FERROCINS, NEW IRON-CONTAINING PEPTIDE ANTIBIOTICS PRODUCED BY BACTERIA

TAXONOMY, FERMENTATION AND BIOLOGICAL ACTIVITY

NOZOMI KATAYAMA, YUKIMASA NOZAKI, KENJI OKONOGI†, SETSUO HARADA and HIDEO ONO†

Discovery Research Laboratories II, Discovery Research Division and †Pharmaceutical Research Laboratories III, Pharmaceutical Research Division, Takeda Chemical Industries, Ltd., 2-17-85 Jusohonmachi, Yodogawa-ku, Osaka 532, Japan

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A Gram-negative bacterium was found to produce new iron-containing peptide antibiotics, ferrocins A, B, C and D, and the producing bacterium was characterized and identified as *Pseudomonas fluorescens* YK-310. These new antibiotics showed antibacterial activity against Gram-negative bacteria *in vitro*. Although the ferrocins showed similar antibacterial activity against both *Escherichia coli* and *Pseudomonas aeruginosa* on the standard assay media, they showed strong therapeutic effects selectively against *P. aeruginosa* in experimentally infected mice.

Many antibiotics with antibacterial activity against *Pseudomonas aeruginosa* have been developed. However, the mortality rate for *P. aeruginosa* infections is still high because of the increased incidence of resistance of *P. aeruginosa* to these antibiotics[1]. Therefore, it is important to develop new antipseudomonal antibiotics.

In the course of a screening for new antipseudomonal antibiotics using strain NS[2] as the test organism, we discovered ferrocins A, B, C and D[3] (formerly called TAN-866[4]) in the culture filtrate of *Pseudomonas fluorescens* YK-310. The ferrocins were found to be iron containing cyclic decapeptides (Fig. 1[3]) having a strong therapeutic effect against *P. aeruginosa* infection. This paper deals with the taxonomy of the producing organism and the fermentation and biological activities of the ferrocins. The physico-chemical properties and structure elucidation of the ferrocins will be reported elsewhere.

Materials and Methods

Taxonomy

Taxonomical and physiological characteristics were determined by the methods of COWAN and STEEL[5], and utilization of carbohydrates, production of soluble pigments and accumulation of poly-β-hydroxybutyrate were examined by the methods of STANIER *et al.*[6].

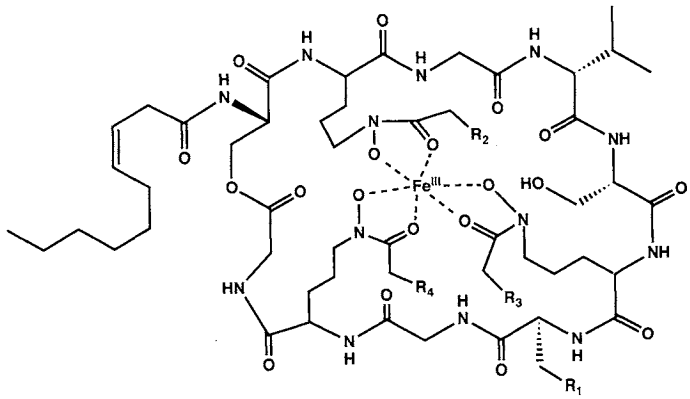
Assay of Ferrocins

The content of ferrocins was determined by a paper-disk method using *P. aeruginosa* C141[7] or NS[2] as the test organism, or by reverse-phase HPLC using a YMC-pack A-312 column (Yamamura Chem. Lab.) with a mobile phase of 36% aqueous CH_3CN.

Preparation of Iron-deficient Medium

A methanol solution of 8-hydroxyquinoline (10 ml, 30 mg/ml) was added to 1 liter of DYAB medium[8], and the mixture was stirred for 1 hour at room temperature. This medium was washed three times with

Fig. 1. Structures of ferrocins A, B, C and D.



