FORMADICINS, NEW MONOCYCLIC β-LACTAM ANTIBIOTICS OF BACTERIAL ORIGIN

I. TAXONOMY, FERMENTATION AND BIOLOGICAL ACTIVITIES

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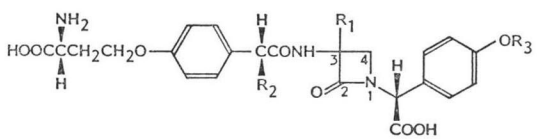
A Gram-negative bacterium produces new monocyclic β-lactam antibiotics with a formylamino substituent, named formadicins A, B, C and D. The producing bacterium was taxonomically characterized and designated as *Flexibacter alginoliquefaciens* sp. nov. YK-49. Formadicins have narrow antibacterial spectra. They are highly active against some species of *Pseudomonas*, *Proteus* and *Alcaligenes*. Of the four, formadicin C shows the most potent antibacterial activity. Several amino acids such as glycine, D-alanine and D-leucine were antagonistic against formadicins. Formadicins, especially formadicins A and C having the formylamino substituent bound to the 3-position of a β-lactam nucleus, were highly resistant to hydrolysis by various types of β-lactamases. Formadicins A and C showed affinity for penicillin-binding proteins (PBPs) 1A and 1B in *Pseudomonas aeruginosa* IFO 3080, but formadicin B and nocardicin A showed affinity only for PBP 1B. Formadicins A and C did not lyse *Escherichia coli* LD-2 solely at their MICs, but when combined with mecillinam each induced a rapid lysis of this organism.

In the course of our screening program for new natural β-lactam antibiotics, we found a Gram-negative bacterium, strain YK-49, that produces new monocyclic β-lactam antibiotics named formadicins as they have a formylamino substituent. Formadicins consist of components A, B, C and D, and have a nocardicin-like skeleton. Formadicins A and C have a formylamino substituent directly bound to a β-lactam nucleus, whereas formadicins B and D have this substituent in the 3-side chains. Furthermore, formadicins A and B, having a D-glucuronide moiety, are the first sugar-containing β-lactam antibiotics of natural origin (Fig. 1)[1].

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



Formadicin	R_1	R_2	R_3
A	NHCHO	OH	D-Glucuronic acid
B	H	NHCHO	D-Glucuronic acid
C	NHCHO	OH	H
D	H	NHCHO	H

Although 11 monocyclic β-lactam antibiotics have been discovered from bacteria, they are all sulfazecin-type (*N*-sulfonated) antibiotics[2~5]; nocardicin-type monocyclic β-lactam antibiotics having a phenylacetic acid moiety at the 1-position, which were originally found to be produced by a *Nocardia* species[6], have not been discovered from bacteria. Recently, chlorocardicin has been reported to be produced by a *Streptomyces* species[7].

Table 1. Morphological and physiological characteristics of strain YK-49.

Cell shape	Slender rods	Urease	−
Size (μm)	0.4~0.7×2~10[a]	Oxidase	+
Motility[b]	Gliding	Catalase	+
Flagella	None	Oxygen demand	Aerobic
Gram stain	Negative	O-F test	Not reactive
Spore formation	−	Range of growth	
Microcyst formation[c]	−	pH	4.9~8.5 (Optimum 5.4~6.6)
Reduction of nitrate	−		
Denitrification	−	Temperature (°C)	6~35 (Optimum 14~31)
Methyl red test	−		
Voges-Proskauer test	−	Degradation of	
Production of indole	−	colloidal chitin	−
Production of H_2S	−	carboxymethylcellulose	−
Utilization of citrate	+	agar	−
potassium nitrate	−	alginate	+
ammonium sulfate	−	Hydrolysis of starch	+
Production of		Liquefaction of gelatin	+
diffusible pigment	−	Tolerance to NaCl (%)	0~3
Growth factor requirement	−	GC content of DNA (%)	39.0±1.5

[a] Filamentous cells of 50~70 μm length were often observed.
[b] Motility was observed according to the method of GILARDI[10].
[c] Formation of microcyst was tested according to the method of DWORKIN and GIBSON[11].

