FR-900148, A NEW ANTIBIOTIC

I. TAXONOMY, FERMENTATION, ISOLATION AND CHARACTERIZATION

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A strain of *Streptomyces*, isolated from a soil sample and identified as *Streptomyces xanthocidicus*, has been found to produce FR-900148, a new antibiotic containing chlorine in its molecule. The antibiotic inhibits both Gram-positive and negative bacteria. However, it is not effective against wild type of *Pseudomonas aeruginosa*. Its antibacterial action is considered to result from cell wall synthesis inhibition since it causes spheroplast formation from susceptible cells.

In the course of screening for new cell wall-inhibitory antibiotics, we isolated a strain of *Streptomyces* designated No. 301 from a soil sample collected at Osaka City. This strain was found to produce a new antibiotic, FR-900148, which was detected in the fermentation broth by the use of a 7-aminocephalosporanic acid-supersensitive mutant of *Pseudomonas aeruginosa*. This antibiotic proved to be of considerable interest because its chemical structure contains chlorine and it has low toxicity in experimental animals. In this report, we describe the taxonomy of the producing organism, fermentation and isolation procedures for obtaining FR-900148 and chemical and biological properties of the substance.

Taxonomy

Strain No. 301 was isolated from a soil sample collected at Osaka City in Japan. The microorganism was identified as a strain of *Streptomyces xanthocidicus*[1~3]. Its characteristics are as follows:

The aerial mycelium is monopodially branched, generally long and straight to flexuous, with 10 to often more than 50 spores per chain. The spore-chain morphology is classified in the *Rectiflexibilis* section (Fig. 1). Spores are oblong to cylindrical, averaging 0.5~1.0 by 1.0~1.8 μm in size, with a smooth surface (Fig. 2). No sporangium or zoospore was observed. Neither fragmentation nor formation of spores occurred in the vegetative mycelium.

The cultural characteristics and a summary of the physiological properties are shown in Tables 1 and 2, respectively. On most media, strain 301 develops moderate pale-yellow-brown vegetative growth and the aerial mycelium is velvety and light gray. Melanoid pigment is occasionally formed on proteinous media, but this reaction is variable. Starch hydrolysis is strongly positive. The hydrolytic activity on gelatin or milk is good. L-Arabinose, D-glucose, glycerin, and sucrose are utilized well, and D-fructose, D-galactose, maltose and D-xylose are utilized fairly well. A whole cell hydrolysate of strain No. 301 contains L,L-diaminopimelic acid.

The microbiological characteristics of strain No. 301 described above are in good agreement

Fig. 1. Aerial mycelium of strain No. 301.
Yeast-malt extract agar, 7 days at 30°C.

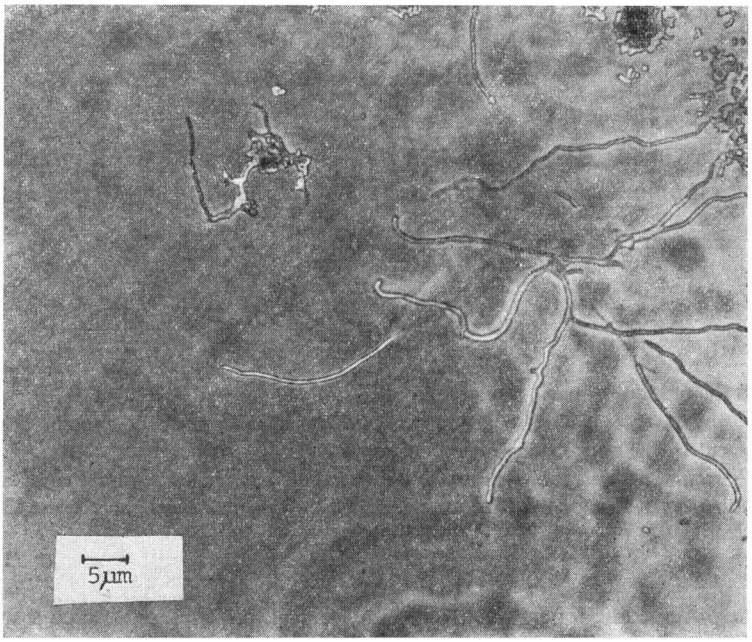
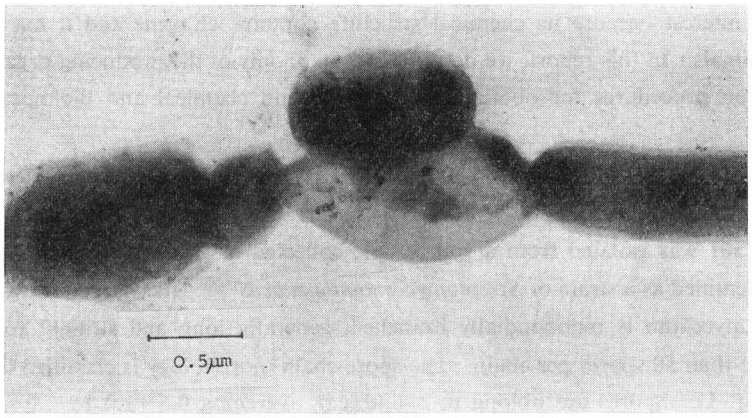


