred to a 200-liter fermentor containing 100 liters of main culture medium consisting of 5% soluble starch, 2% corn steep liquor, 0.5% meat extract, 0.01% $FeSO_4 \cdot 7H_2O$, 0.1% $MgSO_4$ and 1.0% $CaCO_3$ (pH 7.0), and was incubated at 28°C under aeration (100 liters/min) with agitation (200 rpm) for 90~114 hours.

III. Assay Methods

(1) Agar streak method: Antibacterial activity of strain No. T-1124 was measured by the agar streak method and the agar disc method.[8]

(2) Paper disc method: The differential assay of the two antibiotics, juvenimicin and everninomicin, was carried out by using *Azotobacter vinelandii* IFO 12018 and *Bacillus cereus* var. *mycoides* IFO 3040 as test organisms. *Azotobacter vinelandii* was grown on an assay medium consisting of 1% sucrose, 0.2% peptone, 0.1% meat extract, 0.1% yeast extract and 0.8% agar (pH 8.0) at 34°C for 24 hours, and *Bacillus cereus* var. *mycoides* was grown on the nutrient agar (pH 7.0) at 37°C for 18 hours. Juvenimicin A_8 and everninomicin D were used as standard samples.

(3) Agar dilution method: Antimicrobial activity of juvenimicins was determined by the serial agar dilution method on nutrient agar for gram-positive and gram-negative bacteria at 37°C for 18 hours and on glycerol nutrient agar for mycobacteria at 37°C for 42 hours.

Results and Discussion

Morphological Characteristics

Vegetative mycelium develops well and is branched, but no septum is observed, measuring 0.4~0.8 μm wide. Fair growth occurs on various media, but aerial mycelium is not formed. A single spore is formed at the tip of the sporophore branching from the vegetative hypha (Fig. 1). The sporophores are usually 1~3 μm in length, but occasionally 3~6 μm long. Spores are formed, and each spore is spherical or ellipsoidal (0.5~1.0×0.5~1.3 μm), and smooth (Fig. 2).

Cultural Characteristics

The cultural characteristics of strain No. T-1124 on various media are shown in Table 1. The growth is slower than that of *Streptomyces* in general. On synthetic media, the vegetative mycelium is orange yellow to orange, becoming brown or blackish brown to black with moistened to glossy surface. On the organic media, the growth generally exhibits more abundant and folded than on the synthetic media. Colonies are orange to oragne brown, later becoming brown to black. On almost all media, no soluble pigment is produced.

Physiological properties of strain No. T-1124 are shown in Table 2. Hydrolysis of starch, peptonization and coagulation of milk, liquefaction of gelatin, liquefaction of serum and decomposition of chitin are positive, but tyrosinase reaction, nitrate reduction, and decomposition of cellulose are negative. The temperature range for growth is about 20~45°C, with the optimum being 30~37°C. The cultural behaviors at 37°C are similar to those at 28°C, except that the growth occurs more luxuriantly and that the colors are generally darker or blackened.

Utilization of carbon sources of strain No. T-1124 is shown in Table 3. According to the method of PRIDHAM and GOTTLIEB,[6] melibiose, raffinose, galactose, fructose, maltose, sucrose, lactose, trehalose, xylose, glucose and starch are utilized for growth, but arabinose, mannose, rhamnose, inositol, man-

Fig. 1. Microscopic photograph of sporophores of *Micromonospora* sp. strain No. T-1124 (×600)

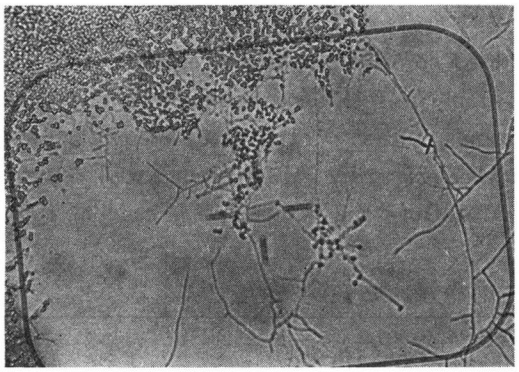


Fig. 2. Electron-microscopic photograph of spores of *Micromonospora* sp. strain No. T-1124 (×6,000)

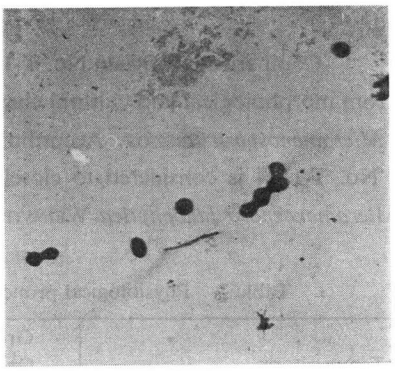


Table 1. Cultural characteristics of *Micromonospora* sp. strain No. T-1124.

