CHARACTERISTICS OF AZTREONAM AS A SUBSTRATE, INHIBITOR AND INDUCER FOR β-LACTAMASES†

YUKO SAKURAI, YUKIE YOSHIDA, KEIKO SAITOH, MAYUMI NEMOTO, AKIHITO YAMAGUCHI and TETSUO SAWAI*

Division of Microbial Chemistry, Faculty of Pharmaceutical Sciences, Chiba University, 1-33 Yayoi-cho, Chiba 260, Japan

(Received for publication November 16, 1989)

Aztreonam was investigated as to its characteristics as a substrate, inhibitor and inducer for the well-defined β-lactamases of Gram-negative bacteria, and its antibacterial efficacy as to bacterial cells producing eight types of β-lactamases was also evaluated. Aztreonam was hydrolyzed at measurable rates by class A β-lactamases, a TEM-2 type penicillinase and the *Proteus vulgaris* cephalosporinase with a broad substrate range. However, the affinity of aztreonam for the class A enzymes was low, this property being well reflected by its high antibacterial activity toward producers of class A β-lactamases. Aztreonam was extremely stable as to the typical class C cephalosporinase of *Citrobacter freundii*, and acted as a competitive and progressive inhibitor for the β-lactamase. While the MICs of aztreonam in the cases of the constitutive producers of class C β-lactamases were evidently affected by enzyme production. An experiment involving aztreonam as a inhibitor in combination with a hydrolyzable β-lactam gave ambiguous results, however, a strong synergistic effect was found in combination with mecillinam. Using *Pseudomonas aeruginosa*, aztreonam was confirmed to be a poor inducer of β-lactamases.

Aztreonam was the first monocyclic β-lactam antibiotic to be clinically used. This antibiotic shows high antibacterial activity toward Gram-negative pathogens including *Pseudomonas aeruginosa*, and exhibits good stability to hydrolysis by many β-lactamases[1]. In addition to these desirable properties, aztreonam is known to act as a β-lactamase inhibitor[2]. β-Lactamases comprise a large family of enzymes exhibiting a variety of substrate profiles[3]. The interaction between β-lactam antibiotics and β-lactamases differs markedly with respect to the β-lactam and the enzyme type. In order to evaluate aztreonam as a substrate and inhibitor for typical β-lactamases of Gram-negative bacteria, we selected three different types of β-lactamases as test enzymes, *i.e.*, the TEM-2 type penicillinase, the *Proteus vulgaris* cephalosporinase with a relatively broad substrate range and the *Citrobacter freundii* cephalosporinase with a typical substrate profile for a cephalosporinase. We also evaluated the antibacterial efficacy of aztreonam as to the β-lactamase-producing bacteria, using a set of Gram-negative bacterial strains producing eight types of β-lactamases constitutively[4]. The enzymological and physical properties of these enzymes have been examined in detail by us[5]. The characteristics of aztreonam as a β-lactamase inducer were also examined, using *P. aeruginosa*.

In this paper, we present the results of a comprehensive survey performed with the use of the well-defined β-lactamases and β-lactamase-producing organisms.

† A part of this work was preliminarily reported at the Symposium entitled "A look to the future: Novel approaches to the treatment of bacterial and fungal infections". Broadbeach, Australia, Nov. 29~30, 1988.