Ferrocin	R_1	R_2, R_3, R_4
A	H	H×3
B	OH	H×3
C and D	H	H×2, CH_3×1

$CHCl_3$ (450, 150, 225 ml). A methanol solution of 8-hydroxyquinoline (10 ml, 18 mg/ml) was added again, and the mixture was stirred for 20 minutes and then washed twice with $CHCl_3$ (225, 225 ml). The medium was incubated in a hot water bath for 30 minutes to remove the $CHCl_3$. A methanol solution of 8-hydroxyquinoline (10 ml, 20 mg/ml) was added to an agar suspension (20 g, 0.1 g/ml), and the mixture was stirred for 1 hour. MeOH (100 ml) was added to this suspension, which was then centrifuged at 3,000 rpm for 5 minutes. The agar was washed twice with MeOH (300, 300 ml) and distilled water (300, 300 ml) successively. Using the iron-deficient broth and agar described above, we prepared the iron-deficient DYAB medium.

Results

Producing Organism

Strain YK-310 was isolated from a soil sample collected in Zentsuji-city, Kagawa-prefecture, Japan. The morphological and physiological characteristics of strain YK-310 are summerized in Table 1. Strain YK-310 is a Gram-negative rod, aerobic, motile by several polar flagella, metabolizes glucose oxidatively and does not require any growth factors. The mol% G+C of the DNA is 65.9. Based upon these characteristics, strain YK-310 is regarded to belong to the genus *Pseudomonas*. Strain YK-310 does not accumulate intracellular poly-β-hydroxybutyrate, and it produces fluorescent pigments. By comparing these characteristics with those of the species described in BERGEY's Manual of Systematic Bacteriology[9], strain YK-310 was identified as *Pseudomonas fluorescens* and designated *P. fluorescens* YK-310. *P. fluorescens* YK-310 has been deposited in the Institute for Fermentation, Osaka, under the accession number IFO 14516, and in the Fermentation Research Institute, Agency of Industrial Science and Technology, Japan, under the accession number FERM BP-1369.

Fermentation

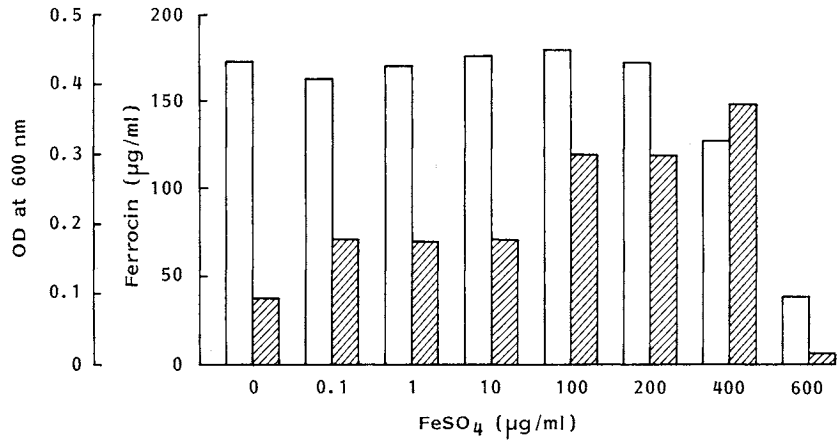
Seed medium (500 ml) consisting of glucose 2%, soluble starch 3%, soy-bean flour 1%, corn-steep liquor 0.3%, Polypepton (Nihon Pharmaceutical Co.) 0.5%, NaCl 0.3% and $CaCO_3$ 0.5% (pH 7.0) in a

Table 1. Morphological and physiological characteristics of strain YK-310.

