FR-900137, A NEW ANTIBIOTIC

I. TAXONOMY AND FERMENTATION OF THE ORGANISM, AND ISOLATION AND CHARACTERIZATION OF THE ANTIBIOTIC

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A strain of *Streptomyces*, isolated from a soil sample and identified as *Streptomyces unzenensis* sp. nov. has been found to produce FR-900137, an interesting new antibiotic, containing phosphorus in its molecule. The antibiotic, obtained as white powder, was shown to inhibit a wide variety of Gram-positive and Gram-negative bacteria except *Pseudomonas aeruginosa*.

During the course of a screening program directed toward the isolation and evaluation of new cell wall-inhibitory antibiotics, we isolated a strain of *Streptomyces* designated strain No. 2050, which was found to produce a new antibiotic, FR-900137. The antibiotic was detected in the fermentation broth by the use of 7-aminocephalosporanic acid-supersensitive mutant of *Pseudomonas aeruginosa*. This new antibiotic proved to be of considerable interest because of its chemical structure containing phosphorus and its potent activity against *Escherichia coli*.

In this report, we describe characterization of the producing organism, fermentation and isolation procedures, and chemical and biological properties of FR-900137.

Taxonomic Studies on Strain No. 2050

Strain No. 2050 which produces FR-900137 is an actinomycete isolated from a soil sample obtained at Unzen, Nagasaki Prefecture, Japan.

The methods and media of taxonomic studies recommended by the International Streptomyces Project (ISP)[1] were used primarily, along with several supplementary tests. All tests were run at 30°C.

Microscopic observations were made on cultures that were grown from 7 to 10 days on sucrose-nitrate agar, glycerin-asparagine agar, starch-inorganic salts agar, yeast-malt extract agar and oatmeal agar. Sporophores are compact and/or closed spirals with 10 to more than 50 spores per chain. The spore chain morphology is classified in the *Retinaculiaperti* section (Fig. 1). The spores are oval, averaging $0.5 \sim 1.0$ by $1.1 \sim 2.0$ μ in size, with smooth surface (Fig. 2). Sporangia or zoospores are not observed. Neither fragmentation of hyphae nor formation of spores occurred in the substrate mycelium.

Colony characteristics were observed on slant cultures after 7 and 14 days of incubation. The cultural characteristics of strain No. 2050 is presented in Table 1. On most media, pale yellow vegetative growth develops moderately and the aerial mass is powdery and light gray. No soluble pigment is formed in the media except yeast-malt extract agar.

Fig. 1. Aerial mycelium of strain No. 2050. Yeast-malt extract agar, 7 days at 28°C.

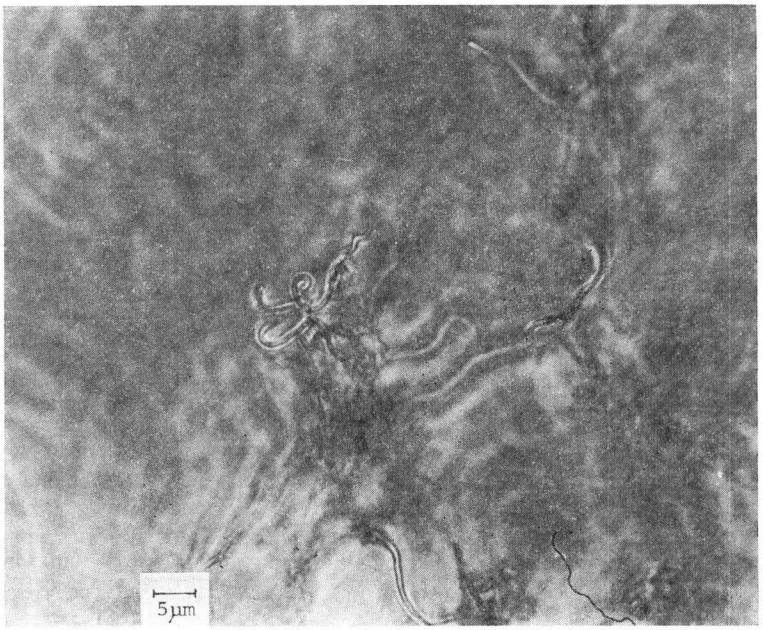
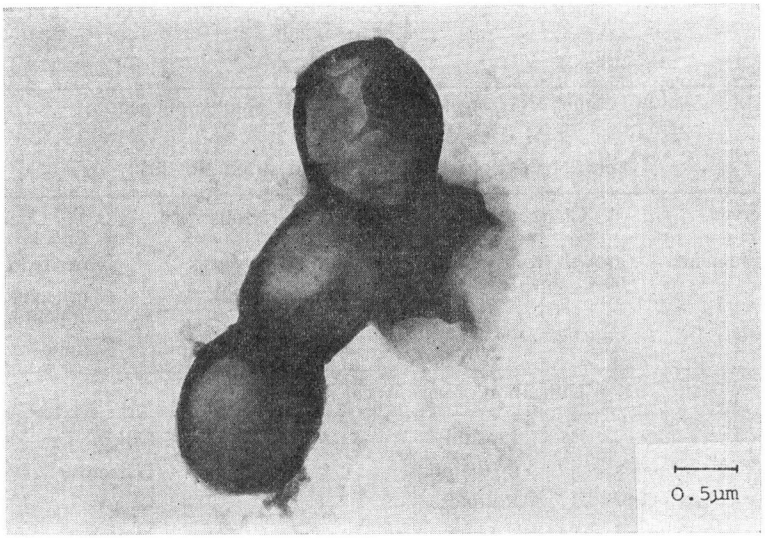