Table 2. Acid and gas formation from sugars and utilization of sugars by strain YK-49.

Sugar	Acid[a]	Gas[a]	Growth[b]
L-Arabinose	±	−	+
D-Xylose	−	−	+
D-Glucose	±	−	+
D-Mannose	+	−	+
D-Fructose	−	−	+
D-Galactose	−	−	+
Maltose	−	−	+
Sucrose	−	−	+
Lactose	−	−	+
Trehalose	−	−	+
D-Sorbitol	−	−	−
D-Mannitol	−	−	+
Inositol	−	−	−
Glycerol	−	−	+
Starch	−	−	+

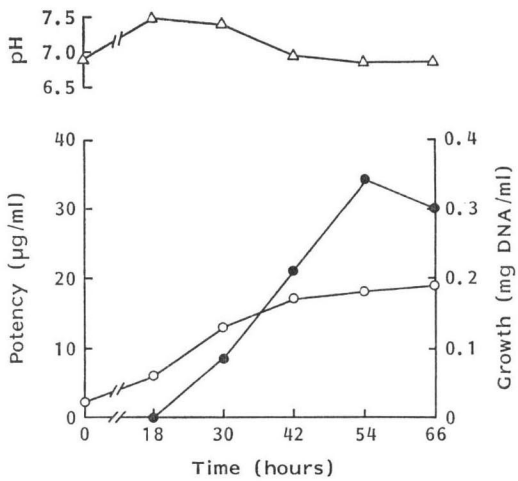
[a] Acid and gas formation from sugars were examined in peptone-water containing 0.1% of a single carbon source.
[b] Sugar utilization was tested using Davis agar containing 1% of a single carbon source, supplemented with 0.005% each of L-cysteine and L-tryptophan.

Fig. 2. Time-course of large-scale fermentation for formadicin production.

Potency (●); calculated as formadicin A using *Pseudomonas aeruginosa* C141, a β-lactam hypersensitive mutant as a test organism[9].

Growth (○); the DNA content was determined by the method of BURTON[14] after extraction with 5% perchloric acid.

pH (△).



In this and the accompanying paper[1], we report on the first members of bacterially produced nocardicin-type antibiotics. This paper deals with taxonomy of the producing organism, fermentation, and biological activities of formadicins.

Discovery and Taxonomy of the Producing Organism

Strain YK-49 was isolated on a starch-casein agar[8] plate from a soil sample collected in the Yoshino district, Nara Prefecture, Japan. It was selected as a β-lactam antibiotic producer based on following observations; its culture filtrate showed higher antibacterial activity against a β-lactam hypersensitive mutant, *Pseudomonas aeruginosa* C141[9], than its parental strain IFO 3080, and induced the formation of spheroplasts from the mutant.

Colonies of strain YK-49 were semitransparent, white-cream, circular, convex, and entire-edged on nutrient agar. It was Gram-negative, slender rods or sometimes filaments, motile by gliding, did not require any growth factors, and did not form spores or microcysts. The GC content of DNA was 39% by the thermal denaturation method in 0.1×standard saline citrate. The other cultural and physiological characteristics are listed in Tables 1 and 2. The following key characteristics such as non-flagellated slender rods, gliding motility, aerobic, a low GC content of DNA, microcyst-forming ability negative, sheaths and sulfur granules negative, decomposing ability of carboxymethylcellulose, colloidal chitin and agar negative, indicate that it belongs to the genus *Flexibacter*. Then its characteristics were compared with those of six species of *Flexibacter* described in BERGEY's Manual of Determinative Bacteriology (8th ed.) and seven species cited in the approved list[12] and the validation lists of the International Journal of Systematic Bacteriology. Strain YK-49, however, did not coincide with any of them. Therefore, strain YK-49 was assigned a new species of *Flexibacter*, named as *F. alginoliquefaciens* to denote its strong degrading activity of alginate.

