A NEW ANTIBIOTIC SF-2185 PRODUCED BY *DACTYLOSPORANGIUM*

I. TAXONOMY, FERMENTATION AND BIOLOGICAL PROPERTIES

KUNIOMI MATSUMOTO*, TAKASHI SHOMURA, MASARU SHIMURA, JUNKO YOSHIDA, MITSUGU ITO, TETSURO WATANABE and TATSUO ITO

Pharmaceutical Research Laboratories, Meiji Seika Kaisha, Ltd., Kohoku-ku, Yokohama 222, Japan

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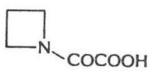
A new antibiotic SF-2185 was found active against plant pathogen, particularly the causal organisms of cucumber downy mildew and rice blast. The producing organism, strain SF-2185, is a novel actinomycete and has been identified as *Dactylosporangium aurantiacum* subsp. *gifuense*.

In the course of our screening program for antibiotics for agricultural application, the culture filtrate of strain SF-2185 was found active in our screening system. Further evaluation showed that the antibiotic was effective against cucumber downy mildew and rice blast diseases. The producing organism was isolated from a soil sample collected at Gifu Prefecture, Japan. This report deals with the taxonomy and fermentation of strain SF-2185, and the biological activity of antibiotics SF-2185. The antibiotic has been characterized as an azetidine (Fig. 1), and detailed chemical description will be reported elsewhere.

Fig. 1. Structure of antibiotic SF-2185.



Taxonomy of Strain SF-2185

The taxonomic characterization was carried out according to the methods described by SHIRLING and GOTTLIEB[1], WAKSMAN[2], and LUEDEMANN and BRODSKY[3]. The color descriptions used in this study were based on the Color Harmony Manual[4]. Analyses of whole-cell hydrolysates were carried out adopting the methods of BECKER *et al.*[5] and LECHEVALIER[6].

A summary of the cultural characteristics of strain SF-2185 is shown in Table 1. Strain SF-2185 formed finger-shaped sporangia abundantly on oatmeal agar (ISP medium 3), Ca-malate agar, and Bennett agar. The sporangium ($1.0 \sim 1.5 \times 4.5 \sim 8.0$ μm in size) occurred singly or in tufts from the vegetative mycelium on the surface of agar media (Plates 1 and 2). Each sporangium contained three to four sporangiospores arranged in a single row. After dehiscence from the sporangium, the spores became highly motile. Formation of globose bodies, which have been described by THIEMANN *et al.*[7] and SHARPLES and WILLIAMS[8], was observed abundantly on oatmeal agar and inorganic salt - starch agar (ISP medium 4) (Plate 2).

The vegetative mycelium ($0.5 \sim 0.7$ μm in diameter) was well developed and branched. The hyphae

Plate 1. Photomicrograph of strain SF-2185 showing finger-shaped sporangia, 1,000×.

The organism was grown on oatmeal agar, 20 days at 28°C.

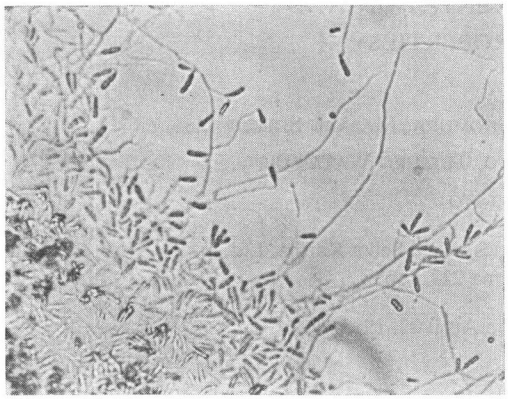


Plate 2. Scanning electron micrograph of strain SF-2185 showing the sporangia and globose bodies, 10,000×, grown on oatmeal agar, 14 days at 28°C.