Materials and Methods

Bacterial Strains and β-Lactam Antibiotics

Three types of β-lactamases were extracted from the following strains and purified by the procedure reported previously[5]. *Escherichia coli* ML1410 RGN823 harbors an R plasmid, RGN823, that mediates the constitutive production of a TEM-2 type penicillinase[6]. *P. vulgaris* GN76/C-1 produces a constitutive cephalosporinase with a broad substrate range, which is active on even penicillins and cephalosporins, including methoxyimino cephalosporins[5]. These two β-lactamases are class A β-lactamases according to AMBLER's classification based on the amino acid sequence around the active site[7]. *C. freundii* GN346 produces a species-specific β-lactamase with the substrate profile of a typical cephalosporinase semiconstitutively[5,8], the cephalosporinase being a class C β-lactamase. Other strains of *Proteus mirabilis*, *E. coli*, *Klebsiella pneumoniae*, *P. vulgaris*, *Morganella morganii*, *C. freundii* and *Enterobacter cloacae* were selected from a set of bacterial strains for evaluation of the β-lactamase stability of β-lactams, see Table 4. For measurement of the ability of aztreonam to induce β-lactamase production, *P. aeruginosa* strains PAO1 and PAO4054 were used. Strain PAO1 is a high producer of an inducible cephalosporinase and strain PAO4054, derived from PAO1, is a defective mutant as to the β-lactamase gene. The mutant strain was kindly provided by Dr. H. MATSUMOTO. The β-lactam antibiotics were kindly provided by the following pharmaceutical companies: Aztreonam, Eisai Co., Tokyo, Japan; benzylpenicillin, Meiji Seika Kaisha Ltd., Tokyo, Japan; 6-aminopenicillanic acid, Toyo Jozo Co., Ltd., Tokyo, Japan; mecillinam, Takeda Chemical Industries Co., Osaka, Japan; cephalothin and cephaloridine, Shionogi & Co., Osaka, Japan; cefoxitin, Merck Sharp and Dohme Research Laboratories, NJ, U.S.A.; and cefuroxime, Nippon Glaxo Co., Ltd., Tokyo, Japan.

Measurement of β-Lactamase Activity, *Ki* Values, IC_{50} Values and Progressive Inhibition

β-Lactamase activity was determined by the microiodometric method devised by NOVICK[9] with slight modification or a spectrophotometric method[10] at 30°C in 50 mM phosphate buffer (pH 7.0). The absorbance at 235, 265 and 318 nm was employed, with the spectrophotometric method, for assaying the hydrolysis of benzylpenicillin, cephalothin and aztreonam, respectively.

One U of β-lactamase was defined as the amount of enzyme which hydrolyzed 1 μmol of substrate per minute at pH 7.0 and 30°C. The *Ki* value of β-lactamase for aztreonam and the inhibitor concentration abolishing 50% of the enzyme activity (IC_{50}) were determined with benzylpenicillin (for the TEM-2 type penicillinase) or cephalotin (for the cephalosporinases) as a substrate, by the spectrophotometric method. The progressive inhibition of β-lactamases by aztreonam was assayed by a modification of the dilution assay method of FISHER *et al.*[11], as follows. β-Lactamase in 50 mM phosphate buffer (pH 7.0) was incubated with various concentrations of aztreonam at 30°C. After different times, 5 μl of each mixture was withdrawn and added to 3 ml of 100 μM benzylpenicillin (for the penicillinase) or cephalothin (for the cephalosporinases) in the same buffer. The hydrolysis rates for the favorable substrates were determined by the microiodometric method.

Measurement of Bacterial Sensitivity to β-Lactams and the Synergistic Effect of Aztreonam in β-Lactamase-producing Bacteria

The antibacterial activity of β-lactams was determined by means of a serial agar dilution method and expressed as the MIC (μg/ml). The procedure was reported in detail previously[4]. The synergistic effect of aztreonam in combination with mecillinam on the β-lactamase-producing bacteria was measured by the method previously reported[12]. The effect was expressed as the fractional inhibitory concentration (FIC) index[13].

Results and Discussion

Stability of Aztreonam as to Three Types of β-Lactamases and its Inhibitory Effect on the Enzymes

The hydrolysis of aztreonam by three β-lactamases was measured by the spectrophotometric method, with substrate concentration of 100 μM. The results are expressed as the number of molecules of the

Table 1. Stability of aztreonam and cephaloridine as the three types of β-lactamases.

β-Lactamase	Class of β-lactamase[a]	Rate of hydrolysis[b]		Ratio[c] (%)
		Aztreonam	Cephaloridine	
TEM-2 type penicillinase	A	4.0	51.7	8
Proteus vulgaris cephalosporinase	A	57.5	346.7	17
Citrobacter freundii cephalosporinase	C	<0.6	165.7	<0.4

[a] AMBLER's classification based on the amino acid sequence around the active site[7].
[b] μmol of β-lactam/μmol enzyme minute^{-1}.
[c] Ratio of aztreonam hydrolysis to cephaloridine hydrolysis at the substrate concentration of 100 μM.