Medium	Characteristics
CZAPEK's agar	G* : moderate to abundant, Apricot (4 ia)** later becoming Dark Olive (1 pn) R* : Apricot SP* : none
Glucose asparagine agar	G : abundant, Apricot to Chocolate (4 nl) R : Beaver (4 li) SP : none
Glycerol asparagine agar (ISP–5)	no growth
Nutrient agar	G : poor, Orange (4 la) R : Brite Melon Yellow (3 ia) SP : none
Yeast malt extract agar (ISP–2)	G : abundant, folded, Orange (4 la) to Copper Persimon (5 lc), later becoming black R : Dusty Orange (4 lc) SP : none
Oatmeal agar (ISP–3)	G : abundant, folded, Orange (4 la) SP : none
Inorganic salts-starch agar (ISP–4)	G : abundant, Apricot (4 la) R : Apricot SP : none
Peptone yeast extract iron agar (ISP–6)	G : poor to moderate, folded, Melon Yellow (3 ga) R : Brite Maize (3 ia) SP : none
Tyrosine agar (ISP–7)	G : poor to moderate, Apricot (4 ia) R : colorless to faint Apricot SP : none
BENNETT's agar	G : abundant, Dusty Orange, later becoming black R : Dusty Orange SP : none
Potato plug (without $CaCO_3$)	G : abundant, lichenoid, wrinkled, Orange to Oak Brown (4 pi) SP : faint gray to dark grayish brown
Cellulose	G : moderate, DK Olive Green (24 1/2 pl), later grayish black no decomposition

* G: Growth, R: Reverse, SP: Soluble pigment
** Color Harmony Manual

nitol and glycerol are not utilized. On the contrary, according to the method of LUEDEMANN,[7] arabinose and mannose are also utilized, although rhamnose, inositol, mannitol and glycerol are doubtfully utilized.

Comparison of Strain No. T-1124 with Other Known *Micromonospora* species

From morphological and cultural characteristics, strain No. T-1124 is considered to belong to the genus *Micromonospora* ØRSKOV. According to the taxonomic criteria[9~12] of the genus *Micromonospora*, strain No. T-1124 is considered to closely resemble *Micromonospora chalcea* (FOULERTON) ØRSKOV and *Micromonospora halophytica* WEINSTEIN, LUEDEMANN, ODEN and WAGMAN.[13] Therefore, com-

Table 2. Physiological properties of *Micromonospora* sp. strain No. T-1124

Temperature	Growth range, 20°~45°C Optimum growth range, 30°~37°C Spores resist 80°C for 5 minutes
pH range	Growth range, pH 5~9 Optimum range, pH 6.5~7.5
Gelatin liquefaction	positive (very slow)
Serum liquefaction	positive
Starch hydrolysis	positive, hydrolyzed zone/growth zone=27~32 mm/9~10 mm
Milk coagulation	positive
peptonization	positive
Nitrate reduction	negative
Cellulose decomposition	negative
Chitin decomposition	positive
Chromogenicity	negative

Table 3. Utilization of carbon sources by *Micromonospora* sp. strain No. T-1124.

Carbon source	Growth	
	A*	B**
i-Inositol	−	±
D-Mannitol	−	±
D-Xylose	+++	+++
L-Arabinose	−	+++
D-Galactose	+++	+++
D-Glucose	+++	+++
D-Fructose	+	+++
D-Mannose	−	+++
Rhamnose	−	±
Melibiose	++	+++
Maltose	+++	+++
Sucrose	++	+++
Lactose	++	+++
Raffinose	+~++	++
Trehalose	++	+++
Soluble starch	+++	+++
Glycerol	−	±
None (control)	−	±

+++: Abundant growth ++: Moderate growth +: Growth
±: Poor growth −: No growth
A*: The method of PRIDHAM & GOTTLIEB.
B**: The method of LUEDEMANN & BRODSKY.

Table 4. Comparison of the cultural characteristics of *Micromonospora* sp. strain No. T-1124 with those of *Micromonospora chalcea* IFO 12135.