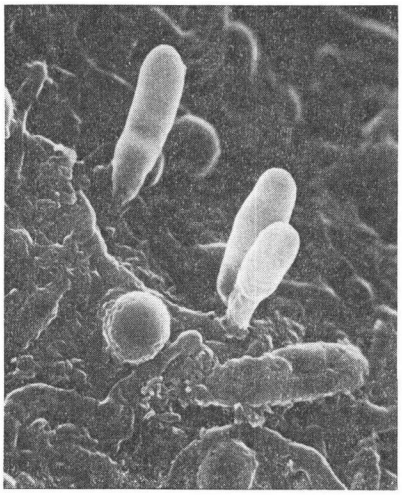


Table 1. Cultural characteristics of strain SF-2185.

Medium	Growth	Sporangia	Globose bodies	Color of colonies	Soluble pigment
Sucrose - nitrate agar	Poor	Moderate	Trace	Light apricot (4ea)	None
Glucose - asparagine agar	Poor	None	None	Pearl (2ba) to light ivory (2ca)	None
Glycerol - asparagine agar (ISP-5)	Poor	Trace	Moderate	Hyaline	None
Inorganic salt - starch agar (ISP-4)	Good	Moderate	Abundant	Light mellon yellow (3ea) to amber (31c)	None
Ca-malate agar	Poor	Abundant	Moderate	Hyaline	None
Oatmeal agar (ISP-3)	Good	Abundant	Abundant	Light apricot (4ea)	None
Yeast extract - malt extract agar (ISP-2)	Good	Moderate	Trace	Light tan (3gc) to amber (31c)	None
Tyrosine agar (ISP-7)	Moderate	None	None	Russet orange (4nc) to bisque (4ec)	Faint grayish pink
Nutrient agar	Poor	None	None	Bamboo (2gc)	None
Bennett agar	Good	Abundant	None	Amber (31c)	None

True aerial mycelium is not formed on all media in this table.
For color designations, see ref 4).

did not fragment into bacillary or coccoid elements. Aerial mycelium was not observed on the agar media tested. The physiological properties and carbon utilization pattern of the organism are shown in Tables 2 and 3. Analyses of whole-cell hydrolysate revealed 3-hydroxydiaminopimelic acid, xylose and a trace of arabinose.

The formation of finger-shaped sporangia containing motile spores, the absence of true aerial mycelium, and the characteristic cell wall composition place strain SF-2185 in the genus *Dactylosporangium* THIEMANN, PAGANI and BERETTA[7]. For this reason, strain SF-2185 was compared with those of known *Dactylosporangium* species. It was found that strain SF-2185 resembled *D. aurantiacum*[7]:

Table 2. Physiological properties of strain SF-2185.

Temperature range for growth	15~40°C (optimum 26~36°C)
Hydrolysis of starch	Positive
Liquefaction of gelatin	Negative
Reduction of nitrate	Negative
Peptonization of skim milk	Negative
Coagulation of skim milk	Negative
Formation of melanoid pigment	Negative
NaCl tolerance 1.5%:	Poor growth
3.0%:	No growth

Table 3. Carbon source utilization of strain SF-2185.

Utilization	Carbon source
Positive	D-Glucose, D-xylose, D-fructose, L-arabinose, D-mannitol, sucrose, L-rhamnose
Negative	*i*-Inositol, raffinose, glycerol

Basal medium: LUEDEMANN's medium[3].

Table 4. Differences between strain SF-2185 and *Dactylosporangium aurantiacum*.

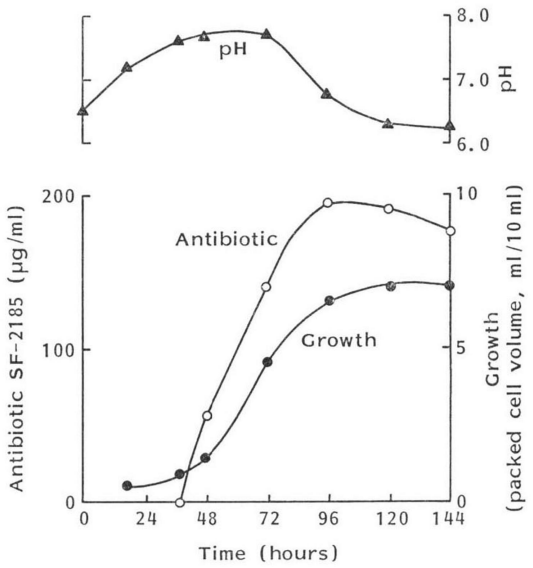
Test	Strain SF-2185	*D. aurantiacum* KCC A-0083
Reduction of nitrate	Negative	Positive
Liquefaction of gelatin	Negative	Positive
Growth at 42°C	None	Poor
Growth on 3% NaCl	None	Poor
Antibiotic produced	SF-2185	None

Table 5. Media used for production of antibiotic SF-2185.