Fermentation

Seed culture was initiated by transferring a loopful of cells grown on a plate count agar[13] slant into a 2-liter Sakaguchi flask containing 500 ml of the following seed medium; glucose 2%, soluble starch 3%, corn-steep liquor 0.3%, soybean flour 1%, Polypepton (Daigo Nutritive Chemicals, Ltd.) 0.5%, NaCl 0.5%, $CaCO_3$ 0.5%, pH 7.0. The culture broth was transferred to 120 liters of the seed medium supplemented with 0.05% of Actocol (an antiform, Takeda Chem. Ind.) in a 200-liter fermentor, and cultivation was carried out at 24°C for 2 days with aeration (100 liters/minute) and agitation (150 rpm). Sixty liters of this culture broth was transferred to 1,200 liters of the large-scale fermentation medium consisting of glycerol 3%, soybean flour 2%, corn gluten meal 1%, Polypepton 0.2%, $CaCO_3$ 0.5% and Actocol 0.05%, in a 2,000-liter fermentor, and cultivation was carried out at 20°C for 66 hours with aeration (1,200 liters/minute) and agitation (120 rpm).

A typical large-scale fermentation profile is shown in Fig. 2. The titer of formadicins calculated as formadicin A was about 35 μg/ml at 54 hours under this condition.

Antibacterial Activity

Formadicins showed comparatively narrow antibacterial spectra. They showed high activity against some species of *Pseudomonas*, *Proteus*, and *Alcaligenes* (Table 3). Their antibacterial spectra were similar to that of nocardicin A[6]. There were such tendencies in the antibacterial potency that formadicins C and D were more active respectively than formadicins A and B, which contained a D-glucuronide moiety, and formadicins A and C which have the formylamino substituent bound to

Table 3. Antibacterial activity of formadicins and nocardicin A.

Organism	MIC (μg/ml) at 10^6 cfu/ml				
	Formadicin				Nocardicin A
	A	B	C	D	
Escherichia coli NIHJ JC2	>100	>100	>100	>100	>100
Salmonella typhimurium IFO 12529	>100	>100	100	100	>100
Citrobacter freundii IFO 12681	>100	>100	>100	>100	>100
Klebsiella pneumoniae IFO 3317	>100	>100	>100	>100	>100
Enterobacter cloacae IFO 12937	>100	>100	>100	>100	>100
Serratia marcescens IFO 12648	>100	>100	>100	>100	>100
Proteus mirabilis ATCC 21100	50	100	50	50	12.5
P. vulgaris IFO 3988	25	50	25	50	3.13
P. morganii IFO 3168	>100	>100	>100	>100	>100
Pseudomonas aeruginosa IFO 12689	100	>100	25	>100	50
P. aeruginosa IFO 3080	50	>100	12.5	>100	12.5
Alcaligenes faecalis IFO 13111	12.5	25	6.25	12.5	1.56
Acinetobacter calcoaceticus IFO 12552	100	>100	25	>100	100
A. calcoaceticus IFO 13006	50	>100	6.25	>100	12.5
Staphylococcus aureus FDA 209P	>100	>100	>100	>100	100
Bacillus subtilis PCI 219	>100	>100	50	50	50
B. thiaminolyticus IFO 3115	50	50	25	12.5	0.78
Brevibacterium thiogenitalis ATCC 19240	>100	50	25	6.25	6.25

MICs were determined at 37°C by the conventional agar dilution method as described previously[15].

Table 4. Antibacterial activity of formadicins and nocardicin A against *Pseudomonas* and *Proteus* species.