Medium	Characteristics	
	Strain No. T-1124	*M. chalcea* IFO 12135
CZAPEK's agar	G* : moderate to abundant, Apricot, later becoming Dark Olive	abundant, Dusty Orange to Luggage Tan
	R* : Apricot	Dusty Orange to Orange Rust
	SP* : none	faint yellowish orange
Glycerol asparagine agar	G : no growth	moderate, Dusty Orange to Orange Rust
	R :	Dusty Orange to Orange Rust
	SP :	none
Tyrosine agar	G : poor to moderate, Apricot	abundant, Orange
	R : colorless to faint Apricot	Orange
	SP : none	none
Yeast malt extract agar	G : abundant, folded, Orange to Copper Persimon, later becoming black	abundant, folded, Copper Brown to black
	R : Dusty Orange	Dk Spice Brown
	SP : none	none
Cellulose	G : poor, Apricot	poor, Apricot
	decom- negative position	positive

*G: Growth R: Reverse SP: Soluble pigment.

Table 5. Antibacterial activity of *Micromonospora* sp. strain No. T-1124 by cross streak method and agar disc method.

	Test organism	Inhibition length (mm)	
		Nutrient agar	Glycerol nutrient agar
Cross streak method	*Escherichia coli*	0	0
	Staphylococcus aureus	16	10
	Staphylococcus aureus OE–R*	6	5
	Bacillus subtilis	20	12
	Bacillus brevis	11	9
	Bacillus megatherium	16	11
	Mycobacterium sp. TAKEO	0	0
Agar disc method	*Bacillus subtilis* pH 6	20	14
	pH 8	25	22

* OE–R: Oleandomycin-erythromycin resistant.

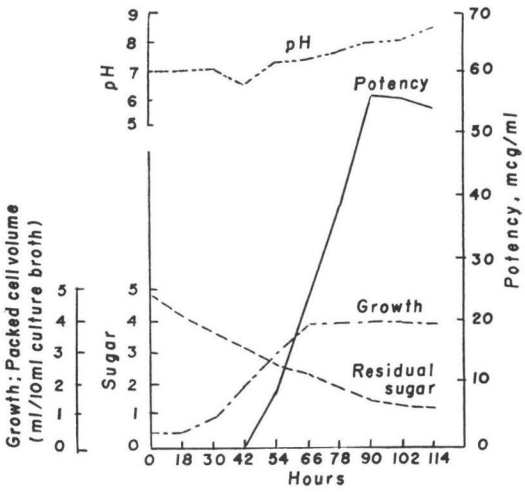
parisons of the cultural characteristics of strain No. T-1124 with those of the related species were carried out and good agreements were obtained in the color of colonies, coagulation and peptonization of milk, hydrolysis of starch, liquefaction of gelatin and utilization of melibiose and raffinose. However, strain No. T-1124 differed from *Micromonospora halophytica* by the fact that strain No. T-1124 showed good growth on potato slice, poor growth on tyrosine agar and did not reduce nitrate. On the other hand, comparison of the cultural characteristics and physiological properties between strain No. T-1124 and *Micromonospora chalcea* IFO 12135 were made under the same condition. As shown in Table 4, the two strains differed in the cultural characteristics on CZAPEK's agar, glycerol asparagine agar and tyrosine agar and in the ability to decompose cellulose. From these results, strain No. T-1124

Table 6. Fermentation medium for the production of juvenimicin by *Micromonospora chalcea* var. *izumensis*

Soluble starch	5%
Proflo	2%
Peptone	0.5%
Meat extract	0.5%
$FeSO_4.7H_2O$	0.05%
$MgSO_4.7H_2O$	0.1%
$CaCO_3$	1.0%

pH 7.0

Fig. 3. Time course of juvenimicin production by *Micromonospora chalcea* var. *izumensis*



was considered to be a variety of *Micromonospora chalcea* and the name *Micromonospora chalcea* var. *izumensis* is proposed. Among the species of *Micromonospora* producing macrolide antibiotics, *Micromonospora megalomiciae*,[14] *Micromonospora rosaria*[15] and *Micromonospora inositola*[16] have been reported. *M. megalomiciae* exhibited good growth and produced brown soluble pigment on tyrosine agar. *M. rosaria* produced wine-red color soluble pigment on various media and spores were blunt spines. *M. inositola* did not grow on glucose asparagine agar but utilized inositol for growth. *M. carbonacea*[17] producing everninomicin showed that the sporophores were of the sympodial type. Based on the above-mentioned characteristics, these species clearly differed from strain No. T-1124.