Seed medium (%)		Production medium (%)	
Starch	2.0	Starch	3.0
Glucose	1.0	Soybean meal	1.5
Wheat germ	0.6	Distiller's solubles	0.8
Polypepton	0.5	Corn steep liquor	1.0
Yeast extract	0.3	$CaCO_3$	0.3
Soybean meal	0.2	NaCl	0.1
$CaCO_3$	0.1	$MgSO_4 \cdot 7H_2O$	0.05
pH	7.0	$CoCl_2 \cdot 6H_2O$	0.001
		pH	7.0

Fig. 2. Time course of antibiotic SF-2185 production in a 30-liter jar fermentor.

Antibiotic was quantitated by the control effect bioassay against downy mildew.



Morphological and cultural characteristics of strain SF-2185 were closely similar to those of *D. aurantiacum*, strain KCC A-0083 (=ATCC 23491, type strain), however differences were found in several respects as shown in Table 4.

Based on the above comparison, it was concluded that strain SF-2185 is a new subspecies of *D. aurantiacum*, designated as *D. aurantiacum* subsp. *gifuense* subsp. nov., referring to Gifu Prefecture where the organism was isolated. Strain SF-2185 is the type strain of *D. aurantiacum* subsp. *gifuense*. A culture of this strain has been deposited in the Fermentation Research Institute, Agency of Industrial Science and Technology, Japan, with an accession number of FERM-P 6308.

Fermentation

Two seed flasks (100 ml) containing 20 ml of the seed medium (Table 5) were inoculated with the mycelia of the organism grown on agar slant. The inoculated flasks were incubated at 28°C for 72

hours on a rotary shaker (model GR-3, Iwashiya Bio-Science Co., Ltd.) (200 rpm) after which the contents were pooled and used to inoculate a second seed consisting of 600 ml of the same medium in a 5-liter flask. The inoculated 5-liter flask was incubated with shaking at 28°C for 48 hours and the microbial growth was used to inoculate a 30-liter fermentor containing 20 liters of the production medium (Table 5). Fermentation was carried out at 28°C with an air-flow rate of 20 liters per minute and an agitation at 250 rpm. Antibiotic production in the culture filtrate was determined by the bioassay using the control effect test against downy mildew of cucumber *in vivo*. A maximum titer (about 200 μg/ml as antibiotic SF-2185) was achieved after 96-hour fermentation (Fig. 2).

Biological Activity

Antimicrobial Activity *In Vitro*

Antimicrobial activity of antibiotic SF-2185 was examined by the agar dilution method. Antibiotic SF-2185 showed weak activities against plant pathogenic bacteria and fungi, such as *Xanthomonas campestris* pv. *oryzae*, *X. campestris* pv. *citri* and *Pseudomonas syringae* pv. *tabaci* on MUKOH and WATANABE's agar medium, and *Pyricularia oryzae* and *Rhizoctonia solani* (*sasakii* type) on CZAPEK's agar medium (Table 6). The antibiotic was inactive against the above organisms on potato dextrose agar (PDA). In a preliminary experiment, the antibiotic had no effect on the movement of zoospores of *Pseudoperonospora cubensis* in the aqueous solution of the antibiotic.

Also, SF-2185 was inactive against 27 strains of Gram-positive or Gram-negative bacteria, including *Staphylococcus, Streptococcus, Bacillus, Escherichia, Salmonella, Proteus, Serratia, Klebsiella, Enterococcus, Shigella* and *Pseudomonas* on Mueller-Hinton agar medium.