Cell shape	Rods	Range of growth	
Size (μm)	0.6~1.2×0.8~2.1	pH	4.7~10.0 (Optimum 7.2~8.4)
Flagella	+	Temperature (°C)	10~34 (Optimum 10~30)
Gram stain	Negative	Production of fluorescent pigment	+
Oxygen demand	Aerobic	Liquefaction of gelatin	+
O-F test	Oxidative	Degradation of Tween 80	+
Reduction of nitrate	−	Accumulation of poly-β-hydroxybutyrate	−
Denitrification	−	Hydrolysis of poly-β-hydroxybutyrate	−
Methyl red test	−	GC content of DNA (%, Tm method)	65.9±1.0
Voges-Proskauer test	−	Utilization of sugars	
Production of indole	−	+: L-Arabinose, D-glucose, D-mannose, D-fructose, D-galactose, sucrose, D-mannitol, inositol, glycerol, starch	
Production of H_2S	−	Acid formation from sugars	
Utilization of		+: L-Arabinose, D-xylose, D-glucose, D-mannose, D-galactose	
Citrate	+		
Potassium nitrate	+		
Ammonium sulfate	+		
Catalase	+		
Oxidase	+		
Urease	+		

Fig. 2. Effect of iron on the ferrocin production.

Open bars: OD at 600 nm, hatched bars: titer of ferrocins measured by a microbiological method.



2-liter Sakaguchi flask was inoculated with a loopful cells of strain YK-310 grown on a nutrient agar slant. After incubation at 24°C for 48 hours on a reciprocal shaker, the seed culture was transferred to a 200-liter fermenter containing 120 liters of the same seed medium supplemented with 0.05% Actcol (Takeda Chemical Ind.) as an antifoaming agent. Cultivation was carried out at 24°C for 48 hours with aeration (120 liters/minute) and agitation (180 rpm). Fifty liters of this culture broth was transferred to a 2,000-liter fermenter containing 1,200 liters of the following fermentation medium: Glycerol 2%, glucose 0.5%, Polypepton 0.5%, meat extract 0.5%, NaCl 0.1% and yeast extract 0.1% (pH 6.5). The fermentation was carried out at 17°C for 42 hours with aeration (1,200 liters/minute) and agitation (150 rpm). Under these conditions, the ferrocins were first detected after 18 hours and reached a maximum at about 42 hours. The titer of ferrocins measured by a microbiological assay was about 60 μg/ml at 42 hours.

Table 2. Antibacterial activities of ferrocins A, B, C and D.

Test organism	MIC (μg/ml) at 10^6 cfu/ml			
	A	B	C	D
Escherichia coli NIHJ JC 2	3.13	6.25	12.5	3.13
Salmonella typhimurium IFO 12529	0.39	0.39	0.39	0.39
Citrobacter freundii IFO 12681	6.25	12.5	12.5	25
Klebsiella pneumoniae IFO 3317	0.78	0.78	12.5	3.13
Serratia marcescens IFO 12648	>100	>100	>100	>100
Proteus mirabilis ATCC 21100	>100	>100	>100	>100
Pseudomonas aeruginosa IFO 3080	3.13	3.13	12.5	3.13
Alcaligenes faecalis IFO 13111	>100	>100	>100	>100
Staphylococcus aureus FDA 209P	>100	>100	>100	>100
Micrococcus luteus IFO 12708	>100	>100	>100	>100
Bacillus subtilis NIHJ PCI 219	>100	>100	>100	>100
B. cereus FDA 5	>100	>100	>100	>100

MICs were determined by an agar dilution method using DYAB medium[11].

Table 3. Antibacterial activity of ferrocin A on the iron-sufficient and iron-deficient media.

Organism	MIC (μg/ml) at 10^6 cfu/ml	
	DYAB[a]	$-$Fe[b]
Escherichia coli NIHJ JC 2	3.13	6.25
Salmonella typhimurium IFO 12529	0.39	0.78
Citrobacter freundii IFO 12681	6.25	6.25
Serratia marcescens IFO 12648	>100	>100
Proteus mirabilis ATCC 21100	>100	>100
Pseudomonas aeruginosa IFO 3080	3.13	0.39
Alcaligenes faecalis IFO 13111	>100	>100
Staphylococcus aureus FDA 209P	>100	>100
Micrococcus luteus IFO 12708	>100	NG[c]
Bacillus subtilis NIHJ PCI 219	>100	>100
B. cereus FDA 5	>100	50

MICs were determined by an agar dilution method.
[a] DYAB medium was used as the assay medium.
[b] Iron-deficient DYAB medium prepared by the method described in the text was used as the assay medium.
[c] No growth.