Fig. 2. Spores of strain No. 301.
Yeast-malt extract agar, 10 days at 30°C.



with those of *Streptomyces xanthocidicus*[1~3]. *Streptomyces xanthocidicus* No. 301 has been deposited with the Fermentation Research Institute, Agency of Industrial Science and Technology, Japan, as FERM-P 4302.

Fermentation

A mature slant culture of *Streptomyces xanthocidicus* was used to inoculate flasks containing 100 ml each of sterile growth medium. The flasks were shaken (220 rpm, 2-inch throw) for three days and the contents were used to inoculate 20 liters of fermentation medium in a stainless steel fermentor. Media compositions are given in Table 3. The culture was incubated at 30°C for 3 days,

Table 1. Cultural characteristics of strain No. 301.

Medium	Characteristics	Medium	Characteristics
Sucrose-nitrate agar	AM: gray, powdery VG: pale yellowish brown to light brown, small colonies SP: none	Yeast-malt extract agar	AM: white to gray, powdery to short cottony VG: pale yellowish brown, small colonies, slightly wrinkled SP: none
Glucose-asparagine agar	AM: light gray, powdery VG: pale yellow, small colonies SP: none or trace	Oatmeal agar	AM: gray, powdery VG: colorless to pale yellow, small colonies SP: none or trace
Glycerin-asparagine agar	AM: light gray, powdery to velvety VG: pale yellow, small colonies SP: none or trace	Peptone-yeast iron agar	AM: none VG: colorless, flat to small colonies SP: trace of brown
Starch-inorganic salts agar	AM: gray, velvety VG: pale yellow, small colonies SP: none	BENNETT agar	AM: gray, powdery VG: pale yellowish brown, small colonies SP: none
Tyrosine agar	AM: light gray, powdery VG: pale yellowish brown, small colonies SP: none or trace of brown	Glucose-peptone gelatin stab	AM: white, thin powdery VG: pale yellow, wrinkled colonies SP: none or brown
Nutrient agar	AM: none or very thin, white VG: pale yellow to cream, small colonies SP: none	Milk	AM: none VG: scant growth SP: none or trace of brown

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

Table 2. Physiological properties of strain No. 301.

Property observed	Characteristics	Utilization of various carbon compounds			
Temperature requirements	growth from 15°C to 40°C optimum 30°C	L-Arabinose	++	D-Mannitol	−
		D-Xylose	+	Mannose	−
Gelatin liquefaction	strongly liquefied	D-Glucose	++	Salicin	−
Starch hydrolysis	hydrolyzed	D-Fructose	+	Galactose	+
		Sucrose	++	Lactose	−
Action on milk	coagulation, no peptonization	Inositol	−	Maltose	+
		L-Rhamnose	−	Glycerin	++
Melanin production	variable	Raffinose	±		

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

aerated at 20 liters per minute and agitated at 300 rpm. The progress of the fermentation was monitored by paper disc agar-diffusion assay of the filtrate for inhibition of *Pseudomonas aeruginosa* IV, a 7-aminocephalosporanic acid-supersensitive mutant grown on MUELLER-HINTON medium (Eiken Kagaku Co.).

Table 3. Media used for the production of FR-900148.

