**Identification of Strain MH193-16F4,
a Benanomycin-Producing Actinomycete,
to *Actinomadura spadix***

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(Received Sep. 14, 1994 / Accepted Oct. 18, 1994)

In search for new antibiotics, we isolated an actinomycete strain MH193-16F4 that produce novel antifungal and antiviral antibiotics, benanomycins A and B¹⁻³). These antibiotics showed excellent activities against a wide range of fungi *in vitro* and *in vivo* and also against *de novo* infection of human T-cells with human immunodeficiency virus type 1 (HIV 1). In this communication, we report the taxonomic properties of the strain MH193-16F4 that turns out to be closely related to those of *Actinomadura spadix*.

The strain MH193-16F4 was isolated from a soil sample collected at the ground of Institute of Microbial Chemistry, Shinagawa-ku, Tokyo, Japan. This strain was compared taxonomically with *Actinomadura spadix* IMC A-0014^T [= KCC A-0146^T = Nonomura, A-116^T (T: type strain)] that had been maintained in our institute.

Morphological observations under a scanning electron microscope (model Hitachi S-570) were made on cultures grown on yeast extract-malt extract agar (ISP No. 2), oatmeal agar (ISP No. 3), and on yeast-starch agar (soluble starch 1%, yeast extract 0.2%, agar 2%, pH 7.0), incubated at 30°C for 2 to 4 weeks.

Cultural and physiological characteristics were determined using methods of Shirling and Gottlieb⁴), Waksman⁵), and Gordon *et al.*⁶). Permissive temperatures for growth were determined on yeast-starch agar at 10°C, 20°C,

24°C, 27°C, 30°C, 37°C and 50°C. Carbohydrate utilization was tested in Pridham and Gottlieb Medium⁷) supplemented with a vitamin B mixture to make final concentrations (µg/ml) of thiamine hydrochloride 0.5, riboflavin 0.5, nicotinic acid 0.5, pyridoxine hydrochloride 0.5, inositol 0.5, calcium pantothenate 0.5, *p*-aminobenzoic acid 0.5, and biotin 0.25.

Whole-cell hydrolysates were prepared according to the method of Becker *et al.*⁸) and analyzed for diaminopimelic acid isomers using high performance liquid chromatography (HPLC) according to the method of Takahashi *et al.*⁹). Whole-cell sugars were prepared by the method of Lechevarier and Lechevalier¹⁰) and analyzed using HPLC according to the method of Yokota and Hasegawa¹¹). Phospholipids and mycolic acids were analyzed by the methods of Minnikin *et al.*¹²) and Minnikin *et al.*¹³), respectively. Menaquinones were extracted and purified by the method of Collins *et al.*¹⁴) and analyzed using HPLC and mass spectrometry, as described by Tamaoka *et al.*¹⁵), except that the solvent system for HPLC was replaced by methanol-isopropyl alcohol (2:1). Methyl esters of cellular fatty acids were prepared by the method of Suzuki and Komagata¹⁶) and analyzed using a set of Hewlett-Packard model 5890 gas chromatography consisting of a Megabore DB-1 column (15 m by 0.53 mm, film 1 µm), a flame ionization detector, and a Hewlett-Packard model 3392A integrator.

Total DNA was prepared by the method of Hotta *et al.*¹⁷) with the substitution of proteinase K for pronase E. The G+C content of DNA was determined using HPLC according to the method of Tamaoka and Komagata¹⁸). Southern hybridization was carried out as described by Hotta and Ishikawa¹⁹), using 250 ng of *Sph*I-digested total DNAs from the organisms tested. Following the prehybridization in 3×SET (0.45 M NaCl, 6 mM EDTA and 90 mM Tris-HCl, pH 8.0) supplemented with 5% Irish Cream Liquor at 60°C overnight, hybridization was

performed in a solution of the same components but including 5~10 ng/ml of a probe (see below), at the same temperature for 48 hrs. As the probe, total DNA from Strain MH193-16F4 was fragmented by sonication to 4 Kb or smaller and labeled with α - ^{32}P -dCTP by nick translation. The specific activity of the probe was 0.5×10^8 cpm/ μg . After the hybridization, the filter was washed 3 times with 2 $\times$ SSC (200 ml) at 80°C for 1 hr each. Autoradiography was carried out by exposing X-ray film (Fuji RX) to the filter at -80°C and the film was scanned on GC 300 Scanning Densitometer (Hoefer Scientific Instruments).

Two-dimensional polyacrylamide gel electrophoresis (PAGE) analysis of ribosomal proteins was performed by the methods of Ochi *et al.*^{20).}

Fig. 1. Scanning electron micrograph of strain MH193-16F4 on yeast starch agar after incubation at 30°C for 25 days. Bar refers to 1 μm .