Table 2. Kinetic constants for inactivation of the three β-lactamases by aztreonam.

β-Lactamase	*Ki* (μM)	IC_{50} (μM)		
		0[a]	5	10
TEM-2 type penicillinase	303	305	280	280
Proteus vulgaris cephalosporinase	124	61	85	104
Citrobacter freundii cephalosporinase	0.32	0.021	0.009	0.007

[a] Preincubation period (minutes).

substrate hydrolyzed per minute per molecule of the enzyme, and are shown in Table 1 together with the hydrolysis rate for cephaloridine, which is a common and favorable substrate of the three β-lactamases. Two class A β-lactamases, particularly the *P. vulgaris* enzyme, showed measurable activity toward aztreonam, although the hydrolysis rate was evidently lower than that for a favorable substrate. On the other hand, aztreonam showed marked stability as to the *C. freundii* enzyme, which is a class C β-lactamase. The *P. vulgaris* enzyme has a few characteristics that are common to class C β-lactamases, such as high activity toward cephalosporins and a high isoelectric point, however, it should be emphasized that the species-specific β-lactamase of *P. vulgaris* was recently identified as a class A β-lactamase on the basis of its entire amino acid sequence[14].

In order to evaluate the efficacy of aztreonam as a competitive inhibitor for the three β-lactamases, the *Ki* values and the aztreonam concentrations required for 50% inhibition of the β-lactamase activity (IC_{50}) were measured (Table 2). Aztreonam showed high affinity for the *C. freundii* enzyme, the *Ki* value being 0.32 μM. On the contrary, aztreonam showed lower affinity for the two class A enzymes, the *Ki* values being more than 380 times that for the class C enzyme. These properties of aztreonam were well reflected in the IC_{50} values. The IC_{50} value was estimated after three different preincubation periods with aztreonam. In the case of the TEM-2 type penicillinase, the preincubation period had no significant influence on the IC_{50} value, but the IC_{50} value for the *P. vulgaris* enzyme increased with an increase in the preincubation period. This is attributed to hydrolysis of the inhibitor by the *P. vulgaris* enzyme. On the other hand, the IC_{50} value for the *C. freundii* enzyme significantly decreased with an increase in the preincubation period, indicating the progressive inhibition of the class C enzyme by aztreonam.

The progressive inhibition of the *C. freundii* enzyme by aztreonam was confirmed experimentally (data not shown). When the two β-lactamases belonging to class A were incubated with a large excess of

aztreonam (1 mM), we could not detect progressive inhibition even through the enzymes were incubated at 30°C for 60 minutes. In contrast, complete inactivation of the *C. freundii* enzyme was caused by 0.5 μM aztreonam within 5 minutes preincubation. However, the inhibition was essentially reversible and the $T_{1/2}$ of the reactivation was estimated to be 50 hours. BUSH *et al.* reported the properties of aztreonam as a substrate and inhibitor for a TEM-2 type β-lactamase, K1 β-lactamase and P99 β-lactamase[2,15]. Our results are essentially consistent with theirs. However, the $T_{1/2}$ period of 50 hours is markedly longer than the 6.8 hours for P99 β-lactamase, a typical cephalosporinase belonging to the class C β-lactamases.