Seed medium	% (w/v)
Potato starch	1
Glycerin	1
Cotton seed meal	1
Dried yeast	1
Production medium	**% (w/v)**
Soluble starch	2.00
Corn steep liquor	0.25
Dried yeast	0.25
Cotton seed meal	0.25
Wheat germ	0.50
KH_2PO_4	0.50
$Na_2HPO_4 \cdot 12H_2O$	0.50
$CoCl_2 \cdot 6H_2O$	1.25×10^{-4}

Fig. 3. Isolation procedure of FR-900148.

Filtrate
↓ acidified with HCl to pH 2
Carbon column
↓ eluted with 70% aqueous acetone
concentrated
DEAE Sephadex (buffered, pH 6.5)
↓ eluted with 0.2 M NaCl
Carbon column
↓ eluted with water
CM Sephadex (H^+ cycle)
↓ eluted with water
concentrated
Sephadex G-15
↓ developed with water
Active fraction
↓ adjusted to pH 6.5
freeze-dry
White powder

Isolation Procedure

The purification method is outlined in Fig. 3. The broth filtrate (18 liters) was adjusted to pH 2.0 with 6 N HCl. The acidified solution was passed through a column of activated charcoal (4 liters) and the adsorbent was washed with water. The active principle was eluted with 5 liters of 70% aqueous acetone. Active fractions were concentrated to give 1.5 liters of aqueous solution which was passed through a column of DEAE-Sephadex (buffered at pH 6.5, 1 liter). The antibiotic was eluted with 0.2 M NaCl solution and again adsorbed on to a column of activated charcoal (2 liters). The active substance was eluted with water (4 liters) in this case, and the active fractions were concentrated to a volume of 100 ml. This concentrate was passed through a column of CM-Sephadex (H^+ cycle, 1 liter) and the antibiotic was eluted with water. After adjustment of the pH to 6.5, the eluate, concentrated to 200 ml, was applied to Sephadex G-25 (2 liters) and the antibiotic was eluted with water. The active fractions adjusted to pH 6.5, were concentrated and freeze-dried to give a white powder. From 20 liters of fermentation broth, 500 mg of pure antibiotic FR-900148 was obtained.

Physico-chemical Properties

FR-900148 melts from 143~147°C (decomp.). Antibiotic activity is lost when solutions in 0.1 N HCl or 0.1 N NaOH are kept at 20°C for 48 hours. The antibiotic is not extractable from water into organic solvents. It is soluble in water and methanol, slightly soluble in ethanol and insoluble in acetone, ethyl acetate or chloroform.

Measurements of optical rotation gave $[\alpha]_D^{20}$ +77.1 (*c* 0.75, H_2O). Potentiometric titration gave an equivalent weight of 330 with pKa values of 3.25 and 7.90. Elemental analysis indicated the following composition:

Calcd. for $C_{10}H_{12}N_2O_4Cl\,Na \cdot 2H_2O$
C 37.68, H 5.02, N 8.79, Cl 11.15, Na 7.22
Found C 37.48, H 5.32, N 8.71, Cl 10.55, Na 7.54

Observation of ten carbon signals in the ^{13}C-nmr spectrum supported the proposed formula. Color reactions were as follows: positive in ninhydrin, negative in EHRLICH and DRAGENDORFF tests. Thin-layer chromatography (TLC) of FR-900148 gave the results summarized in Table 4. In the ultraviolet region, the spectrum in water shows end-absorption. The infrared absorption spectrum shown in Fig. 4, has the following significant absorption maxima (KBr): 3250, 3050 (C=C–H), 2900, 1700~1500 (amides and C=C), 1370, 1250, 670 (C–Cl) cm^{-1}. As shown in Fig. 5, the ^1H-nmr spectrum has the

Fig. 4. IR spectrum of FR-900148 (KBr).