Table 1. Cultural characteristics of strain MH193-16F4

Medium	Aerial mycelium	Growth	Soluble pigment
Yeast starch agar	White	Dk Red	Red
Yeast extract-malt extract agar (ISP 2)	Scant, grayish white [3cb, Sand]	Dull red [7 1/2pe, Dk Red] ~ brown purple [7pi, Dk Wine]	Dull red [6 1/2pe, Tomato Red]
Oatmeal agar (ISP 3)	Scant, grayish white [3cb, Sand]	Pale pink [5gc, Peach Tan] ~ dark red [7pe, Wine Red]	Slightly reddish
Inorganic salts-starch agar (ISP 4)	None	Colorless	None
Glycerol asparagine agar (ISP 5)	None	Colorless	None
Tyrosine agar (ISP 7)	None	Colorless ~ pale yellowish brown [3ic, Lt Amber]	None
Sucrose nitrate agar	None	Colorless	None
Glycerol nitrate agar	None	Colorless	None
Glucose asparagine agar	None	Colorless	None
Starch agar	None	Colorless	None
Calcium malate agar	None	Colorless	None
Nutrient agar	None	Pale yellow [2gc, Bamboo]	None

Observations after incubation at 27°C for 2 to 3 weeks.

Color names and numbers were taken from Color Harmony Manual ²¹⁾.

Taxonomic Characteristics of Strain MH193-16F4

Morphological characteristics: Vegetative mycelia were well developed and irregularly branched. Fragmentation of hyphae was not observed. Aerial mycelia were formed on yeast extract-malt extract agar (ISP No. 2), oatmeal agar (ISP No. 3) and yeast-starch agar. Short spore chains were formed into straight, loop-like or small round masses on aerial mycelia. Mature spore chains comprised of 3 to 7 spores each. The spores were cylindrical (0.8 by 1.0 to 1.2 μm) or spherical (0.8 to 1.2 μm), and the spore surface was smooth. The spores were not motile. No synnemata, sclerotia or sporangia were observed (Fig. 1).

Cultural and physiological characteristics:

The cultural characteristics of strain MH193-16F4 are shown in Table 1. This strain grew poorly on most agar media except on yeast extract-malt extract agar (ISP No. 2), oatmeal agar (ISP No. 3) and yeast-starch agar. Addition of thiamine (vitamin B₁) improved growth on most agar media. Aerial mycelia were scant, grayish white [3cb, Sand], while vegetative mycelia were pale pink [5gc, Peach Tan] to dark red [7pe, Wine Red]. Soluble pigment was dull red [6¹/₂ pe, Tomato Red]. The colors of the vegetative mycelia and the soluble pigment changed to orange by the addition of HCl. Physiological characteristics and carbohydrate utilization are shown in Table 2. Permissive temperature range for growth was 20°C to 37°C,

Table 2. Physiological characteristics of strain MH193-16F4 and *A. spadix* IMC A-0014^T

	MH193-16F4	<i>A. spadix</i>		MH193-16F4	<i>A. spadix</i>
Requirement for thiamine	+	-	Acid from:		
Formation of melanoid pigment	-	-	Adonitol	+	+
Liquefaction of gelatin	-	-	L-Arabinose	+	+
Coagulation of milk	+	+	Cellobiose	+	+
Peptonization of milk	(+)	(+)	Erythritol	-	-
Reduction of nitrate	+	+	Glucose	+	+
Hydrolysis of:			Glycerol	+	+
Aesculin	+	+	Inositol	-	(+)
Starch	+	+	Lactose	+	+
Decomposition of:			Mannitol	+	+
Adenine	-	-	Mannose	+	+
Casein	+	+	Melezitose	+	+
Hypoxanthine	-	-	Melibiose	+	+
Tyrosine	-	-	Methyl α -glucoside	-	-
Urea	-	-	Raffinose	+	+
Xanthine	-	-	L-Rhamnose	+	+
Utilization of:			Sorbitol	-	+
Benzoate	-	-	Trehalose	+	+
Citrate	+	+	D-Xylose	+	+
Mucate	+	-	Utilization of:		
Succinate	+	+	L-Arabinose	+*	+*
Tartrate	-	-	D-Fructose	+*	+*
Resistance to:			D-Glucose	+*	+*
Lysozyme (0.01%)	-	-	Inositol	v*	-*
NaCl 3%	(+)	+	D-Mannitol	+*	+*
5%	-	$\pm$	Raffinose	+*	+*
Survival at 50°C	+	+	Rhamnose	+*	+*
			Sucrose	+*	+*
			D-Xylose	+*	+*

Symbols: +; positive, (+); weakly positive, $\pm$; doubtful, -; negative
+*; utilization, v*; variable utilization, -*; no utilization

Table 3. Menaquinone composition, fatty acid composition and G+C contents of strain MH193-16F4 and *A. spadix* IMC A-0014^T.

