CEFOXITIN RESISTANCE BY A CHROMOSOMAL CEPHALOSPORINASE IN *ESCHERICHIA COLI*

Ikuko Takahashi, Tetsuo Sawai, Tohru Ando and Saburo Yamagishi

Faculty of Pharmaceutical Sciences, Chiba University, Chiba, Japan

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Cefoxitin resistance, an unique property found in clinical isolates of *Escherichia coli* was investigated. Cefoxitin resistant strains, 255 and GN206, produced cephalosporinase constitutively. The cephalosporinase was located in the periplasm, and its production was considered to be mediated by chromosomal gene(s). Cephalosporinase-less mutants from both strains were susceptible to cefoxitin as well as other β-lactam antibiotics, suggesting that the cephalosporinase was responsible for the resistance to β-lactam antibiotics including cefoxitin. The cephalosporinases from the *E. coli* strains were partially purified and their enzymological properties were compared with those of cephalosporinases of *Citrobacter freundii* and *Proteus morganii*. Although the cephalosporinases of *E. coli*, as well as other cephalosporinases, showed little activity for cefoxitin-hydrolysis, the *E. coli* cephalosporinases exhibited a significantly higher affinity for cefoxitin than other cephalosporinases. It was assumed that the *E. coli* enzyme located around the targets of cefoxitin protected the targets from the antibiotic by its high affinity for the antibiotic.

We have classified 416 clinical isolates of *Escherichia coli* on the basis of susceptibility to ampicillin and cephaloridine[1]. The investigation revealed that about 9% of the collection was resistant to both ampicillin and cephaloridine. Furthermore, a fraction of those strains exhibited resistance to 12.5 μg/ml or more concentrations of cefoxitin which is known to be resistant to hydrolysis by many β-lactamases[2]. These cefoxitin resistant strains were found to produce cephalosporinase without exception, though the cephalosporinase was unable to hydrolyze cefoxitin *in vitro*. The incapability of transferring the cefoxitin resistance to a sensitive *E. coli* strain, absence of loss of the resistance after ethidium bromide or acridine orange curing, and the stability of the resistance for prolonged storage of culture suggested that the cephalosporinases of these strains were coded for by chromosomal genes[1]. Thereafter, we found that an *E. coli* strain isolated in 1965 also produced cephalosporinase and exhibited the characteristic of cefoxitin resistance.

The present investigation was undertaken to determine a possible role of these cephalosporinases in the bacterial resistance to β-lactam antibiotics especially to cefoxitin.

Materials and Methods

Bacterial strains and media

E. coli strains GN206 and GN86 were isolated from clinical sources in 1965[3], and *E. coli* 255 in 1975[1]. *E. coli* W3630, a derivative of K-12, is from our stock. The strains GN206 and 255 are resistant to ampicillin, cephaloridine and cefoxitin. The strains GN86 and W3630 are susceptible to those antibiotics. *Citrobacter freundii* strains GN346 and GN346/16–10[4,5], and *Proteus morganii* 1510[6] produce their species-specific cephalosporinases constitutively.

The organisms were grown in nutrient broth or heart infusion broth at 37°C. Heart infusion agar was employed for the determination of bacterial susceptibility to antibiotics. The media used were obtained from Eiken Chemical Co., Tokyo, Japan.

β-Lactam antibiotics

β-Lactam antibiotics were kindly provided by the following pharmaceutical companies: benzylpenicillin and ampicillin, Meiji Seika Co., Tokyo, Japan; cephaloridine, Glaxo Laboratories Ltd., Greenford, Middlesex, England; cefazolin, Fujisawa Pharmaceutical Co., Osaka, Japan; cephalothin, Shionogi Pharmaceutical Co., Osaka, Japan, and cefoxitin, Merck Sharp & Dohme Research Laboratories, Rahway, N. J.