Potency of Aztreonam as an Inducer for Class C β-Lactamase

Most class C β-lactamases produced by Gram-negative bacteria are inducible and β-lactam compounds act as inducers. It is well known that β-lactam compounds differ from one another in the ability to induce enzyme synthesis, and the inducibility is not necessarily correlated with the antibacterial potency. 6-Aminopenicillanic acid is known to be a good inducer for class C β-lactamases from the experience of many workers. While the low activity of aztreonam as to β-lactamase induction is well known, we reexamined the inducibility of aztreonam quantitatively using *P. aeruginosa* PAO1, a producer of a inducible class C β-lactamase. The experimental results are shown in Table 3 together with the reference data obtained for 6-aminopenicillanic acid. The results indicate the very poor ability of aztreonam as to induction. The same conclusion can be also drawn on comparison of the MICs of *P. aeruginosa* PAO1 and its β-lactamase defective mutant, PAO4054 (*bla*$^-$). The MICs of aztreonam for the two strains are identical indicating its poor inducibility. This is also in striking contrast to the results for 6-aminopenicillanic acid, cephaloridine and cefoxitin (Fig. 1). It should be noted that the same conclusion was drawn with the use of the inducible cephalosporinase of *P. vulgaris*, a class A β-lactamase (data not shown). Recently, OLIVA *et al.* have proposed the interesting hypothesis that inducer binding to penicillin-binding protein 2 (PBP2) is involved in the induction of the class C β-lactamase in *E. coli*[16]. Aztreonam has a very limited range as to PBP binding, binding tightly only

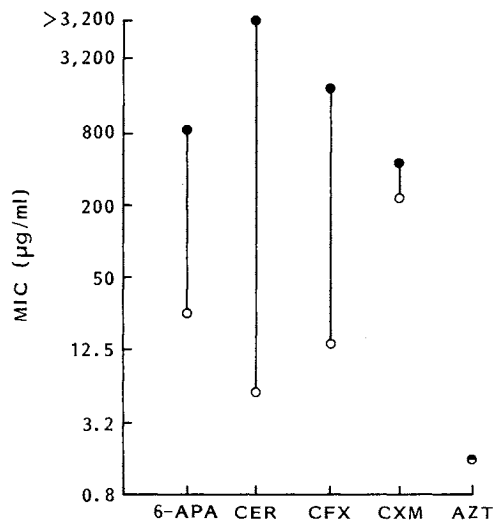
Table 3. Cephalosporinase activity of *Pseudomonas aeruginosa* PAO1 in the presence or absence of 6-aminopenicillanic acid or aztreonam.

Inducer	Inducer concentration (μg/ml)		Cephalosporinase activity (U/mg protein)
6-Aminopenicillanic acid	0		ND
	500	(0.63)[a]	5.70×10^{-1}
	1,000	(1.25)	5.72×10^{-1}
	2,000	(2.5)	2.76×10^{-1}
Aztreonam	0		ND
	0.5	(0.32)	ND
	1.0	(0.63)	ND
	2.0	(1.25)	ND
	5.0	(3.13)	ND

[a] Ratio to MIC in parenthesis.
ND: Not detectable.

Fig. 1. Difference in the MIC level of a β-lactam between wild and mutant (*bla*$^-$) strains of *Pseudomonas aeruginosa* PAO1.

MICs as to strains PAO1 (●) and PAO4054 (*bla*$^-$) (○) were determined as to aztreonam (AZT), cephaloridine (CER), cefoxitin (CFX), cefuroxime (CXM) and 6-aminopenicillanic acid (6-APA). The gap between the MICs for the two test strains is indicated by a solid line.



to PBP3[15]. The lack of inducibility may be attributed to the lack of binding ability as to PBP2.

Antibacterial Activity of Aztreonam toward Gram-negative Bacteria Producing Eight Types of β-Lactamases

In order to evaluate the stability of β-lactams as to various types of β-lactamases located in the periplasmic space of Gram-negative bacteria, we proposed a set of bacterial strains[4]. The set was composed of eight bacterial groups and each group was made up of 3 or 4 strains. The strains in the same group produce the same type of β-lactamase constitutively, however, the amount of the enzyme produced in cells differs with the strain. From the MICs of antibiotics for the strains of a given group, we can estimate the relative stability of an antibiotic as to an enzyme. By using the set of strains listed in Table 4, the stability of aztreonam as to eight types of β-lactamases, comprising four class A β-lactamases and four class C β-lactamases, was estimated. The results are shown in diagrammatically in Fig. 2 together with those for cephaloridine, cefoxitin and cefuroxime. Cephaloridine is one of the traditional cephalosporins. Cefoxitin and cefuroxime are the prototypes of cephamycins and methoxyimino cephalosporins, respectively.