Organism	MIC (μg/ml) at 10^6 cfu/ml			
	Formadicin			Nocardicin A
	A	B	C	
Pseudomonas aeruginosa IFO 12689	>100	>100	25	>100
P. aeruginosa IFO 3080	50	>100	12.5	25
P. aeruginosa IFO 3445	25	>100	6.25	12.5
P. aeruginosa IFO 3449	50	>100	25	50
P. aeruginosa PAO-1	50	>100	25	50
P. acidovorans IFO 13582	50	>100	3.13	>100
P. maltophilia IFO 12020	50	>100	6.25	100
P. putida IFO 13696	>100	>100	>100	>100
P. stutzeri IFO 12510	>100	>100	100	100
P. stutzeri IFO 3773	12.5	12.5	6.25	12.5
Proteus mirabilis ATCC 21100	50	50	25	12.5
P. mirabilis PPG-38	3.13	12.5	3.13	0.78
P. morganii IFO 3168	>100	>100	>100	>100
P. rettgeri IFO 13501	50	>100	25	12.5
P. vulgaris IFO 3045	12.5	100	12.5	3.13
P. vulgaris IFO 3988	12.5	25	12.5	3.13

MICs were determined at 28°C by the conventional agar dilution method as described previously[15].

P. mirabilis PPG-38 is a β-lactam-sensitive mutant derived from *P. mirabilis* ATCC 21100 and is defective in PBP4.

the 3-position of a β-lactam nucleus were more active respectively than formadicins B and D having the substituent in the side chains.

The antibacterial activity of formadicins against a wide range of *Pseudomonas* and *Proteus* species

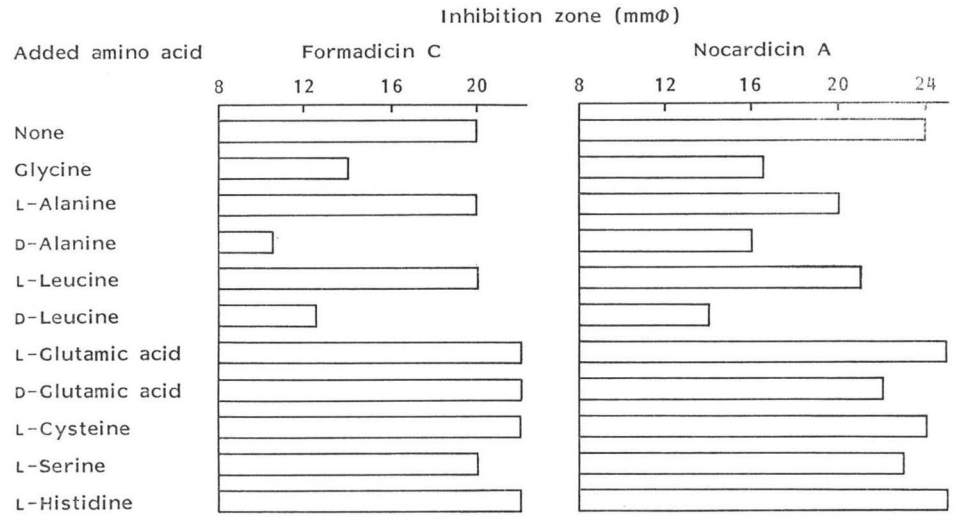
Table 5. Protective effect of formadicins and nocardicin A in mice.

Compound	*Escherichia coli* O-111		*Proteus vulgaris* GN4712
	MIC (μg/ml) at 10^8 cfu/ml	ED_{50} (mg/kg, sc)	ED_{50} (mg/kg, sc)
Formadicin A	>100	119	33.1
Formadicin B	>100	nd	>200
Formadicin C	>100	308	89.8
Formadicin D	>100	>400	>400
Nocardicin A	50	130	65

Mice were infected intraperitoneally with 0.5 ml of a suspension of *E. coli* O-111 or *P. vulgaris* GN4712. Groups of five mice at each dose level were subcutaneously given 0.2 ml of an antibiotic solution immediately after infection. The 50% effective dose (ED_{50} mg/kg) was calculated by the conventional method from the survival rate at 5 days after infection.
nd: Not determined.

Fig. 3. Effects of amino acids on the activity of formadicin C and nocardicin A against *Pseudomonas aeruginosa* IFO 3080.

