NEW ANTIBIOTICS PRODUCED BY BACTERIA GROWN ON *n*-PARAFFIN (MIXTURE OF C_{12}, C_{13} AND C_{14} FRACTIONS)

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Pseudomonas fluorescens KY 4032, when grown on *n*-paraffin (mixture of C_{12}, C_{13} and C_{14} fractions) as the sole source of carbon, produced antibacterial substances in the culture medium. Two kinds of new substances named as fluopsins C and F, $(C_2H_4NOS)_2Cu$ and $(C_2H_4NOS)_3Fe$, were isolated through the extraction of the culture broth with chloroform and the chromatography on silicic acid column. Fluopsin C contains copper atom in the molecule while fluopsin F does iron instead of copper. The biological activities of these compounds were remarkable against most of both gram-positive and gram-negative bacteria. For the production of these antibiotics by this microorganism, *n*-paraffin was the preferable carbon source. The production of fluopsin C was apparently dependent on the quantity of copper ion added to the culture medium. In the limited concentration of copper ion the major product was replaced by fluopsin F.

Recently, there have been many reports concerning with the microbial production of useful materials from hydrocarbons. However, little is known about the production of antibiotics by *n*-paraffin-grown bacteria except for that of pyocianine.[1]

In the course of screening studies, we found that *Pseudomonas fluorescens* grown on *n*-paraffin as the sole source of carbon produced some antibacterial materials in the aqueous layer of the culture medium. This was noted particularly when the cells were incubated under comparatively higher concentration of copper ions.

The present paper deals with the isolation of these new antibiotics (fluopsins C and F) and their biological activities. Effects of copper ion in the medium on the production of fluopsins are also described.

Materials and Methods

1. Growth of Microorganism

Pseudomonas fluorescens KY 4032 was incubated in the following culture medium: *n*-paraffin 10 % w/v; KH_2PO_4 0.2 %, $Na_2HPO_4 \cdot 12H_2O$ 0.2 %, $MgSO_4 \cdot 7H_2O$ 0.1 %, ammonium sulfate 0.5 %, corn steep liquor 0.3 %, yeast extract 1.0 %, $CuCl_2 \cdot 2H_2O$ 0.01 %, $MnSO_4 \cdot 4H_2O$ 0.001 %, $FeSO_4 \cdot 7H_2O$ 0.001 %, $ZnSO_4 \cdot 7H_2O$ 0.001 % in tap water. The pH was adjusted to 6.5. For the production of fluopsins, 30-liter jar fermentor containing 15 liters of the medium was used under the condition of agitation of 450 r.p.m. and aeration of 15 liters per minute at 30°C. In the other experiments, 5-liter jar fermentors were also used by the same procedure reported previously[2]. In each run, the inoculum was

prepared by the previous method[2] and transferred to the growth medium at the ratio of 5~10 % by volume. The pH of the medium was adjusted to pH 6.5~7.0 with ammonia water during the incubation.

2. Estimation of Biological Activity

Antibacterial activities of fluopsins C and F were estimated by a conventional paper disc method using *B. subtilis* ATCC 6633 grown on a nutrient agar at pH 7 as a test organism. The linear correlation was demonstrated between the quantity of fluopsins and the diameter of the growth-inhibited zone. Other tests for the biological activity are described in the text.

3. Paper and Thin-layer Chromatography

Toyoroshi No. 51A papers and silica gel G plates were applied for this work. The following solvent systems were used for paper chromatography: Butanol - acetic acid - water (4 : 1 : 2), and for thin-layer chromatography; chloroform - methanol (95 : 5). The detection of materials on papers or plates was done by exposing them over ultraviolet lamp or spraying anisidine-HCl reagent.

Results and Discussion

1. Isolation of Antibacterial Substances, Fluopsins C and F

When *Pseudomonas fluorescens* KY 4032 was incubated with *n*-paraffin as the sole source of carbon, antibacterial substances appeared in the aqueous layer of the culture medium usually one day later than the cell growth occurred and reached to the maximum production in 3 or 4 days. The isolation of these antibiotics were attempted using 16 liters of the culture broth obtained after 3-day incubation in 30-liter jar fermentor. A typical isolation procedure is given below for a starting volume of 10 liters of the supernatant which was gained by centrifugation of the broth after adjusting pH at 3.0~3.5.

The supernatant adjusted again to pH 5.0 was extracted with chloroform (5 liters) twice at room temperature and the combined extract was evaporated at 40°C *in vacuo* after dehydration with sodium sulfate. The residue was dissolved in chloroform (100 ml) and submitted to the chromatography on a 13 $cm^2 \times 12$ cm column of silicic acid (Kokusan Chemical Works Limited). *n*-Paraffin was first separated by eluting with excessive amount of *n*-hexane. Subsequently, chloroform was used to flow out one of the antibacterial substances. Another compound was finally eluted with chloroform - methanol (95 : 5). Chloroform fractions having brown coloration were concentrated to dryness. The residue was dissolved in warm ethanol. Black crystals came out after keeping it in cold room overnight. These were recrystallized from ethanol twice. Dark brown prisms with metalic luster (fluopsin C 425 mg) were obtained. Chloroform-methanol fractions were similarly treated and deep violet plates (fluopsin F 160 mg) were isolated. Both compounds thus isolated are soluble in ethanol and chloroform but not soluble in water, ether, ethyl acetate nor benzene. Their chemical structures will be described in detail by SHIRAHATA *et al.*[3] in which fluopsin C is recognized to contain copper atom in its molecule while fluopsin F does iron instead of copper.

2. Culture Conditions Essential for Fluopsin Production

The production of fluopsin was discovered firstly in the medium in which *n*-paraffin was used as the sole source of carbon as described above. Therefore, other carbon

Fig. 1. Relationship between the concentration of cupric chloride and the production of fluopsin C.