Measurement of bacterial susceptibility to β-lactam antibiotics

Antibacterial activity of β-lactam antibiotics was determined by a serial agar dilution method, and the activity was expressed by minimal inhibitory concentration (MIC). Overnight culture of the bacterial strain in nutrient broth was diluted 100-fold with fresh broth and 5 μl of the bacterial suspension (about 3×10^6 cells per ml) was spotted on heart infusion agar plates by utilizing an inoculum replicating device (Microplanter, Sakuma Manufactory Ltd., Tokyo, Japan). The plates were incubated at 37°C for 18 hours, and MICs were determined.

Isolation of mutants lacking cephalosporinase activity

Bacteria in the exponential growth phase in 10 ml of heart infusion broth were harvested by centrifugation, washed once with 0.1 M citrate buffer (pH 6.0) and resuspended in the same volume of the buffer containing 100 μg of N-methyl-N′-nitro-N-nitrosoguanidine (NTG) per ml. After incubation for 10 minutes at 30°C, the cell suspension was centrifuged to remove NTG and the bacterial cells were resuspended in 10 ml of fresh broth. The culture was incubated at 37°C for two to three doubling times with shaking, and a portion of the culture was inoculated onto an agar plate to make 100~200 colonies. After incubated for 16 hours at 37°C, the agar plate was overlaid with 6.5 ml of the solution containing 5 ml of 1.2% melted agar (55°C), 1 ml of 0.1% phenol red adjusted to pH 8.7 and 0.5 ml of 5% cephalothin. Since the colonies producing β-lactamase turned to yellow by hydrolysis of β-lactam ring of the antibiotic within a few minutes, the cephalosporinase negative colonies (red) were scored.

Preparation of crude cell extract

Bacterial cells growing exponentially in heart infusion broth were harvested by centrifugation and resuspended in 0.1 M phosphate buffer, pH 7.0. The cells were subjected to sonic oscillation at 20 kc for 3 minutes (Ohtake Works, Tokyo, Japan) in an ice-water bath and centrifuged at $100,000\times g$ for 30 minutes at 4°C after removing unbroken cells or large fragments by centrifugation at $10,000\times g$ for 20 minutes. The supernatant fluid was used as crude enzyme preparations.

Enzyme assay

β-Lactamase activity was determined iodometrically at 30°C in 0.1 M phosphate buffer (pH 7.0) by a colorimetric assay[7] or the method of PERRET[8]. For kinetic measurements of the enzyme reaction, the microiodometric assay method devised by NOVICK[9] was employed with a slight modification. One unit of the β-lactamase activity was defined as that enzyme activity which hydrolyzes 1 μmol of substrate per minute.

β-Galactosidase activity was assayed by hydrolysis of *o*-nitrophenyl-β-D-galactopyranoside according to the procedures described by SMITH and WYATT[10]. One unit of the β-galactosidase activity was defined as that amount of the enzyme which releases 1 μmol of *o*-nitrophenol per minute.

Estimation of molecular weight of β-lactamase

The molecular weight of β-lactamase was estimated by gel filtration through a Sephadex G-75 column according to the method of ANDREWS[11]. About one unit of the enzyme dissolved in 0.5 ml of 0.15 M phosphate buffer (pH 7.0) containing 0.5 mg of cytochrome C (Mol. Wt. 12,400; C. F. Boehringer & Soehne GmbH Mannheim, Germany), 2 mg of α-chymotrypsinogen (Mol. Wt. 25,000; Sigma Chemical Co., St. Louis, Mo.) and 3 mg of ovalbumin (Mol. Wt. 45,000; Sigma Chemical Co.) was loaded onto a Sephadex G-75 superfine column (1.5 by 85 cm) equilibrated with 0.15 M phosphate buffer (pH 7.0). The column was eluted with the buffer at a flow rate of 7 ml/hour at 5°C. Each of 1.5-ml fractions of the column effluent was used for determination of β-lactamase activity. Reference proteins and cytochrome C were located by absorption at 280 nm and 412 nm, respectively.