Assay plates were M-9 agar containing 1 mg/ml of an amino acid, seeded with *P. aeruginosa* IFO 3080. Paper disks (8 mm; diameter) dipped with a solution (1 mg/ml) of formadicin C or nocardicin A were placed on the plates and incubated overnight at 37°C.



was compared with that of nocardicin A. In comparison of formadicin C, which was most active among formadicins, and nocardicin A, the former was more active against *Pseudomonas*, but less active against *Proteus* than the latter (Table 4).

Formadicins A and C showed fairly good protective effect in mice from intraperitoneal infection by *E. coli* O-111 and *P. vulgalis* GN4712 (Table 5). No acute toxicity was observed in mice (>1,000 mg/kg, sc).

Amino Acids Antagonism

Since several amino acids are known to antagonize nocardicin A[16] and chlorocardicin[7], we examined the effect of several amino acids on the antibacterial activity of formadicin C. Of amino acids tested, glycine, D-alanine and D-leucine were markedly antagonistic to these antibiotics. L-

Table 6. Stability of formadicins and nocardicin A to β-lactamases.

Source of enzyme	Relative rate of hydrolysis[a]				
	Formadicin				Nocardicin A
	A	B	C	D	
Penicillinase					
Staphylococcus aureus 1840	<0.01	0.33	<0.01	0.69	<0.01
Escherichia coli TN 713	<0.01	1.11	<0.01	1.59	0.11
Klebsiella oxytoca TN 1719	<0.01	0.28	<0.01	0.41	3.52
Cephalosporinase					
Enterobacter cloacae TN 1282	<0.01	0.01	<0.01	0.08	0.01
Pseudomonas aeruginosa U 31	<0.01	0.65	<0.01	1.45	0.01
Proteus vulgaris GN 4413	<0.01	6.33	<0.01	6.73	6.55

The rate of hydrolysis by β-lactamases was determined spectrophotometrically or microbiologically as described previously[17,18].

[a] Expressed as relative rate of hydrolysis, taking the rate for benzylpenicillin (penicillinase), or cephaloridine (cephalosporinase) as 100.

Alanine and L-leucine were partially antagonistic to nocardicin A, but not to formadicin C (Fig. 3).

Stability to Hydrolysis by β-Lactamases

Formadicins and nocardicin A were compared in their stability to six types of β-lactamases. These antibiotics were stable to hydrolysis by the β-lactamases. In particular, formadicins A and C were far more stable than formadicins B and D, and nocardicin A (Table 6), indicating that the formylamino substituent directly bound to a β-lactam nucleus confers the monocyclic β-lactam antibiotics extreme resistance to the β-lactamases, as is the case with the cephem antibiotics[18].

β-Lactamase Inhibitory Activity

β-Lactamase inhibitory activity of formadicins and nocardicin A was assayed as described previously[19]. None of these antibiotics showed inhibitory activity against penicillinases of *Staphylococcus aureus* 1840 and *E. coli* TN713, and cephalosporinases of *Enterobacter cloacae* TN1282 and *P. vulgaris* GN4413 (I_{50} values, >100 μg/ml).

Lytic Activity against *P. aeruginosa* IFO 3080

Lytic activity of formadicins A, B and C, and nocardicin A against *P. aeruginosa* IFO 3080 was examined as described previously[18]. Their lytic activity was weak when compared with their antibacterial activity against this organism; they did not lyse this organism at their MICs under the experimental conditions used (Fig. 4). The order of potency of lytic activity was as follows; nocardicin A>formadicin A>formadicin C>formadicin B. The lytic activity of these antibiotics did not necessarily parallel their antibacterial activity (Table 3).