Fig. 2. Spores of strain No. 2050. Yeast-malt extract agar, 10 days at 28°C.



Summarized physiological properties are shown in Table 2. Starch is not hydrolyzed by strain No. 2050. The hydrolytic activity on gelatin or milk is good. Carbon utilization tests were made according to PRIDHAM-GOTTLIEB method[2]. Maltose and glycerin are well utilized, and D-glucose and galactose are fairly utilized for growth of the organism. The procedure of BECKER *et al.*[3] was used for preparation of cells and chromatographic detection of the isomers of diaminopimelic acid. Whole cell hydrolysates contain L,L-diaminopimelic acid.

Microscopic studies and cell wall components of strain No. 2050 indicate that this strain belongs to the genus *Streptomyces*. Accordingly, a comparison of this organism was made with published

Table 1. Cultural characteristics of strain No. 2050.

Medium	Characteristics	Medium	Characteristics
Sucrose-nitrate agar	AM: very thin, gray, powdery VG: colorless, small colonies SP: none	Nutrient agar	AM: none VG: pale yellow, small colonies SP: none
Glucose-asparagine agar	AM: thin, white, powdery VG: colorless to pale yellow, small colonies SP: none	Yeast-malt extract agar	AM: gray, powdery VG: pale yellow, slightly wrinkled SP: olive green
Glycerin-asparagine agar	AM: grayish white, powdery GV: pale yellow, small colonies SP: none	Oatmeal agar	AM: light gray, powdery VG: colorless, small colonies SP: none
Starch-inorganic salts agar	AM: thin powdery, light gray VG: colorless, small colonies SP: none	Glucose-peptone gelatine stab	AM: light gray, powdery VG: colorless to pale yellow SP: none
Tyrosine agar	AM: gray, powdery VG: pale yellow, small colonies SP: none	Milk	AM: thin, white powdery VG: colorless to pale yellow SP: none
		Peptone-yeast iron agar	AM: none VG: colorless to pale yellow, small colonies SP: none

Symbols: AM: aerial mycelium; VG: vegetative growth; SP: soluble pigment.

Table 2. Physiological properties of strain No. 2050

Property observed	Characteristics	Property observed	Characteristics
Temperature requirements	growth from 15°C to 40°C, opt. 28°C	Starch hydrolysis	no hydrolyzed
		Action on milk	no coagulation, rapid peptonization
Gelatin liquefaction	liquefied	Melanin production	none

Utilization of various carbon compounds

L-Arabinose	−	Inositol	−	Salicin	±
D-Xylose	−	L-Rhamnose	−	Galactose	+
D-Glucose	+	Raffinose	−	Lactose	−
D-Fructose	−	D-Mannitol	−	Maltose	++
Sucrose	±	Mannose	±	Glycerin	++

Symbols: ++, good utilization; +, fair utilization; ±, doubtful utilization; −, no utilization.

descriptions[4~8] of *Streptomyces* species. From the above-mentioned information, strain No. 2050 is considered to resemble *Streptomyces amakusaensis*, *Streptomyces omiyaensis*, and *Streptomyces viridifaciens*. It was found, however, that these species were differentiated from strain No. 2050 as follows:

Streptomyces amakusaensis: Melanoid pigment is formed. Aerial mycelium is poor on oatmeal agar. Aerial mass color is blue on starch-inorganic salts agar.