Spheroplast formation

Spheroplast formation was carried out according to the procedures described by OSBORN *et al.*[12]

Results

Resistance to β-Lactam Antibiotics and Cephalosporinase Activity in *E. coli* Strains 255 and GN206

MIC values of five β-lactam antibiotics to *E. coli* strains 255 and GN206, and cephalosporinase activity in these *E. coli* strains are shown in Table 1 together with those properties of *C. freundii* and *P. morganii*. The *E. coli* strains produced constitutively cephalosporinase which was considered to be mediated chromosomally. The *C. freundii* and the *P. morganii* strains are also constitutive producers of their species-specific cephalosporinases[5,6]. When strains 255 and GN206 were compared with the *C. freundii* and the *P. morganii* strains, the *E. coli* strains showed higher cefoxitin-resistance than the strains of other species though the *E. coli* strains produced less cephalosporinase activity. However, the cephalosporinases of the *E. coli* strains as well as the enzymes of other species could hardly hydrolyze cefoxitin.

Table 1. Levels of resistance to β-lactam antibiotics and β-lactamase activity in *E. coli* strains in comparison with the corresponding properties of the strains of *C. freundii* and *P. morganii*.

Strain	MIC (μg/ml)					Cephalosporinase activity*	Substrate specificity**				
	APC	CER	CEZ	CET	CFX		APC	CER	CEZ	CET	CFX
E. coli 255	400	50	100	800	100	0.72	<1	268	809	633	<1
E. coli GN206	200	25	25	400	25	0.25	1	264	444	343	<1
E. coli W3630	1.6	1.6	1.6	6.3	1.6	<0.003					
C. freundii GN346/16–10	100	100	200	800	25	4.0	4	6,600	8,000	1,200	<1
P. morganii 1510	400	400	200	1,600	12.5	2.7	7	1,220	832	412	<1

* Units per mg of dry weight of bacteria; Substrate, cephaloridine.
** Rates of hydrolysis of the substrates are expressed in per cent of hydrolysis of benzylpenicillin.
Abbreviations: APC, ampicillin; CER, cephaloridine; CEZ, cefazolin; CET, cephalothin; CFX, cefoxitin.

Cephalosporinase-less Mutants from *E. coli* 255 and *E. coli* GN206

As shown in Table 1, the levels of resistance to β-lactam antibiotics except cefoxitin were closely correlated to cephalosporinase activity in the bacteria examined. In order to clarify whether the cephalosporinase productivity can be attributed to the cefoxitin resistance in the *E. coli* strains or not, cephalosporinase-less mutants were isolated from the strains 255 and GN206. The MIC values of β-lactam antibiotics to the mutant strains, 255/L-7 and GN206/L-8, are listed in Table 2. These results strongly suggest that the resistance to β-lactam antibiotics including cefoxitin in the *E. coli*

Table 2. Cephalosporinase activity and sensitivity to β-lactam antibiotics of the mutant strains of *E. coli* defective in cephalosporinase-production.

Strain	MIC (μg/ml)					Cephalosporinase activity (units/mg of dry weight of bacteria)
	APC	CER	CEZ	CET	CFX	
E. coli 255/L–7	3.1	3.1	1.6	1.6	3.1	0.003
E. coli GN206/L–8	3.1	3.1	1.6	1.6	3.1	<0.003

strains can be attributed to cephalosporinase productivity. No significant differences in susceptibility to other antibiotics, *i.e.*, tetracycline, streptomycin and chloramphenicol, and in other biochemical properties between the mutant strains and their parental strains were observed (data not shown).

Properties of Cephalosporinases Produced by *E. coli* 255 and *E. coli* GN206 and Comparison with those of Cephalosporinases f om *C. freundii* and *P. morganii*

The molecular weights of the cephalosporinases of the *E. coli* strains were estimated to be 32,000 by gel filtration. The value is about the same as those of typical cephalosporinases produced by the *C. freundii* and the *P. morganii* strains, which were estimated to be 34,000[5] and 38,000[6], respectively.