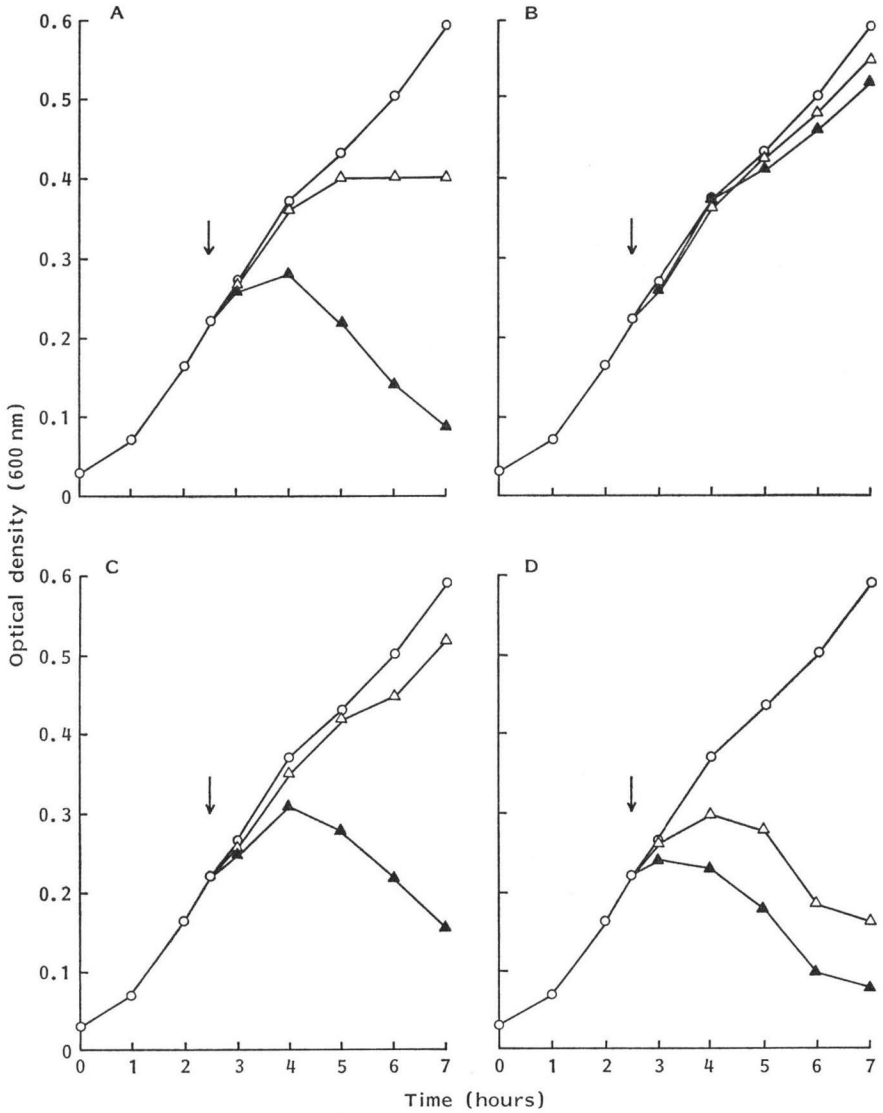
Binding Affinity for Penicillin-binding Proteins (PBPs) in *P. aeruginosa* IFO 3080

Affinities of formadicins A, B and C, and nocardicin A for PBPs were compared by a competitive binding experiment with [^{14}C]benzylpenicillin using envelopes of *P. aeruginosa* IFO 3080 as described previously[15]. All the antibiotics showed affinity for PBP 1B in the organism, but only formadicins A and C, the compounds with the formylamino substituent directly bound to a β-lactam nucleus, also

Fig. 4. Lytic activity of formadicins A, B and C, and nocardicin A against *Pseudomonas aeruginosa* IFO 3080.

A culture of *P. aeruginosa* IFO 3080 grown in DYAB medium[15] to the exponential phase was diluted 5-fold with fresh medium. A portion (4.5 ml) of the diluted culture was delivered into sterilized tubes which were then incubated at 37°C with reciprocal shaking. After 2.5 hours of incubation, 0.5 ml of a solution of formadicins A (A), B (B), C (C), or nocardicin A (D) was added to the culture. Growth was followed by measuring the absorbance at 600 nm with a Spectronic 20 colorimeter (Shimadzu, Baush & Lomb).