Streptomyces omiyaensis: Spore chain morphology is classified in the *Rectiflexibilis* section. D-Xylose and L-rhamnose are utilized for growth.

Streptomyces viridifaciens: L-Arabinose, D-xylose, and D-fructose are well utilized for growth.

As a result of the above comparisons, strain No. 2050 is considered a new species of genus *Streptomyces*. The name *Streptomyces unzenensis* is proposed for strain No. 2050, referring to the source of the soil from which the organism was isolated. This strain has been deposited in Fermentation Research Institute, Agency of Industrial Science and Technology, Japan as FERM-P 4385.

Fermentation

The growth of *Streptomyces unzenensis* sp. nov. on the mature slant culture was used to inoculate four 500-ml flasks containing 100 ml of sterile growth medium. The flasks were shaken on a rotary shaker (220 rpm, 2 inch throw) for three days. The content of the flasks was used to inoculate 20 liters of fermentation medium in stainless steel fermentor. The composition of the media are as follows: Seed medium, potato starch 1%, glycerin 1, cotton seed meal 1, dried yeast 1; Production medium, soluble starch 5.0%, cotton seed meal 0.5, gluten meal 2.5, $MgSO_4 \cdot 7H_2O$ 1.0, KH_2PO_4 1.0, $Na_2HPO_4 \cdot 12H_2O$ 0.7.

The culture was incubated at 30°C for three days, aerated at 20 liters per minute and agitated at 300 rpm. Progress of fermentation was monitored by paper disc-agar diffusion assay of the supernatant fluid from centrifuged broth sample (3,000 rpm for 30 minutes). *Pseudomonas aeruginosa* IV, a 7-aminocephalosporanic acid-supersensitive mutant, was used as a test organism with MUELLER-HINTON medium (Eiken Kagaku Co.,) for the bioassay.

Isolation Procedure

The broth filtrate (150 liters) was concentrated to 20 liters at pH 8.0 and 80 liters of methanol were added. The precipitate formed was discarded. The supernatant was concentrated to 30 liters of aqueous solution. The solution was passed through a column containing 15 liters of activated carbon. The column was then eluted with 70% aqueous methanol (50 liters). After concentration to 20 liters, the concentrate was applied to a column of DEAE Sephadex (OH^- form) and the resin was eluted with 0.1 N NaOH solution (30 liters). The eluate was adjusted to pH 8.0 with Duolite C-20 (H^+ form) and was concentrated to 10 liters. The concentrate was applied to a column of Diaion HP-20 (10 liters) and the activity was eluted with water. The active fractions were then adsorbed onto a column of CM Sephadex (pH 6.5, buffered) and the antibiotic was eluted with water. The eluate was concentrated to a volume of 500 ml, which was applied to a column of Sephadex G-15 (5 liters). The antibiotic was developed with water. The active fractions were collected and lyophilized to give white powder. One gram of pure antibiotic FR-900137 was obtained from 150 liters of fermentation broth.

Physicochemical Properties

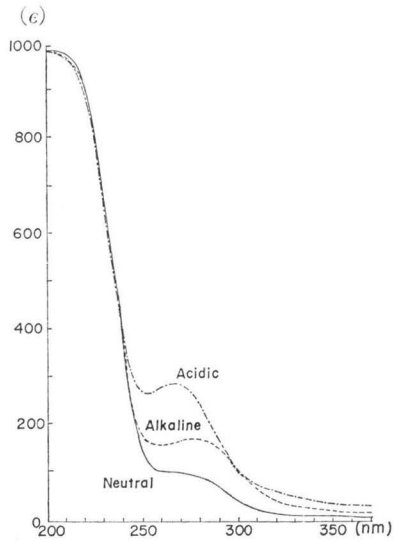
FR-900137 is a white powder which melts at 134°C (decomp.). The antibiotic activity is unstable in acidic solution. It is soluble in methanol, water, slightly soluble in ethanol, insoluble in acetone, ethyl acetate, and chloroform. The optical rotation is $[\alpha]_D^{20}$ +31.6 (*c* 1, H_2O). Potentiometric titration gave an equivalent weight of 270. Elemental analysis gave the following composition.

Calcd. for $C_8H_{20}N_3O_4P$:	C 37.94, H 7.91, N 16.60, P 12.25
Found:	C 38.23, H 8.18, N 16.50, P 11.78

Table 3. Chromatographic behavior of FR-900137.

T.L.C.	Solvent system	Rf
Cellulose	*n*-Butanol saturated with water	0.25
	n-Propanol - water (7: 3)	0.70
Alumina	Chloroform - methanol (2: 1)	0
Silica gel	Chloroform - methanol (1: 1)	0.15

Fig. 3. UV spectrum of FR-900137 (H_2O).