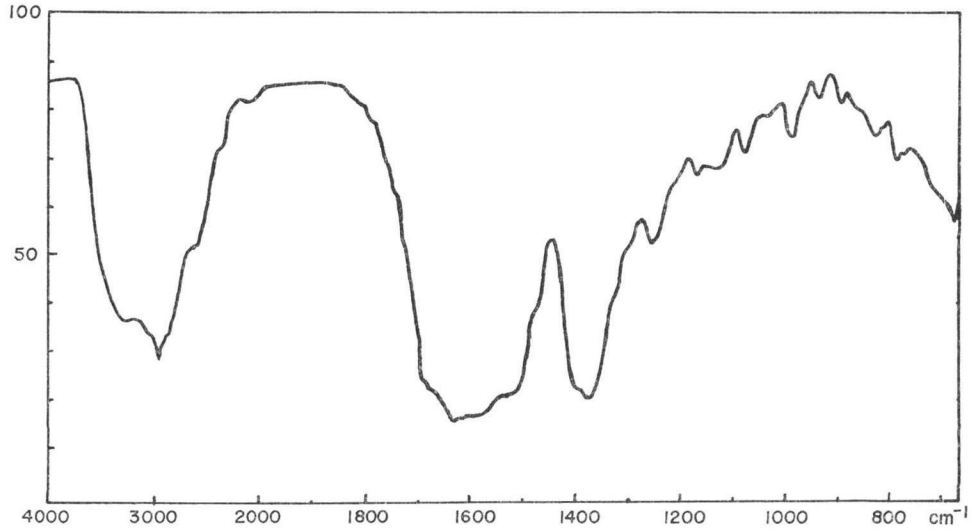
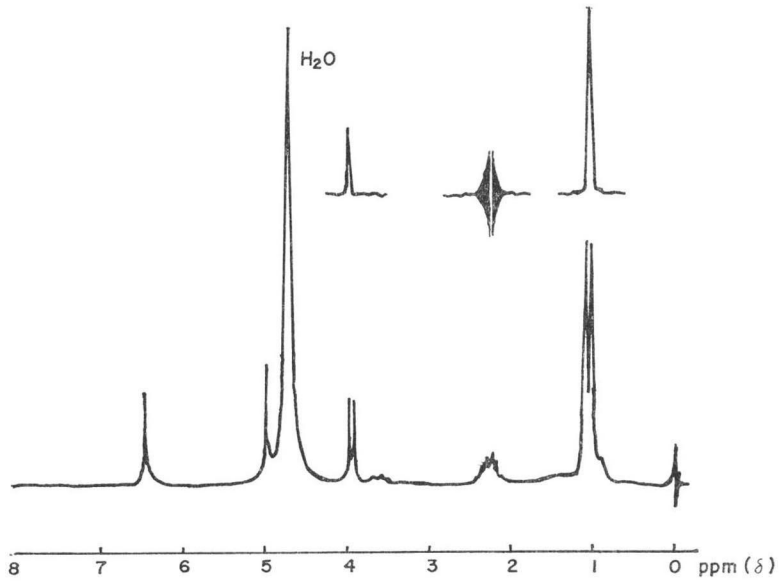


Fig. 5. ^1H-NMR spectrum of FR-900148 (D_2O)



following characteristics: δ 1.04 (6H, d, J=7 Hz), δ 2.25 (1 H, m), δ 3.92 (1 H, d, J=7 Hz), δ 4.98 (1 H, s), δ 6.46 (1 H, s). Decoupling experiment indicated the presence of valine in the molecule.

Biological Properties

The antibacterial activity of FR-900148 is shown in Table 5. One loopful of inoculum of approximately 10^5 cells in one ml of medium was streaked on agar plates containing two-fold decremental dilutions of the antibiotics. The inoculated plates were incubated for 18 hours to 24 hours at 37°C. FR-900148 showed antibacterial activity against Gram-positive and Gram-negative bacteria but the spectrum did not include a wild-type strain of *Pseudomonas aeruginosa*. The antibiotic has no activity against *Mycoplasma* or *Acholeplasma* which lack cell walls. When cells of *Escherichia coli* were treated with a lethal concentration of FR-900148 in hypertonic medium, most of them were transformed into spheroplasts (Fig. 6). Thus, it seems likely that the antibiotic inhibits bacterial cell wall synthesis.

To each of five ICR mice (20~25 g), was given a single intravenous dose of 80 mg (3~4 g/kg body weight) and no toxic symptoms were observed.

Table 4. Chromatographic behavior of FR-900148.

T.L.C.	Solvent system	Rf
Silica gel	*n*-Propanol - water (6: 4)	0.65
Cellulose	*n*-Butanol saturated with water	0
	n-Butanol - acetic acid - water (4: 1: 1)	0.45
	n-Propanol - water (6: 4)	0.60

Table 5. Antibacterial spectrum of FR-900148.