	Menaquinone composition(%)			Fatty acid composition(%)								G+C contents (mol%)	
	MK-9			Straight chain			Branched chain				10-Methyl branched chain		
	H4	H6	H8	16:0	17:0	18:0	i-16:0	i-17:0	i-18:0	ai-17:0	10Me-17:0	10Me-18:0	
MH193-16F4	6	43	49	2	3	5	32	2	4	26	6	4	69.3
<i>A.spadix</i>	3	42	54	1	1	5	36	1	4	16	10	7	69.9

with the optimal temperature for growth between 30°C and 37°C.

Chemotaxonomic characteristics: Whole-cell hydrolysates contained *meso*-diaminopimelic acid, glucose, mannose, galactose, rhamnose, xylose and madurose. These data indicate that strain MH193-16F4 has a type III cell wall and a type B whole-cell sugar pattern. Phospholipid pattern was type PI; phosphatidylinositol but none of phosphatidylmethylethanolamine, phosphatidylethanolamine or unknown glucosamine-containing phospholipid was found. Mycolic acids were absent. Major menaquinones were MK-9(H₈) and MK-9(H₆). Cellular fatty acids consisted of *iso*-branched 14-methylpentadecanoic acid (*i*-16:0), *anteiso*-branched

14-methylhexadecanoic acid (*ai*-17:0), 10-methyl-branched heptadecanoic acid (10Me-17:0) and other minor components. G+C content of DNA was 69.3 mol% (Table 3).

Identity of strain MH193-16F4: The taxonomic properties given above suggested that strain MH193-16F4 belonged to the genus *Actinomadura*²². Among the known species of *Actinomadura*, *A. spadix*²³⁻²⁵ was most closely related to strain MH193-16F4: *A. spadix* had MK-9(H₈) and MK-9(H₆) as major menaquinones, and had *i*-16:0 and *ai*-17:0 as main components of cellular fatty acids. Accordingly, strain MH193-16F4 was compared with *A. spadix* IMC A-0014^T in more details. As shown in Table 4, morphological characteristics of

Table 4. Comparison of strain MH193-16F4 and *A. spadix* IMC A-0014^T

	MH193-16F4	<i>A. spadix</i>
Spore chain	Straight, loops, small round mass	Loops, spirals, small round mass
Spore surface	Smooth	Smooth
Aerial mycelium	Grayish white	Brownish white
Growth	Pale pink ~ dark red	Pale yellowish brown ~ pale brown
Soluble pigment	Dull red	Brownish

strain MH193-16F4 closely resembled *A. spadix* which also had either loop-like, spiral, or small round masses of spore chains and had the smooth spore surface. Cultural characteristics of strain MH193-16F4 also resembled those of *A. spadix* except that the former produced a red pigment. Physiological characteristics of strain MH193-16F4 were similar to those of *A. spadix*; differences were in the requirement for thiamine, the utilization of mucate, acid formation from sorbitol and the production of benanomicin (Table 2). Major menaquinone and fatty acid compositions of both strains were also similar (Table 3). DNA homology between the two strains was very high (>80%). The two-dimensional PAGE pattern of ribosomal proteins of Strain MH193-16F4 was similar to that of *A. spadix*, and the ribosomal AT-L30 proteins²⁰⁾ of the two strains showed the same mobility (relative electrophoretic mobility, or REM, 14.0).

Taken together, we concluded that strain MH193-16F4 is a member of *A. spadix* and propose to name it *Actinomadura spadix* MH193-16F4. We consider this strain differs from *Actinomadura* sp. AB1236, reported by Furumai *et al.*²⁶⁾, which also produces benanomicins A and B but has low DNA homology (21 %) to *A. spadix*.

A subculture of strain MH193-16F4 was submitted to the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, Tsukuba-shi, Ibaragi, Japan, under the accession No. FERM P-9529.

ACKNOWLEDGMENTS

The authors are grateful to Dr. K. Hotta of National Institute of Health for his kind suggestions on DNA homology studies and to Dr. K. Ochi of National Food Research Institute for conducting the experiments on ribosomal proteins and to Dr. T. Tamamura of Central Research Laboratories, Hokko Chemical Industries Co. Ltd. for the analyses of fatty acids.

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