Table 4. Therapeutic effect of ferrocins A, B, C and D in experimentally infected mice.

Organism	Ferrocin	MIC (μg/ml)	ED_{50} (mg/kg)
P. aeruginosa P9	A	>100	0.57
	B	>100	0.593
	C	>100	0.590
	D	>100	0.197
E. coli O-111	A	>100	>50

MICs were determined by an agar dilution method using Trypticase Soy agar (BBL). The inoculum size was 10^8 cfu/ml. Mice were infected intraperitoneally with 0.5 ml of bacterial suspension (10^8 cfu/ml). Group of five mice at each dosage level were given subcutaneously 0.2 ml of ferrocin solution immidiately after infection. The ED_{50} was calculated from the survival rate 5 days after infection.

We examined the effect of iron supplementation of the fermentation medium on the ferrocin production at the flask level. As shown in Fig. 2, the titer of ferrocins increases with the addition of ferrous sulfate until the growth of strain YK-310 is suppressed. The addition of ferrous sulfate (400 μg/ml) resulted in a more than 3-fold increase in ferrocin production.

Biological Activities

Antibacterial activities of ferrocins are shown in Table 2. Ferrocins A, B, C and D were active against some Gram-negative bacteria *in vitro*. The antibacterial activities and spectra of the four ferrocins are similar.

We examined the effect of the iron content of the assay medium on the antibacterial activity of ferrocin A. As shown in Table 3, ferrocin A shows strong antibacterial activity against *P. aeruginosa* on iron-deficient medium. Similar results were observed with *P. fluorescens* and *Pseudomonas putida* (data not shown), but

not with the other bacteria tested. Supplementing the iron-deficient medium with iron (10 μg/ml) decreased the antibacterial activity of ferrocin A, but supplementing with nickel, cobalt or manganese did not (data not shown).

Ferrocins A, B, C and D showed strong therapeutic effects against *P. aeruginosa* P9 in experimentally infected mice at a dose of less than 1 mg/kg (ED_{50}) when administered subcutaneously (Table 4). The preliminary acute toxicity (LD_{50}) of ferrocin A in mice upon intraperitoneal injection or oral administration was more than 1,000 mg/kg.

Discussion

Many microorganisms including bacteria produce low molecular weight compounds which have a high affinity for ferric ions. These compounds are designated siderophores[10]. Most of them show no antibacterial activity and they are produced in a low-iron environment to take up iron which is essential for the growth of their producers[10,11]. Although siderophores with antimicrobial activity have been discovered, they are mainly produced by actinomycetes[11,12] and only few iron-chelating antibiotics are produced by bacteria[13,14]. These antibiotics produced by bacteria have been reported to act as iron scavengers. On the other hand, the ferrocins may not act as a iron scavenger, because the ferrocins have already chelated iron and can not decrease ionic iron content of assay media. With respect to the mechanism of action, the ferrocins seem to be quite different from the other iron-chelating antibiotics of bacterial origin.

Although the MIC values of ferrocin A for *E. coli* and *P. aeruginosa* on normal medium were the same (Tables 2, 4), ferrocin A showed extremly strong therapeutic effect only against *P. aeruginosa* in mice (Table 4). On the iron-deficient medium, the antibacterial activity of ferrocin A only against *P. aeruginosa* became stronger than that measured on normal medium, and ferrocin A showed higher antibacterial activity against *P. aeruginosa* than against *E. coli* (Table 3). In body fluids, there are some iron binding proteins such as transferrin and lactoferrin, and they create a low ionic iron environment[15]. So, ferrocin A must show higher antibacterial activity against *P. aeruginosa* than against *E. coli* in the body fluids. Therefore, the therapeutic effect of ferrocin A against *P. aeruginosa* infection might be superior to that against *E. coli* infection. It is generally assumed that siderophores are actively taken up into the cells by iron transport systems which are receptor-dependent[16]. So, these findings may reflect the differences in the iron transport systems between *P. aeruginosa* and *E. coli*.