○, Control; △, 100 μg/ml; ▲, 1,000 μg/ml.



showed affinity for PBP 1A (Fig. 5). None of the tested antibiotics had the affinity for the PBPs other than PBP 1A and 1B even at 100 μg/ml, the maximum concentration tested. The D-glucuronide moiety does not seem to affect affinity for the PBPs, since formadicins A and C showed the similar affinity profiles (Fig. 5).

Fig. 5. Affinity of formadicins A, B and C, and nocardicin A for PBPs in *Pseudomonas aeruginosa* IFO 3080.

The assay of affinity for PBPs in *P. aeruginosa* IFO 3080 was carried out as described with *E. coli* LD-2 previously[15].

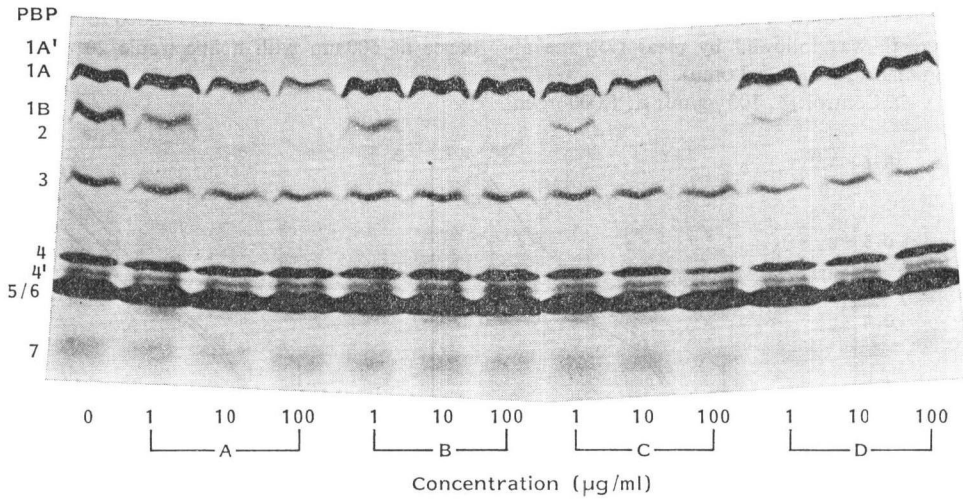
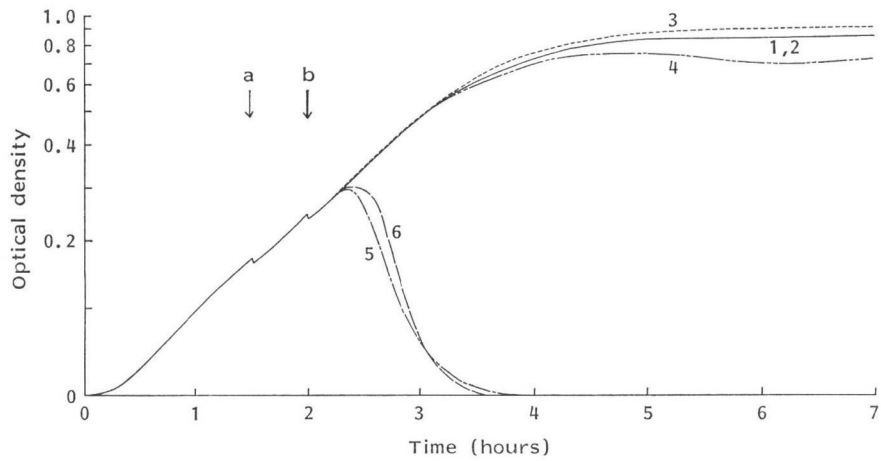


Fig. 6. Combination effect of formadicin A or C, and mecillinam on bacteriolytic activity against *Escherichia coli* LD-2.

Growth of *E. coli* LD-2 on DYAB medium[15] at 37°C was monitored with an automatic growth recorder BIO-LOG II (Jasco Jouan). Mecillinam, and formadicin A or C were added at 1.5 hours cultivation (a) and at 2 hours cultivation (b), respectively. Final concentrations of the antibiotics corresponded to their MICs against this organism; mecillinam, 0.39 μg/ml; formadicins A and C, 100 μg/ml.