The solid line for each group in the figure indicates the range from the highest MIC to the lowest MIC among the strains belonging to that group. The longer the line the lower the efficacy of the antibiotic in β-lactamase producers. The results indicated that aztreonam and cefoxitin are most effective for the

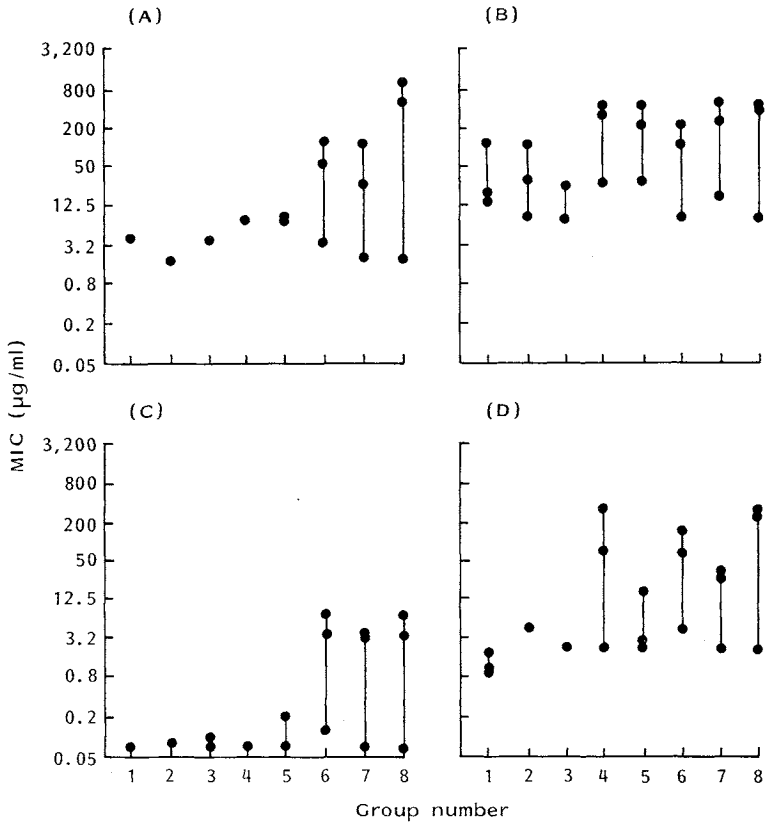
Table 4. Bacterial strains used for evaluation of β-lactamase stability[4].

Group	Strain	β-Lactamase activity (U/mg dry weight cells)[a]	
		Class A β-lactamase	Class C β-lactamase
1	*Proteus mirabilis* N-29	2.0	
	P. mirabilis N-29/2	0.28	
	P. mirabilis N-29/5	0.01	
2	*Escherichia coli* ML1410 RGN823	16.7	
	E. coli ML1410 RGN14	0.6	
	E. coli ML1410	<0.003	
3	*Klebsiella pneumoniae* GN69	1.11	
	K. pneumoniae GN118	0.047	
	K. pneumoniae GN69/2-1	<0.01	
4	*Proteus vulgaris* GN79/C-1	2.4	
	P. vulgaris GN79/C-1/1	1.8	
	P. vulgaris GN79/C-1/3	0.03	
	P. vulgaris GN79/C-1/2	<0.01	
5	*Morganella morganii* 1510		2.68
	M. morganii 1510/3		0.07
	M. morganii 1510/9		0.006
6	*Escherichia coli* 255		0.72
	E. coli GN206		0.25
	E. coli 255/L-7		0.003
7	*Citrobacter freundii* GN346		24.2
	C. freundii GN346/16-10		4.0
	C. freundii GN346/16		0.067
8	*Enterobacter cloacae* 363		24.9
	E. cloacae 363/1-3		10.6
	E. cloacae 363/2		0.05
	E. cloacae 363/1		<0.01

[a] The activity of class A enzymes except for the *P. vulgaris* cephalosporinase, was measured with benzylpenicillin as the substrate, and class C enzymes and the *P. vulgaris* enzyme were assayed with cephalothin as the substrate.