Strain No. T-1124 has been deposited in the Institute for Fermentation, Osaka, under the accession

Table 7. Antimicrobial spectra of juvenimicins and everninomicin produced by *Micromonospora chalcea* var. *izumensis*.

Test organism	Media	*Minimum inhibitory concentration (mcg/ml)					
		Juvenimicins					EVM**
		A_2	A_3	A_4	B_1	B_3	
Staphylococcus aureus 209P	NA	2.0	0.5	2.0	2.0	2.0	0.5
Staphylococcus aureus 209P OE-R***	NA	100	100	100	100	100	1.0
Bacillus subtilis PCI 219	NA	1.0	0.1	0.5	1.0	1.0	0.5
Bacillus cereus	NA	5.0	0.1	2.0	2.0	2.0	0.5
Bacillus brevis	NA	1.0	0.2	5.0	2.0	20.0	1.0
Sarcina lutea	NA	2.0	0.05	1.0	0.2	0.5	0.1
Micrococcus flavus	NA	2.0	0.05	1.0	0.2	0.2	0.01
Escherichia coli NIHJ	NA	>100	5.0	100	100	100	>100
Proteus vulgaris	NA	>100	10.0	100	100	100	>100
Pseudomonas aeruginosa	NA	>100	100	>100	>100	>100	>100
Mycobacterium sp. 607	GNA	>100	>100	>100	>100	>100	>100

* Agar dilution method
Media: NA; nutrient agar (pH 7.0)
GNA; glycerol nutrient agar (pH 7.0)
** EVM: crude everninomicin
*** Oleandomycin-erythromycin resistant

number of IFO 12988 and in the American Type Culture Collection under the accession number of ATCC 21561.

Antibacterial Activities of Strain No. T-1124

Antibacterial activities of strain No. T-1124 by the cross streak and the agar disc method were shown in Table 5. From these results, it was found that strain No. T-1124 inhibited growth of gram-positive bacteria and partially inhibited the growth of macrolide-resistant *Staphylococcus aureus*. Strain No. T-1124 showed stronger activity on the alkaline assay medium than on the acidic assay medium. It was suggested that strain No. T-1124 produced simultaneously a basic macrolide antibiotic and the other antibiotic which inhibited a macrolide-resistant organism.

Fermentation

The various components of the media for the production of juvenimicins by strain No. T-1124 were investigated. Soluble starch and dextrin were superior to other carbon sources such as glucose, sucrose, lactose and maltose. Among various nitrogen sources such as Proflo, corn steep liquor, soybean flour, corn gluten meal and yeast extract, Proflo was the most preferable for the antibiotic production. On the other hand, soybean flour or corn gluten meal supported good growth, but the antibiotic was not produced. Numerous monovalent and divalent cation salts were examined for the production of juvenimicins. The production of juvenimicins was stimulated by addition of 0.05% ferrous sulfate and 0.1% magnesium sulfate in the medium. Ammonium nitrate (0.5~1.0%) inhibited the production of everninomicin remarkably, but did not inhibit the production of juvenimicins. The composition of the most suitable medium for the production of juvenimicins was shown in Table 6. This medium was applied to the fermentation in a 200-liter fermentor and a typical time course of juvenimicins production was shown in Fig. 3.

Antibacterial Activities of Juvenimicins

Juvenimicins were found to be composed of eight components, juvenimicins $A_{1\sim4}$ and $B_{1\sim4}$, based on thin-layer chromatography, and juvenimicins A_2, A_3, A_4, B_1 and B_3 were isolated.[1] Antibacterial activity of each component was determined by the agar dilution method as shown in Table 7. Among the juvenimicin components, juvenimicin A_3 showed the highest antibacterial activity against gram-positive bacteria (0.05~0.5 mcg/ml) and some of gram-negative bacteria, *Escherichia coli* and *Proteus vulgaris* (5~10 mcg/ml). It was not active against macrolide-resistant strain of *Staphylococcus aureus* FDA 209P, mycobacteria, fungi and yeast (100 mcg/ml). Juvenimicin A_3 also inhibited the growth of *Xanthomonas oryzae* (less than 1.0 mcg/ml), *Haemophilus gallinarum* (0.78 mcg/ml) and *Mycoplasma gallisepticum* (less than 0.001 mcg/ml). The other components showed about 1/4~1/20 activity of juvenimicin A_3. Everninomicin was active against gram-positive bacteria including macrolide-resistant strain of *Staphylococcus aureus* FDA 209P.

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