Inspection of the signals observed in ^{13}C-nmr spectrum supported the proposed molecular formula. Color reactions are as follows: positive in ninhydrin, molybdate, iodine, and potassium permanganate tests, negative in MOLISCH and ferric chloride tests. Rf values of FR-900137 on TLC are summarized in Table 3.

The ultraviolet spectrum is shown in Fig. 3. IR spectrum is shown in Fig. 4, with the following significant absorption maxima (KBr): 3200, 2950, 1690 (amide I), 1530 (amide II), 1470, 1230 (P=O), 1070, 1050 (C–O), 790 cm^{-1}. As shown in Fig. 5, the ^1H-nmr spectrum has the following characteristics: methyl protons at δ 0.95 (3H × 2, d, J = 6 Hz), 1.60 (1 H, m), 1.70 (2 H, m), N-methyl protons at δ 2.80 (3 H, d, J = 8 Hz), O-methyl protons at δ 3.56 (3 H, d, J = 11 Hz), and α-methine proton of amino acid at δ 3.90 (1 H, m). This nmr spectrum indicates the presence of leucine moiety.

Biological Properties

The antibacterial activity of FR-900137 is shown in Table 4. One loopful of the culture of test organism (approximately 10^6 cells/ml) was streaked on agar plates containing two-fold decremental

Fig. 4. IR spectrum of FR-900137 (KBr).

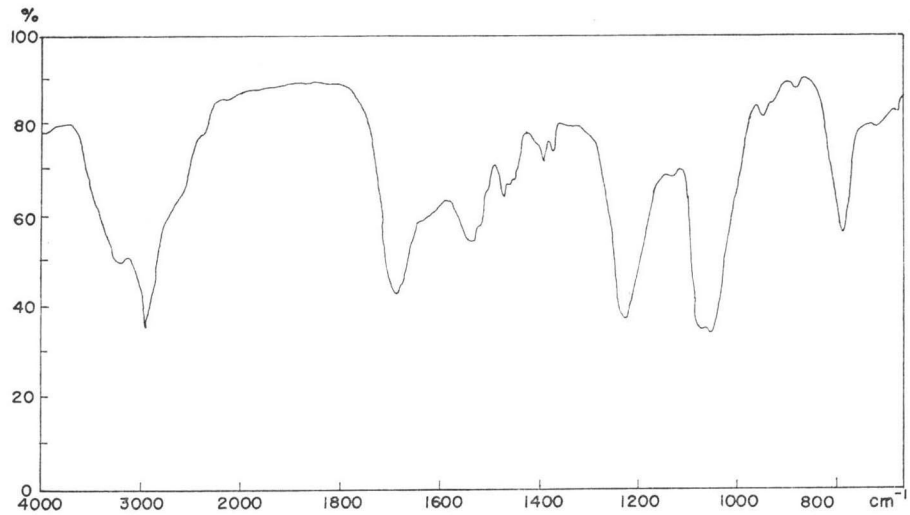
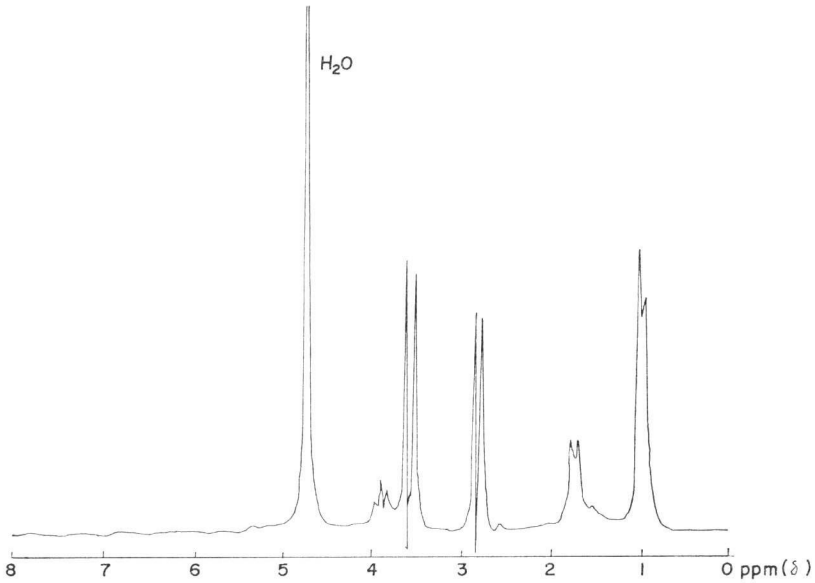