The cephalosporinases of *E. coli* were examined with regard to their activity to hydrolyze cefoxitin at a low substrate concentration. The enzyme activity against cefoxitin was expressed as the percentage of that against cephalothin. The relative activity of the cephalosporinases of the *C. freundii* and the *P. morganii* strains against cefoxitin was also examined under the same experimental conditions. The results confirmed marked stability of cefoxitin to all the cephalosporinases used (Table 3).

Although cefoxitin was highly resistant to hydrolysis by the cephalosporinases, the *Km* values of the enzymes for cefoxitin, which were measured by using a large amount of the enzymes, suggested high affinity of the enzymes for cefoxitin. The dissociation constant of the cephalosporinase-cefoxitin complex was then determined with cephalothin as substrate. Cefoxitin was a competitive inhibitor for the enzyme reaction, and the *Ki* value for cefoxitin varied with each of the cephalosporinases. As shown in Table 3, the cephalosporinases of *E. coli* showed remarkably low *Ki* values when compared with other cephalosporinases.

Table 3. Kinetics of hydrolysis of cefoxitin by cephalosporinases of *E. coli*, *C. freundii* and *P. morganii*.

Source of cephalosporinase	Relative rate of hydrolysis of CFX*	*Km* (μM) Substrate		*Ki* (μM)**
		CET	CFX	
E. coli 255	0.05	45	<1	<1
E. coli GN206	0.09	36	4	<1
C. freundii GN346	0.26	13	<1	18
P. morganii 1510	0.07	12	2	8

* The rates of hydrolysis were determined by a modified microiodometric method with substrate concentration of 100 μM. The rates are relative and expressed as the percentage of hydrolysis of cephalothin.

** *Ki* values for cefoxitin were determined with cephalothin as substrate.

Location of Cephalosporinase in *E. coli* 255 and *E. coli* GN206

The cephalosporinase of *E. coli* was associated with the cells, and little was found in the supernatants from cultures in the logarithmic growth phase. In order to confirm the periplasmic location of the enzymes, cells of the strains 255 and GN206 were converted into spheroplasts with the aid of lysozyme and EDTA. Under conditions where β-galactosidase was little released from the spheroplasts, more than 80% of total cephalosporinase activity was found in a soluble form. The result suggests that the cephalosporinases of *E. coli* were periplasmic enzymes like many other β-lactamases of Gram-negative bacteria.

Properties of a Mutant Strain of *E. coli* Resistant to Ampicillin

E. coli K-12 is known to produce trace amounts of *ampC*-mediated β-lactamase constitutively[18]. It was assumed that clinical isolates of *E. coli* susceptible to ampicillin also revealed the phenotypic expression for production of such a β-lactamase similar to that in *E. coli* K-12. We derived ampicillin-resistant mutants as shown in Fig. 1, and examined whether the mutants showed corresponding levels of resistance to cefoxitin as the result of the acquisition of the higher productivity of β-lactamase.

An *in vitro*-developed ampicillin resistant strain was obtained from *E. coli* GN86 by the serial cultivation of the parental strain on a series of agar media containing increasing concentrations of ampicillin (Fig. 1), and designated as *E. coli* GN86/apr. No difference in biological and biochemical properties except the levels of resistance to β-lactam antibiotics between the parental and the mutant strains was observed, and subculture of the strain GN86/apr on the drug-free medium had essentially no influence on the degree of ampicillin resistance. As shown in Table 4, the strain GN86/apr gained the constitutive productivity of cephalosporinase, and also showed cefoxitin resistance. The cephalosporinase of the mutant strain was similar in substrate profile to those of the *E. coli* strains 255 and GN206.

Fig. 1. Development of ampicillin-resistance in *E. coli* GN86 *in vitro*.

E. coli GN86 was subjected to serial cultures on heart infusion agar plates containing increasing concentrations of ampicillin. Eight subcultures were repeated and the strain GN86/apr was obtained as survivor on the plate containing 800 μg/ml of ampicillin. Ampicillin resistance is expressed as the maximum concentration of the drug which allows visible growth of bacteria after 18 hours of incubation at 37°C.