Fig. 2. Diagram showing the effect of β-lactamase production on the MIC levels of the test strains.

(A) Cefoxitin, (B) cephaloridine, (C) aztreonam, (D) cefuroxime.



The range of MIC variation in the same group of strains is indicated by a solid line. The numbers on the abscissa are the group numbers of the test strains listed in Table 4. 1: *Proteus mirabilis* (class A penicillinase), 2: *Escherichia coli* (class A TEM-2 type penicillinase), 3: *Klebsiella pneumoniae* (class A penicillinase), 4: *Proteus vulgaris* (class A cephalosporinase), 5: *Morganella morganii* (class C cephalosporinase), 6: *E. coli* (class C cephalosporinase), 7: *Citrobacter freundii* (class C cephalosporinase), 8: *Enterobacter cloacae* (class C cephalosporinase).

enzyme-producers among the β-lactams tested, but the antibacterial efficacy of both β-lactams is evidently reduced on high production of class C β-lactamases. Class C β-lactamases produced by clinical isolates are mainly inducible enzymes[17], and so aztreonam is believed to be effective toward these isolates. It should be emphasized, however, that one of the approaches to the gain of aztreonam resistance is constitutive production of class C β-lactamases.

Synergistic Activity of Aztreonam in Combination with Mecillinam

Aztreonam acts on class C β-lactamases as a strong competitive and progressive inhibitor. This led to the idea of using aztreonam as an inhibitor in combination with a hydrolyzable β-lactam. We examined the efficacy of aztreonam as an inhibitor as to the test strains producing three different types of β-lactamases, *i.e.*, a TEM-2 type penicillinase, the *P. vulgaris* cephalosporinase and the *C. freundii* cephalosporinase, in combination with ampicillin, cephalothin and cephaloridine. The results were contrary to our expectation, the synergistic effect of the inhibitor with these traditional β-lactams in β-lactamase-producing bacteria

Fig. 3. Synergistic activity of aztreonam (AZT) in combination with mecillinam (MPC) toward *Proteus vulgaris*.

AZT (μg/ml)	MPC (μg/ml)											AZT alone
	400	200	100	50	25	12.5	6.3	3.2	1.6	0.8	0.4	
0.05												
0.025										+	+	+
0.0125										+	+	+
0.0063									+	+	+	+
0.0032								+	+	+	+	+
0.0016								+	+	+	+	+
0.0008						+	+	+	+	+	+	+
MPC alone		+	+	+	+	+	+	+	+	+	+	+

Bacterial growth with the combination of the two β-lactams is denoted by +, for positive.

not being definite. One of the reasons for these results may be the high antibacterial potency of aztreonam.

However, interestingly, we found that there was a marked synergistic effect when aztreonam was combined with mecillinam. Fig. 3 shows an example of the results, which were obtained for *P. vulgaris* GN76/C-1. The lowest FIC index value was 0.048, which was obtained with the combination of 0.03 MIC aztreonam and 0.016 MIC mecillinam. It is evident from the kinetic properties shown in Tables 1 and 2 that aztreonam is a poor inhibitor for the *P. vulgaris* enzyme. Such a synergistic effect is considered to be independent of the β-lactamase inhibition and may be attributed to the inhibitory effect on the targets, *i.e.*, PBPs. The specific target of attack of aztreonam and mecillinam are PBP3 and PBP2, respectively[15,18], and the cooperative effects on these targets may cause such a synergistic effect. Recently, GRACÍA *et al.* reported that the simultaneous inhibition of PBP1 and PBP2 of *E. coli* efficiently induces cell lysis[19]. A similar effect is assumed in the case of the combination of aztreonam and mecillinam.

