A SET OF BACTERIAL STRAINS FOR EVALUATION OF β-LACTAMASE-STABILITY OF β-LACTAM ANTIBIOTICS

TETSUO SAWAI, TAKASHI YOSHIDA, KIKUO TSUKAMOTO and SABURO YAMAGISHI*

Faculty of Pharmaceutical Sciences, Chiba University, Chiba, Japan

(Received for publication August 12, 1980)

A set of bacterial strains composed of nine bacterial groups, with each made up of three or four strains, was used to estimate the stability of β-lactam antibiotics to nine types of β-lactamases from Gram-negative bacteria. The strains in the same group produced the same kind of enzyme constitutively, but the enzyme activity achieved in the bacterial cell differed with different strains. The difference in antibacterial activity of each antibiotic on the strains of each group, easily measured by a simple plate technique, permits an estimation of its relative stability to each β-lactamase. This method was applied to thirteen β-lactam antibiotics including seven new ones.

One of the principal objective of β-lactam antibiotic research is to improve the β-lactamase-stability of the antibiotic by modification of its chemical structure or introduction of a β-lactam with a novel nucleus. For such research, a simple method for estimating the β-lactamase-stability of the derivatives is desired. In order to evaluate the relative stability of the antibiotic to nine kinds of β-lactamase of Gram-negative bacteria, using a simple plate technique, we selected a set of bacterial strains. The set was composed of nine bacterial groups and each group was generally made up of 3 or 4 bacterial strains. The bacterial strains in the same group produce the same kind of β-lactamases constitutively; however, the intracellular enzyme activity differs with different strains. From minimum inhibitory concentrations measured for each strain of a given group, the relative stability of the antibiotic to the β-lactamase characteristic of the group was estimated. Nine kinds of β-lactamases, of which the enzymological and physiological properties were examined in detail, were used.

Here are described examples of the method applied to thirteen β-lactam antibiotics including seven new ones.

Materials and Methods

Bacterial Strains and R Plasmids

The organisms used in this study are listed in Table 1. The R plasmids RGN14 and RGN823 mediate type Ia and type Ib penicillinase-synthesis in their host bacteria, respectively[1]. Type Ia and Ib penicillinases corresponded to TEM-1-type and TEM-2-type of MATTHEW's classification[2], respectively. The R plasmid RGN238 mediates type II penicillinase(oxacillin-hydrolyzing penicillinase) synthesis in its host bacteria[3,4]. The R plasmid RN-29 mediates carbenicillinase(carbenicillin-hydrolyzing penicillinase) synthesis and a detailed report of this penicillinase will be presented subsequently.

β-Lactam Antibiotics

β-Lactam antibiotics employed in this study were kindly provided by the following pharmaceutical companies: cephalexin (CEX), cefoperazone (CPZ) and piperacillin (PIPC), Toyama Chemical Co., Tokyo, Japan; carbenicillin(CPC) and cefazolin (CEZ), Fujisawa Pharmaceutical Co., Osaka, Japan;

* Died August 16, 1980.

Table 1. Bacterial strains for evaluation of β-lactamase-stability.

Organisms	Description	Reference
E. coli ML1410	A substrain of K12, 58-161 F⁻ *met*, resistant to nalidixic acid	9
E. coli ML1410 RGN823	ML1410 carrying RGN823	1
E. coli ML1410 RGN14	ML1410 carrying RGN14	1, 3
E. coli ML1410 RGN238	ML1410 carrying RGN238	3, 4
K. pneumoniae GN69	Clinical isolate	7, 10, 11
K. pneumoniae GN118	Clinical isolate	7, 10, 11
K. pneumoniae GN69/2-1	Penicillinase less mutant of GN69	This paper
P. mirabilis N-29	Clinical isolate harboring an R plasmid RN-29	This paper
P. mirabilis N-29/2	Penicillinase mutant of N-29	This paper
P. mirabilis N-29/5	Penicillinase mutant of N-29	This paper
E. coli 255	Clinical isolate	12, 13
E. coli 255 RGN823	255 carrying RGN823	This paper
E. coli 255/L-7	Cephalosporinase less mutant of 255	13
E. coli GN206	Clinical isolate	10, 13
C. freundii GN346	Clinical isolate	10, 14
C. freundii GN346 RGN823	GN346 carrying RGN823	This paper
C. freundii GN346/16	Cephalosporinase mutant of GN346	14
C. freundii GN346/16-10	Cephalosporinase mutant of GN346/16	14
Ent. cloacae 363	Clinical isolate	This paper
Ent. cloacae 363/1	Cephalosporinase mutant of 363	This paper
Ent. cloacae 363/2	Cephalosporinase mutant of 363	This paper
Ent. cloacae 363/1-3	Cephalosporinase mutant of 363/1	This paper
P. morganii 1510	Clinical isolate	8
P. morganii 1510 RGN823	1510 carrying RGN823	This paper
P. morganii 1510/3	Cephalosporinase mutant of 1510	This paper
P. morganii 1510/9	Cephalosporinase less mutant of 1510	This paper
P. vulgaris GN76/C-1	Constitutive mutant of cephalosporinase, isolated from clinical isolate GN76	This paper
P. vulgaris GN76/C-1/1	Cephalosporinase mutant of GN76/C-1	This paper
P. vulgaris GN76/C-1/2	Cephalosporinase mutant of GN76/C-1	This paper
P. vulgaris GN76/C-1/3	Cephalosporinase mutant of GN76/C-1	This paper