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References

1) KORVICK, J. A. & V. L. YU: Antimicrobial agent therapy for *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. 35: 2167~2172, 1991
2) KATAYAMA, N.; K. KOYAMA, Y. NOZAKI, S. HARADA & H. ONO: A new sulfazecin-type β-lactam antibiotic, TAN-850. J. Takeda Res. Lab. 46: 62~68, 1987
3) TSUBOTANI, S.; N. KATAYAMA, K. KOYAMA, H. ONO & S. HARADA: New iron-containing peptide antibiotics, ferrocins. Abstracts Papers of 108th Annual Meeting of the Pharmaceutical Society of Japan, No. 4E13 11-2, Hiroshima, 1988
4) HARADA, S.; H. ONO & N. KATAYAMA (Takeda Chem. Ind., Ltd.): TAN-866, production and use thereof. Jpn. Kokai 25795 ('89) Jan. 27, 1989
5) COWAN, S. T. & K. J. STEEL: Manual for Identification of Medical Bacteria. Cambridge University Press, 1965
6) STANIER, R. Y.; N. J. PALLERONI & M. DOUDOROFF: The aerobic pseudomonads; a taxonomic study. J. Gen. Microbiol. 43: 159~271, 1966
7) ONO, H.; Y. HOZAKI, N. KATAYAMA & H. OKAZAKI: Cephabacins, new cephem antibiotics of bacterial origin. I. Discovery and taxonomy of the producing organisms and fermentation. J. Antibiotics 37: 1528~1535, 1984

8) NOZAKI, Y.; A. IMADA & M. YONEDA: SCE-963, a new potent cephalosporin with high affinity for penicillin-binding proteins 1 and 3 of *Escherichia coli*. Antimicrob. Agents Chemother. 15: 20～27, 1979
9) PALLERONI, N. J.: *Genus* I. *Pseudomonas Migula* 1894, 237. *In* BERGEY's Manual of Systematic Bacteriology. Volume 1. *Eds.*, N. R. KRIEG & J. G. HOLT, pp. 141～199, The Williams and Wilkins Co., Baltimore, 1984
10) NEILANDS, J. B.: Microbial iron compounds. Annu. Rev. Biochem. 50: 715～731, 1981
11) ZÄHNER, H.; E. BACHMANN, R. HÜTTER & J. NÜESCH: Sideramine, eisenhaltige Wachstumsfaktoren aus Mikroorganismen. Pathol. Microbiol. 25: 708～736, 1962
12) BÉRDY, J.: Chelate-forming peptides. *In* CRC Handbook of Antibiotic Compounds. Volume IV. Part 1. pp. 439～458, CRC Press, Boca Raton, 1980
13) KUNZE, B.; N. BEDORF, W. KOHL, G. HÖFLE & H. REICHENBACH: Myxochelin A, a new iron-chelating compound from *Angiococcus disciformis* (Myxobacterales). Production, isolation, physico-chemical and biological properties. J. Antibiotics 42: 14～17, 1989
14) KUNZE, B.; W. TROWITZSCH-KIENAST, G. HÖFLE & H. REICHENBACH: Nannochelins A, B and C, new iron-chelating compounds from *Nannocystis exedens* (myxobacteria). Production, isolation, physico-chemical and biological properties. J. Antibiotics 45: 147～150, 1992
15) BULLEN, J. J.; H. J. ROGERS & E. GRIFFITHS: Role of iron in bacterial infection. Curr. Top. Microbiol. Immunol. 80: 1～35, 1978
16) BRAUN, V.: Genetics of siderophore biosynthesis and transport. *In* Biochemistry of Peptide Antibiotics. *Eds.*, H. KLEINKAUF & H. DÖHREN, pp. 103～129, Walter de Gruyter, Berlin, 1990