Test organism	M.I.C. (μg/ml)[1]
Staphylococcus aureus 209P JC-1	800
Staphylococcus aureus Newman	3.1
Staphylococcus aureus ATCC 65389	400
Staphylococcus aureus Smith	3.1
Bacillus subtilis ATCC 6633	200
Sarcina lutea PCI 1001	50
Streptococcus pneumoniae III	100
Streptococcus hemolyticus A-5-8	400
Streptococcus faecalis 6733	50
Corynebacterium diphtheriae M406MGL	50
Escherichia coli NIHJ JC-2	12.5
Escherichia coli K-12	1.6
Klebsiella pneumoniae NCTC 418	800
Proteus vulgaris IAM-1025	400
Salmonella typhi T-287	0.4
Salmonella paratyphi B 8006	100
Shigella flexneri Ia EW-8	50
Pseudomonas aeruginosa IAM-1095	>800
Pseudomonas aeruginosa NCTC 10490	>800
Pseudomonas aeruginosa IV[2]	25
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 of *P. aeruginosa* NCTC 10490 which is supersensitive to 7-aminocephalosporanic acid.
3) PPLO-medium containing 20% serum and 0.1% yeast extract.

Discussion

Discovery of nocardicin A[4] and FR-900098[5] proved the usefulness of a screening system with mutants supersensitive to cell-wall inhibitors. We undertook, therefore, to screen with a mutant strain selected from *Pseudomonas aeruginosa* NCTC 10490 for its sensitivity to 7-aminocephalosporanic acid. FR-900148 has been detected with this organism (Table 6).

The properties described in this paper characterize FR-900148 as an amphoteric, water-soluble, electrophoretically mobile, cell wall-inhibitory, chlorine-containing substance containing valine.

Fig. 6. Morphology of cells treated with FR-900148.
One ml of logarithmic culture of *E. coli* NIHJ JC-2 was transferred to 2 ml of nutrient broth containing 30% sucrose and 1,000 μg/ml of FR-900148.
After incubation for 6 hours, cell morphology was examined under microscope.

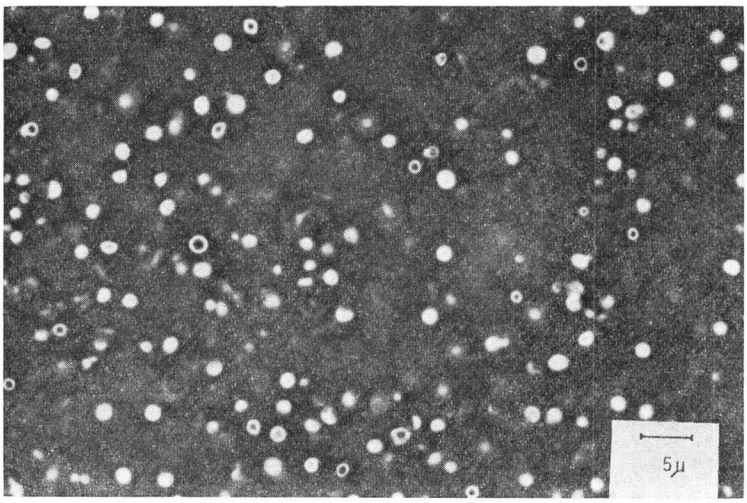
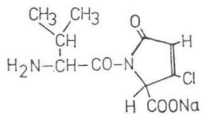


Fig. 7. Structure of FR-900148.



Several chlorine-containing antibiotics have been reported[6~8]. FR-900148 is different from all of them in its chemical properties. Elucidation of its chemical structure (Fig. 7) will be reported in a succeeding paper.

Its activity against bacteria and low toxicity in experimental animals suggest that the antibiotic has potential as an effective chemotherapeutic agent.

Table 6. Susceptibility of strains *Ps.* and *Ps.* IV to various kinds of antibiotics.

Antibiotics	M.I.C. (μg/ml)[1]	
	Ps.[2]	*Ps.* IV[3]
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

1) MIC was determined by the usual serial dilution method using nutrient agar medium.
2) *Pseudomonas aeruginosa* NCTC 10490.
3) A mutant strain of *Pseudomonas aeruginosa* NCTC 10490 which was selected as a supersensitive strain to 7-aminocephalosporanic acid.

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