cephaloridine (CER) and cephalothin(CET), Torii Pharmaceutical Co., Tokyo, Japan; ampicillin(APC), Meiji Seika Co., Tokyo, Japan; apalcillin (APPC), Sumitomo Chemical Ind. Co., Osaka, Japan; cefoxitin (CFX), Merck Sharp & Dohme Research Laboratories, Rahway, N.J., U.S.A.; cefotaxime(CTX), Hoechst Japan, Tokyo, Japan; cefuroxime(CXM), Shin Nihon Jitsugyo Co., Tokyo, Japan; PS-5, Sanraku-Ocean Co., Tokyo, Japan.

β-Lactamase Studies

β-Lactamase activity of bacterial cells was determined by the method of PERRET[5] after sonic treatment of the cells with three one-minute bursts at 5°C, and expressed as units per mg of bacterial dry weight. One unit of penicillinase or cephalosporinase was defined as the activity which hydrolyzed one μmole of benzylpenicillin or cephaloridine, respectively, in 1 minute at pH 7.0 and 30°C.

Nine kinds of β-lactamases, consisting of four penicillinases and five cephalosporinases, were used to study the kinetics of β-lactamase hydrolysis of the antibiotics. An iodometric assay method[6] was used for the kinetic experiments. Type I and type II penicillinases of R plasmids, and penicillinase of *Klebsiella pneumoniae* GN69 were purified by procedure reported previously[1,7]. Cephalosporinase of *Proteus*

morganii 1510 was purifed according to the procedure described by FUJII-KURIYAMA *et al.*[8]. Penicillinase of RN-29 and four other cephalosporinases (the enzymes of *Escherichia coli, Proteus vulgaris, Citrobacter freundii* and *Enterobacter cloacae*) were partially purified by adsorption and elution on a CM-Sephadex column and gel-filtration on a Sephadex G-75 column.

Measurement of Bacterial Sensitivity to β-Lactam Antibiotics

The anti-bacterial activity of β-lactam antibiotics was determined by a serial agar dilution method and the activity was expressed by minimum 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 using an inoculum replicating device (Microplanter, Sakuma Factory, Tokyo, Japan). MICs were measured after incubation at 37°C for 18 hours. All media used were products of Eiken Chemical Co., Tokyo, Japan.

Isolation of Mutant Strains

Isolation of the mutant strains producing less β-lactamase than their parental strain was carried out as follows: The bacterial cells growing exponentially in heart infusion broth were harvested by centrifugation and washed once with 0.1 M citrate buffer (pH 6.0). The washed cells were treated with 100 to 300 μg/ml of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine(NG) in citrate buffer at 30°C or 37°C for 10 to 30 minutes. The concentration of NG, the temperature and the time of exposure varied according to the susceptibility of the bacteria to NG, in order to obtain from 1 to 10% survivors. The conditions for each bacterial species were as follows: *E. coli*, 100 μg/ml, 30°C, 10 minutes; *K. pneumoniae*, 200 μg/ml, 30°C, 15 minutes; *Proteus mirabilis*, 200 μg/ml, 30°C, 15 minutes; *Ent. cloacae*, 200 μg/ml, 37°C, 20 minutes; *P. morganii*, 300 μg/ml, 30°C, 30 minutes; and *P. vulgaris*, 200 μg/ml, 37°C, 30 minutes. The NG-treated cells were spread on MÜELLER-HINTON agar (Difco) and incubated at 37°C for 18 hours. The colonies which had appeared were covered with 5 ml of 1.2% agar, melted and kept at 52~55°C, and 1 ml of 0.1% phenol red (adjusted to pH 9.0 by 0.1 N NaOH) as well as 0.4 ml of either 10% ampicillin, in the case of penicillinase-producing bacteria, or 5% cephalothin for cephalosporinase producers. Non acidifying(red) or slowly acidifying colonies (yellowing slowly) indicative of lower β-lactamase production than the parental strain, were isolated.