1, Control; 2, mecillinam; 3, formadicin A; 4, formadicin C; 5, mecillinam+formadicin A; 6, mecillinam+formadicin C.



Synergistic Effect on Lytic Activity with Mecillinam

BERENGNER *et al.* reported that nocardicin A and mecillinam acted together to induce a fast lytic responce in *E. coli*, although each of them was not bacteriolytic against this organism[20]. Then, we

examined a combination effect of formadicin A or C, and mecillinam on lytic activity against *E. coli* LD-2[15]. As shown in Fig. 6, formadicins A and C, and mecillinam alone did not lyse this organism at their MICs. However, an addition of formadicin A or C at 30 minutes after an addition of mecillinam strongly induced lytic action against this organism.

Discussion

Until recently, bacteria have not been recognized a source of producers of β-lactam antibiotics. However, since the discovery of sulfazecin and isosulfazecin[2], and other monobactams[3], many new β-lactam antibiotics having almost all types of β-lactam nuclei detected from other microorganisms, *i.e.* fungi and actinomycetes, have been isolated from bacteria[4,5,9,21~27]; bacteria have proved to be a fruitful source of β-lactam antibiotics.

In this report, we have described the first members of nocardicin-type antibiotics of bacterial origin. We obtained 13 strains producing formadicins, and examined their taxonomical characteristics. All were found to be the same species as strain YK-49 described in this paper, although there were minor differences in their characteristics. Two species of *Flexibacter* were reported to produce 3 monocyclic β-lactam antibiotics[4,5]. *F. alginoliquefaciens* YK-49 differs from these species in the characteristics such as O-F test, production of H_2S, and utilization of sugars[4,5].

As in the case of 7-formylamino cephem antibiotics produced by *Flavobacterium* sp. SC12154[23], *F. chitinovorum* PB-5016[24], *Lysobacter lactamgenus* YK-90[6], *Xanthomonas lactamgena* YK-278, and YK-280[6], and *Flavobacterium* sp. PB-5246[25], formadicins have the formylamino substituent. Thus, the activity of introducing a formylamino substituent to a β-lactam nucleus seems to be widely distributed in bacteria, but its enzymatic mechanism has not been elucidated yet. The formylamino substituent has proved to confer a high resistance to β-lactamases on β-lactam antibiotics[18,23].

Formadicins were highly active against limited species of bacteria. Comparing the antibacterial activity of formadicin C and nocardicin A, the former was more active against *Pseudomonas* species but less active against *Proteus* species than the latter. It is not clear whether this antibacterial property was caused by the presence of the formylamino substituent at 3-position, or by the difference in the 3-side chains.

The permeability barrier of Gram-negative bacteria sometimes diminishes the antibacterial activity of β-lactam antibiotics. However, formadicins and nocardicin A seem to easily permeate through the outer membrane of *E. coli*, because these antibiotics showed the same MIC values against the permeability mutant (PG-12) and its parental strain (CPC20) of *E. coli*[29] (data not shown). In *E. coli*, PBP 1A and 1B are essential for cell growth and their functions complement each other[30,31]. NOGUCHI *et al.* suggested that PBP 1A and 1B of *P. aeruginosa* corresponded to PBP 1B and 1A in *E. coli*, respectively[32], and CURTIS *et al.* suggested the each of PBPs of *P. aeruginosa* fundamentally performed the same roles of the corresponding PBPs in *E. coli*[33]. In this report, we showed that formadicins A and C had affinity for both PBP 1A and 1B, but formadicin B and nocardicin A only for PBP 1B in *P. aeruginosa*. In contrast, MICs of formadicins A, B and C, and nocardicin A against this organism were 50, >100, 12.5, 12.5 μg/ml, respectively (Table 3). These results indicate that their antibacterial potencies against this organism can not be explained based on their affinity profiles. Therefore, unknown factors may be involved in the antibacterial mechanism of these monocyclic β-lactam antibiotics.

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