•——• Fluopsin C titer of 72-hour culture
o——o Fluopsin C titer of 96-hour culture

Cultivation was carried out in the test tubes containing 10 ml medium at 30°C.

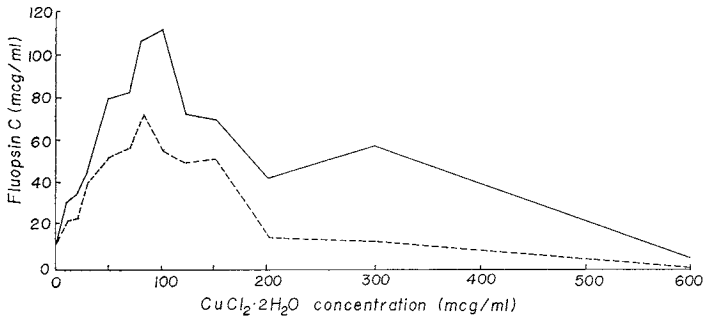


Fig. 2. Time course of fluopsin production by *Pseudomonas fluorescens* grown on *n*-paraffin.

o·····o Bacterial growth (Optical density at 660 mμ of the bacterial culture diluted in one hundred-fold with water.)
•——• Fluopsin C

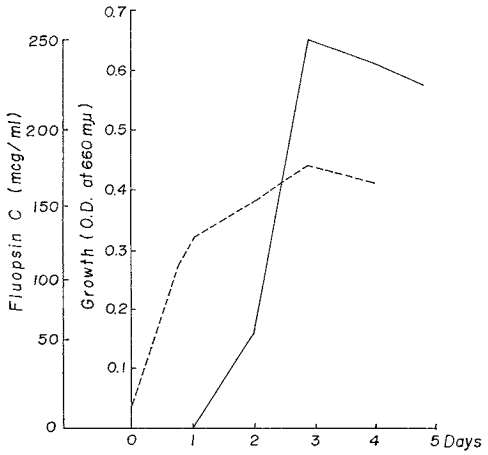


Table 1. Antimicrobial spectra of fluopsins C and F

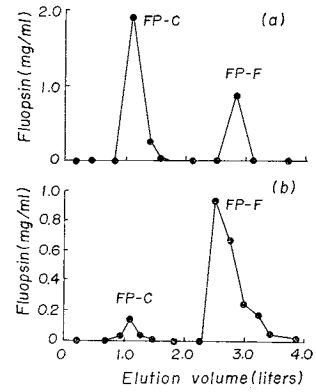
Test organisms	M.I.C.* (mcg/ml)	
	C	F
Escherichia coli (multiple drug resistant)	0.65	1.30
Streptococcus faecalis ATCC 10541	0.65	2.62
Pseudomonas aeruginosa BMH 1	>83	>83
Staphylococcus aureus ATCC 6538	0.16	0.65
Escherichia coli ATCC 26	0.65	2.62
Bacillus subtilis 10707	0.16	0.65
Proteus vulgaris ATCC 6897	0.16	5.22
Shigella sonnei ATCC 9290	0.65	2.60
Salmonella typhosa ATCC 9992	0.65	1.30
Klebsiella pneumoniae ATCC 10031	0.65	1.30

* Minimal inhibitory concentrations of fluopsins by agar dilution method.

Fig. 3. Silicic acid column chromatograms of fluopsins produced under different culture conditions.

(a) When 100 mcg/ml of cupric chloride and 1,000 mcg/ml of ferrous sulfate were added to the medium, fluopsin C was main product.
(b) When 3 mcg/ml of cupric chloride and 1,000 mcg/ml of ferrous sulfate were added, the major product was replaced by fluopsin F.

FP-C : Fluopsin C,
FP-F : Fluopsin F.
cupric chloride : $CuCl_2 \cdot 2H_2O$
ferrous sulfate : $FeSO_4 \cdot 7H_2O$



sources were compared with it in a subsequent experiment. Little production of fluopsin was observed from glucose or sorbitol regardless of the cell growth even in longer period of the incubation (122 hours). Accordingly it is possible to say that *n*-paraffin is the preferable carbon source for the production of fluopsin under the condition described in this paper. Recently, EGAWA *et al.* have shown that a strain of *Pseudomonas* produced the similar substance from sucrose.[4]

In the other experiment, the relation of the concentration of copper ion (as cupric chloride) added into the medium to the production yield of fluopsins was investigated. As presented in Fig. 1, fluopsin C increases almost proportionally to the

concentration of cupric chloride in the range of 3~100 mcg/ml. Actually, with 80 mcg/ml of cupric chloride, approximately 250 mcg/ml of fluopsin C was produced using 5-liter jar fermentor at the agitation of 800 r.p.m. The time course of this fermentation is shown in Fig. 2.

On the other hand, when the concentration of cupric chloride was reduced to approximately 3 mcg/ml, the dominant product was apparently replaced by fluopsin F, of which titer was approximately 200 mcg/ml. Fig. 3 shows the dramatic alteration between fluopsins C and F by copper ion concentration.

3. Biological Activities of Fluopsins

Both fluopsins C and F demonstrated strong antibacterial activities against gram-positive and gram-negative bacteria except for *Pseudomonas*. The minimal inhibitory concentrations of two compounds are listed in Table 1.

The acute toxicities of fluopsins C and F to mice by intraperitoneal injection were: LD_{50} 3~9 mg/kg and 30~50 mg/kg, respectively. In addition, it is realized to have significant activities against EHRLICH ascites type cells, HeLa cells and sarcoma 180 solid type cells *in vitro*, but unfortunately in the *in vivo* test, these two compounds were not so effective possibly because of their strong toxicities.

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