P. vulgaris GN76/C-1, a constitutive mutant of cephalosporinase, was isolated as described above, except that acidifying colonies (yellowing quickly) were isolated.

Results

Preparation and Properties of the Strains for Evaluation

The mode of β-lactamase-synthesis in bacteria, *i.e.*, constitutive or inducible production, influences greatly the resistance level of the β-lactamase-producing bacteria to the antibiotic. In order to exclude such a factor, the microorganisms selected for this study were either constitutive producers of β-lactamase, or constitutive mutants from the inducible wild type.

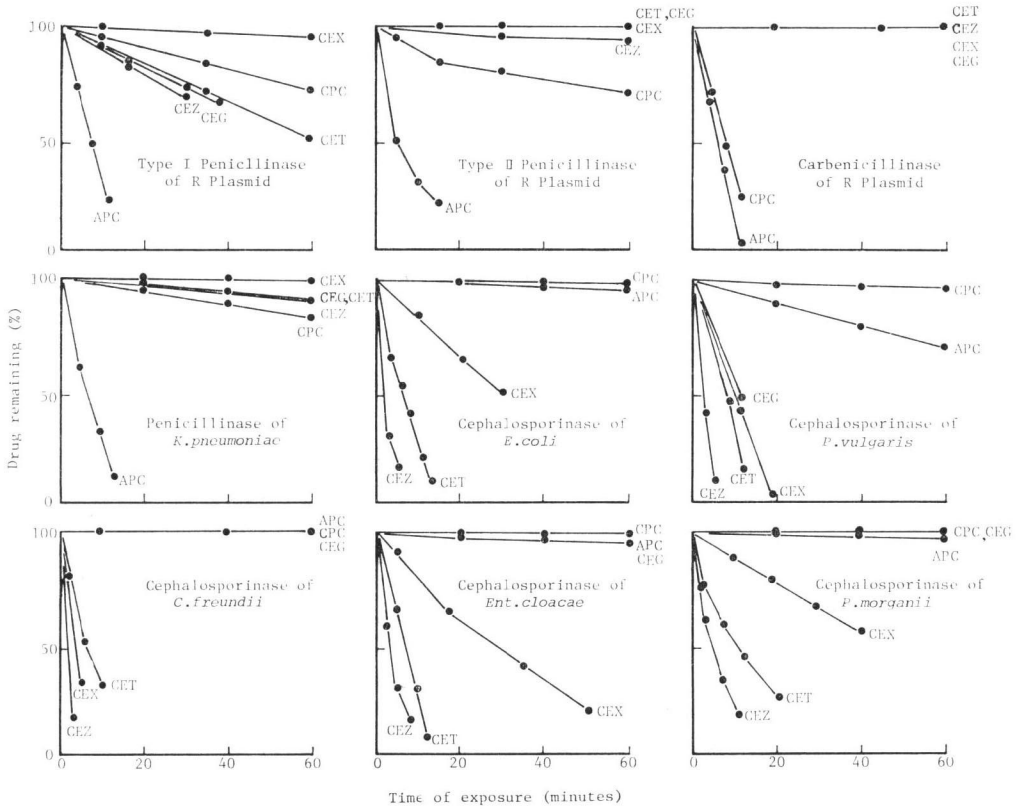
K. pneumoniae strains GN69 and GN118 are producers of the species-specific penicillinase[7,11]. *K. pneumoniae* GN69/2-1 is a mutant strain lacking penicillinase activity.

E. coli strains 255 and GN206 produce a cephalosporinase which is very similar in substrate specificity, molecular weight and constitutive production to a chromosomal β-lactamase of *E. coli* K12[15]. *E. coli* 255/L-7 is a mutant strain from 255[13], and lacks β-lactamase activity.