Fig. 5. ^1H nmr spectrum of FR-900137 (D_2O).



dilutions of the antibiotic. The inoculated plates were incubated for 18~24 hours at 37°C. FR-900137 showed selective antibacterial activity against *Escherichia coli*, and *Bacillus subtilis* and moderate effect on *Staphylococcus aureus* and *Proteus*. It had no inhibitory activity against *Pseudomonas aeruginosa*. *Pseudomonas aeruginosa* IV, a supersensitive mutant to 7-aminocephalosporanic acid, was inhibited at 6 μg/ml of the antibiotic. FR-900137 showed no activity against *Mycoplasma and Acholeplasma*, which have no cell wall. Cells of *Escherichia coli* were treated with a lethal concentration of FR-900137 in a hypertonic medium. All cells were transformed into a spherical form resembling spheroplast caused by penicillin but smaller in size. Though it may have inhibitory activity on bacterial cell-wall metabolism, the precise mechanism of its antibacterial action is unknown.

Table 4. Antimicrobial spectrum of FR-900137.

Test organism	MIC (μg/ml)[1]
Staphylococcus aureus FDA 209P	100
Staphylococcus aureus 279	100
Bacillus subtilis ATCC 6633	3
Escherichia coli NIHJ JC-2	3
Escherichia coli 1341-1	6
Escherichia coli 1341-18	<1.5
Escherichia coli 1341-22	6
Escherichia coli 1341-28	3
Proteus vulgaris IAM 1095	3
Proteus mirabilis 1	50
Proteus rettgeri 15	50
Klebsiella pneumoniae NCTC 418	50
Pseudomonas aeruginosa NCTC 10490	>800
Pseudomonas aeruginosa IV[2]	6
Mycoplasma gallisepticum PG-1[3]	>800
Acholeplasma laidlawii A LD-1[3]	>800

1) MIC test was conducted by the usual serial agar dilution method using nutrient agar
2) A mutant strain supersensitive to 7-aminocephalosporanic acid.
3) PPLO-medium containing 20% serum and 0.1% yeast extract.

Each of five ICR mice (20~25 g in weight) was given a single intravenous dose of 20 mg (0.8~1.0 g/kg) and all survived. During 14 days of observation after injection, no toxic symptom was ob-

served. FR-900137 was found to contain phosphorohydrazidate but it showed no mutagenic effect against *Bacillus subtilis* M-45 in Rec⁻ assay method described by KADA *et al.*[9]

Discussion

The discovery of nocardicin A[10] and FR-900098[11] by the use of the cell-wall inhibitor—supersensitive mutant system proved the usefulness of this screening system. We undertook, therefore, a screening program using a mutant strain of *Pseudomonas aeruginosa* IV, which was selected as a 7-aminocephalosporanic acid-supersensitive mutant (Table 5). FR-900137 was detected by this mutant strain. But the mechanism of the cross-sensitivity between FR-900137 and 7-aminocephalosporanic acid observed in this mutant organism is not obvious.

The data described in this report characterize FR-900137 as an amphoteric, water-soluble, phosphorus-containing substance. Several phosphorus-containing antibiotics have been reported. FR-900137 is distinct from any of these antibiotics[11~15] in its chemical properties. It has been found that the antibiotic contains a phosphorohydrazidate moiety in the molecule as shown in Fig. 6.

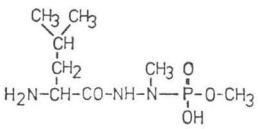
The details of structure elucidation will be the subject of the succeeding paper. FR-900137 shows antibiotic activity particularly against *Escherichia coli*. The activity of this antibiotic may be associated with cell wall metabolism. But the mechanism of its antibacterial action has not been elucidated.

Table 5. Susceptibility of strains *Ps.* and *Ps.* IV to various kinds of antibiotics.

Antibiotics	M.I.C. (μg/ml)	
	Ps.	*Ps.* IV
7-Aminocephalosporanic acid	3,000	0.8
Nocardicin A	25	0.2
Cephalosporin C	3,000	0.4
Penicillin G	3,000	0.2
Cephamycin C	800	0.2
Fosfomycin	50	0.4
FR-900098	200	0.8
Chloramphenicol	100	50.0
Kanamycin	50	12.5

MIC was determined by usual serial agar dilution method using nutrient agar medium.

Fig. 6. Structure of FR-900137.



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