Control of Plant Disease

Downy Mildew of Cucumber: Cucumber seedlings (*Cucumis sativus* L. cultivar. Tokiwa Jibai) grown in unglazed pot (9 cm in diameter, 3 seedlings/pot) filled with clayish loam soil were used in this study. The antibiotic was applied to the seedlings by the foliar or drench application technique

Table 6. Antimicrobial activity of antibiotic SF-2185 against phytopathogenic bacteria and fungi.

Test organisms	Media*	MIC (μg/ml)
Xanthomonas campestris pv. *oryzae*	A	50
X. campestris pv. *citri*	〃	400
Pseudomonas syringae pv. *tabaci*	〃	200
P. syringae pv. *lacrymans*	〃	>400
Erwinia carotovora subsp. *carotovora*	〃	>400
Corynebacterium michiganense pv. *michiganense*	〃	>400
Pyricularia oryzae	B	400
Alternaria kikuchiana	〃	>400
Botrytis cinerea	〃	>400
Fusarium oxysporum f. sp. *lycopersici*	〃	>400
Rhizoctonia solani	〃	>400
R. solani (*sasakii* type)	〃	400
Gibberella fujikuroi	〃	>400
Cochliobolus miyabeanus	〃	>400
Colletotrichum lagenarium	〃	>400
Diaporthe citri	〃	>400

* A: MUKOH and WATANABE's agar medium, B: CZAPEK's agar medium.

using 5 pots in a treatment.

Thus, in the foliar application technique, cucumber seedlings (1-leaf stage) were sprayed with the aqueous solution of the antibiotic containing 0.05% of Tween-20 in a final volume at a rate of 10 ml/pot. Then the seedlings were inoculated with conidial suspension of *P. cubensis* (about 10 conidia in a microscopic field at 150×) collected from diseased leaves. The inoculated seedlings were kept overnight in a moist chamber at 24~26°C and subsequently transferred in a green house (20~28°C).

In the drench application technique, cucumber seedlings were treated by pouring 40 ml of the aqueous solution of the antibiotic onto each pot, then the treated pots were kept in a green house (20~28°C). Seven days after the treatment, the seedlings were inoculated by spraying with conidial suspension to the leaves.

The degree of disease outbreak was graded seven days after inoculation according to the percentage of leaf area infected by the pathogen. The grading system is as follows;

Disease degree	Infected leaf area (%)	Disease degree	Infected leaf area (%)
Grade 0	0	3	21~ 40
1	1~ 5	4	41~ 70
2	6~20	5	71~100

The protective value of the antibiotic was calculated using the following equation.

$$\text{Protective value (\%)} = \left(1 - \frac{\text{Average of disease degree of the treated seedlings}}{\text{Average of disease degree of the non-treated seedlings}}\right) \times 100$$

To evaluate the effectivity of the antibiotic, TPN (daconil wettable powder containing 75% of tetrachloroisophthalonitrile, Takeda Chem. Ind., Ltd.) was used as a reference chemical. Antibiotic SF-2185 protected the cucumber seedlings from downy mildew at ≧12.5 μg/ml by the foliar application, and at 25 μg/ml by the drench application (Table 7). The protective dose for TPN was 375 μg/ml in the foliar application.

Blast of Rice: Rice seedlings (*Oryza sativa* L. cultivar. Jukkoku) grown in a plastic pot (6 cm in diameter, 8 seedlings/pot) filled with clayish loam soil, were used in this study. Five pots of the seedlings were used in a treatment.

In the foliar application technique, the rice seedlings (4-leaf stage) were sprayed with the aqueous solution of the antibiotic containing 0.05% of Tween-20 in a final volume at a rate of 10 ml/pot. In

Table 7. Control effect of antibiotic SF-2185 against downy mildew of cucumber.

Sample	Concentration (μg/ml)	Protective value (%)	
		Application	
		Foliar	Drench
SF-2185	100	100	100
	50	100	100
	25	100	92
	12.5	94	50
TPN	375	94	
Control (No treatment)		0	

Table 8. Control effect of antibiotic SF-2185 against blast of rice.