References

1) SYKES, R. B.; D. P. BONNER, K. BUSH & N. H. GEORGOPAPADAKOU: Azthreonam (SQ 26,776), a synthetic monobactam specifically active against aerobic Gram-negative bacteria. Antimicrob. Agents Chemother. 21: 85～92, 1982
2) BUSH, K.; J. S. FREUDENBERGER & R. B. SYKES: Interaction of azthreonam and related monobactam with β-lactamases from Gram-negative bacteria. Antimicrob. Agents Chemother. 22: 414～420, 1982
3) RICHMOND, M. H. & R. B. SYKES: The β-lactamases of gram-negative bacteria and their possible physiological role. Adv. Microb. Physiol. 9: 31～88, 1973
4) SAWAI, T.; T. YOSHIDA, K. TSUKAMOTO & S. YAMAGISHI: A set of bacterial strains for evaluation of β-lactamase-stability of β-lactam antibiotics. J. Antibiotics 34: 1318～1326, 1981
5) SAWAI, T.; M. KANNO & K. TSUKAMOTO: Characterization of eight β-lactamases of Gram-negative bacteria. J. Bacteriol. 152: 567～571, 1982
6) SAWAI, T.; K. TAKAHASHI, S. YAMAGISHI & S. MITSUHASHI: Variant of penicillinase mediated by an R factor in *Escherichia coli*. J. Bacteriol. 104: 620～629, 1970
7) AMBLER, R. P.: The structure of β-lactamases. Philos. Trans. R. Soc. Lond. (Biol.) 289: 321～331, 1980
8) SAWAI, T.; S. NAKAJIMA, T. MOROHOSHI & S. YAMAGISHI: Thermolabile repression of cephalosporinase synthesis in *Citrobacter freundii*. Microbiol. Immunol. 21: 631～638, 1977
9) NOVICK, R. P.: Micro-iodometric assay for penicillinase. Biochem. J. 83: 236～240, 1962
10) YAMAGUCHI, A.; T. HIRATA & T. SAWAI: Kinetic studies on inactivation of *Citrobacter freundii* cephalosporinase by sulbactam. Antimicrob. Agents Chemother. 24: 23～30, 1983
11) FISHER, J.; R. L. CHARNAS & J. R. KNOWLES: Kinetic studies on the inactivation of *Escherichia coli* RTEM β-lactamase by clavulanic acid. Biochemistry 17: 2180～2184, 1978
12) SAWAI, T. & T. YOSHIDA: A simple method for testing the efficacy of a β-lactamase inhibitor against β-lactamase-producing Gram-negative bacteria. J. Antibiotics 35: 1072～1077, 1982

13) DOUGHERTY, P. F.; D. W. YOTTER & T. R. MATTHEWS: Microdilution transfer plate technique for determining in vitro synergy of antimicrobial agents. Antimicrob. Agents Chemother. 11: 225~228, 1977
14) OKUGUCHI, M.; S. NAKATA, N. NAKAJIMA & K. SUGIMOTO: Cloning and nucleotide sequence of the *bla* gene of *Proteus vulgaris*. Proceedings of the 9th Annual Meeting of the Japanese Society of Molecular Biology, p. 309, Nagoya, Dec. 4~7, 1986
15) BUSH, K.; F. Y. LIU & S. A. SMITH: Interactions of monobactams with bacterial enzymes. Dev. Ind. Microbiol. 27: 153~164, 1987
16) OLIVA, B.; P. M. BENNETT & I. CHOPRA: Penicillin-binding protein 2 is required for induction of the *Citrobacter freundii* class I chromosomal β-lactamase in *Escherichia coli*. Antimicrob. Agents Chemother. 33: 1116~1117, 1989
17) SAWAI, T.; S. MITSUHASHI & S. YAMAGISHI: Drug resistance of enteric bacteria. XIV. Comparison of β-lactamases in gram-negative rod bacteria resistant to α-aminobenzylpenicillin. Jpn. J. Microbiol. 12: 423~434, 1968
18) SPRATT, B. G.: Distinct penicillin-binding proteins involved in the division, elongation and shape of *Escherichia coli* K12. Proc. Natl. Acad. Sci. U.S.A. 72: 2999~3003, 1975
19) GARCÍA DEL PORTILLO, F.; M. A. DE PEDRO, D. JOSELEAU-PETIT & R. D'ARI: Lytic response of *Escherichia coli* cells to inhibitors of penicillin-binding proteins 1a and 1b as a timed event related to cell division. J. Bacteriol. 171: 4217~4221, 1989