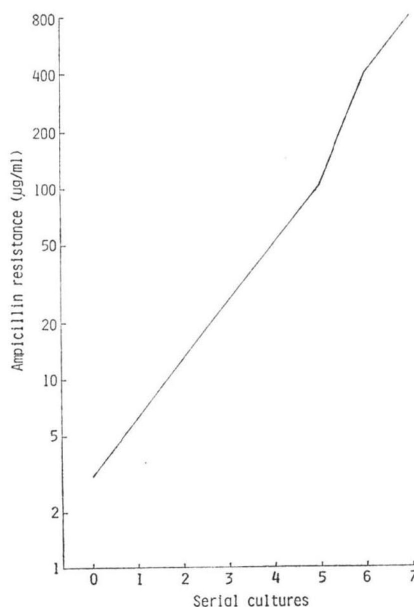

Table 4. Levels of resistance to β-lactam antibiotics and β-lactamase activity in *E. coli* strain GN86 and its mutant resistant to ampicillin.

Strain	MIC (μg/ml)					Cephalosporinase activity*	Substrate specificity**			
	APC	CER	CEZ	CET	CFX		APC	CER	CEZ	CFX
E. coli GN86	3.2	3.2	<3.2	6.3	<3.2	<0.003				
E. coli GN86/apr	400	100	50	>800	100	0.57	<1	250	346	<1

* Units per mg of dry weight of bacteria.

** Rates of hydrolysis of the substrates are expressed in per cent of hydrolysis of benzylpenicillin.

Discussion

Cefoxitin was very stable to hydrolysis by the cephalosporinases tested in this study, and this result was consistent with the observations of other workers[2]. However, it was demonstrated that there is a significant positive correlation between the cephalosporinase activity and the cefoxitin resistance in the *E. coli* strains 255 and GN206, and their cephalosporinase-less mutants. When com-

pared the cephalosporinase of *E. coli* with those of other bacterial species in enzymological property, one of marked differences was very lower *Ki* value of the *E. coli* enzyme for cefoxitin, suggesting very high affinity of the enzyme to the antibiotic. Therefore, an assumption is provided that the *E. coli* cephalosporinase locating around the targets of cefoxitin protects the targets from access of the antibiotic by its high binding affinity. The similar explanation may be applicable to ampicillin or carbenicillin resistance found in the cephalosporinase-producing strains of Gram-negative bacteria. Although cephalosporinases of Gram-negative bacteria scarcely hydrolyze these penicillins in general, they possess higher affinity for the penicillins than for their favorite substrates, the cephalosporins[5].

A β-lactamase mediated by the *amp* genes on chromosome of *E. coli* K-12 was purified and characterized by LINDSTRÖM *et al.*[14] The enzyme is produced constitutively and believed to locate in the periplasm. On the basis of its substrate profile, the enzyme may be classified into "cephalosporinase" though it was called "penicillinase" by them. The cephalosporinase of *E. coli* K-12 is similar in the properties to the cephalosporinases of our *E. coli* strains. However, the molecular weight of the enzyme of *E. coli* K-12 was reported to be 29,000, and the figure is somewhat smaller than those of the cephalosporinases of *E. coli* 255 and *E. coli* GN206. Although we could not perform direct comparison of the enzymes of our *E. coli* strains with that of *E. coli* K-12, the properties of the cephalosporinase produced by *E. coli* GN86/apr suggested that the enzymes of the clinical isolates were species-specific, that is, chromosomally mediated β-lactamase of *E. coli.*

Recently, we found that the cefoxitin-resistant *E. coli* strains also showed resistance to cefuroxime which was a new cephalosporin stable to hydrolysis by many β-lactamases[15]. It may be reasonable to expect that the clinical isolates producing larger amount of the species-specific β-lactamase will increase in *E. coli* with the introduction of new β-lactam antibiotics such as cefoxitin and cefuroxime.

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