Sample	Concentration (μg/ml)	Protective value (%)	
		Application	
		Foliar	Drench
SF-2185	400	90	
	100	64	94
	50	37	82
EDDP	100	98	
Probenazole	100		95
Control (No treatment)		0	0

the drench application technique, rice seedlings (3-leaf stage) were treated by pouring 40 ml of the aqueous solution of the antibiotic onto each pot. The treated pots were put in the 1-liter beakers filled with water up to the surface of the soil to simulate paddy field conditions. The pots were incubated in a green house (22~35°C) until 4-leaf stage.

The treated seedlings were subsequently inoculated with *P. oryzae* by spraying the seedlings with conidial suspension (about 20 spores in a microscopic field at 150×). The inoculated seedlings were kept in a moist chamber for about 20 hours, then incubated in a green house (22~26°C) for seven days.

The lesions on the leaf were counted and protective values were calculated by the following equation.

$$\text{Protective value } (\%) = \left(1 - \frac{\text{Average lesion number of the treated seedlings}}{\text{Average lesion number of the non-treated seedlings}}\right) \times 100$$

EDDP (Hinosan emulsifiable concentrate containing 30% of *O*-ethyl-*S*,*S*-diphenyldithiophosphate, Nihon Tokushu Noyaku Seizo K.K.) and probenazole (Oryzemate granule containing 8% of 3-allyloxy-1,2-benzisothiazole-1,1-dioxide, Meiji Seika Kaisha, Ltd.) were used as reference chemicals in the foliar application and the drench application experiments, respectively.

Against rice blast, antibiotic SF-2185 was effective at 100 μg/ml in the drench application and at 400 μg/ml in the foliar application, though less effective than EDDP or Probenazole at 100 μg/ml (Table 8).

Other Biological Properties

Antibiotic SF-2185 was not active in mitecidal, anthelmintic, anticoccidial, antiviral and immunoadditive tests. The LD_{50} value tested by oral administration to mice was >415 mg/kg.

Discussion

Antibiotic SF-2185 is a novel azetidine antibiotic produced by a new subspecies of *D. aurantiacum* having a simple chemical structure.

The antibiotic is apparently active in the plant disease protection experiments, and possesses systemic control activity through root system against downy mildew of cucumber and rice blast. The antibiotic is weakly active or inactive *in vitro* against the microorganisms tested including these pathogens. These results indicate that the plant metabolism may convert the antibiotic to a more active substance, or the antibiotic may act against host plant to give protective conditions, although the mechanism of action should be resolved in further studies.

References

1) SHIRLING, E. B. & D. GOTTLIEB: Methods for characterization of *Streptomyces* species. Int. J. Syst. Bacteriol. 16: 313~340, 1966
2) WAKSMAN, S. A.: The Actinomycetes. Vol. II. Classification, Identification and Description of Genera and Species. The Williams & Wilkins Co., Baltimore, 1961
3) LUEDEMANN, G. M. & B. C. BRODSKY: Taxonomy of gentamicin-producing *Micromonospora*. Antimicrob. Agents Chemother.-1963: 116~124, 1964
4) JACOBSON, E.; W. C. GRAUVILLE & C. E. FOGS: Color Harmony Manual. 4th Ed. Container Corporation of America, Chicago, 1958
5) BECKER, B.; M. P. LECHEVALIER, R. E. GORDON & H. A. LECHEVALIER: Rapid differentiation between *Nocardia* and *Streptomyces* by paper chromatography of whole-cell hydrolysates. Appl. Microbiol. 12:

421~423, 1964
6) LECHEVALIER, M. P.: Identification of aerobic actinomycetes of clinical importance. J. Lab. Clin. Med. 71: 934~944, 1968
7) THIEMANN, J. E.; H. PAGANI & G. BERETTA: A new genus of the Actinoplanaceae: *Dactylosporangium*, gen. nov. Arch. Mikrobiol. 58: 42~52, 1967
8) SHARPLES, G. P. & S. T. WILLIAMS: Fine structure of the globose bodies of *Dactylosporangium thailandense* (Actinomycetales). J. Gen. Microbiol. 84: 219~222, 1974
9) GREIG, C. G. & D. H. LEABACK: Use of chroline in the detection of compounds on paper chromatograms. Nature 188: 310~311, 1960