C. freundii GN346 produces a large amount of cephalosporinase constitutively at 37°C, but at 20°C the enzyme is synthesized as a typical inducible enzyme[14]. Strain GN346/16 is a mutant with less ability to produce cephalosporinase[14]. Strain GN346/16-10 is a back mutant of GN346/16 with partially restored cephalosporinase activity.

Ent. cloacae 363 was isolated from a patient in 1977. It produces a typical cephalosporinase constitutively. The mutant strains 363/1 and 363/2 produce smaller amounts of cephalosporinase than

Fig. 1. Kinetics of hydrolysis of six β-lactam antibiotics by nine β-lactamases.



the parental strain. Strain 363/1-3, a back mutant with partially restored cephalosporinase activity, was isolated from the strain 363/1 by selecting growth on cefazolin-containing agar.

P. morganii 1510 produces a typical cephalosporinase constitutively. Strains 1510/3 and 1510/9 are mutant strains with lower cephalosporinase activity.

The cephalosporin-resistant isolates of *P. vulgaris* from clinical sources are generally high producers of an inducible cephalosporinase with relatively broad substrate specificity[10]. *P. vulgaris* GN76/C-1 is a constitutive mutant derived from the wild strain GN76[10]. Other *P. vulgaris* strains with lower enzyme activity were derived from GN76/C-1.

It was also confirmed that there were no essential differences in the substrate profiles and heat-stability between the β-lactamases of the mutants and their respective parental strains.

Substrate Profiles of Nine β-Lactamases

The rate of hydrolysis of six β-lactam antibiotics by the nine β-lactamases was measured, and the relative concentration of the remaining drug was plotted against time of exposure (Fig. 1). These plots give an adequate idea of the characteristics of the β-lactamase produced by each group of strains.

Carbenicillinase showed special activity towards penicillins. The other two penicillinases, especially the type I penicillinase mediated by the R plasmid, had a broad substrate specificity. Cephalosporinases, except the enzyme from *P. vulgaris*, were typical as judged by their substrate profiles. The cephalosporinase of *P. vulgaris* showed the unique property of having a broader substrate profiles. More de-

Table 2. Relationship between penicillinase activity and levels of resistance to β-lactam antibiotics in penicillinase-producing bacteria.

Organisms	Penicillinase activity (units/mg dry weight of bacteria)	MIC (μg/ml)												
		Well-known β-lactams						New β-lactams						
		APC	CPC	CET	CER	CEZ	CEX	PIPC	APPC	CPZ	CFX	CXM	CTX	PS-5
E. coli ML1410 RGN823	16.7	>3,200	>3,200	25	25	25	12.5	800	800	12.5	3.1	3.1	0.05	6.3
E. coli ML1410 RGN14	0.6	800	>3,200	12.5	6.3	3.1	12.5	50	50	0.8	3.1	3.1	0.025	3.1
E. coli ML1410	<0.003	3.1	3.1	6.3	3.1	1.6	12.5	1.6	1.6	≤0.2	3.1	1.6	0.025	3.1
E. coli ML1410 RGN238	0.025	400	200	6.3	3.1	3.1	12.5	25	50	≤0.2	3.1	6.3	0.1	3.1
K. pneumoniae GN69	1.11	400	3,200	3.1	6.3	3.1	6.3	25	50	1.6	3.1	1.6	0.05	3.1
K. pneumoniae GN118	0.047	25	200	1.6	3.1	1.6	3.1	3.1	3.1	≤0.2	1.6	1.6	0.025	3.1
K. pneumoniae BN69/2-1	<0.01	1.6	6.3	1.6	3.1	1.6	6.3	1.6	1.6	≤0.2	3.1	1.6	0.05	3.1
P. mirabilis N-29	2.0	1,600	3,200	12.5	50	6.3	12.5	50	100	25	3.1	1.6	0.025	ND
P. mirabilis N-29/2	0.28	12.5	25	6.3	6.3	3.1	12.5	0.8	1.6	0.8	3.1	1.6	0.025	ND
P. mirabilis N-29/5	0.01	3.1	1.6	6.3	6.3	3.1	12.5	0.8	0.8	0.8	3.1	1.6	0.025	ND

Abbreviations: APC; ampicillin, CPC; carbenicillin, CET; cephalothin, CER; cephaloridine, CEZ; cefazolin, CEX; cephalexin, PIPC; piperacillin, APPC; apalcillin, CPZ; cefoperazone (T-1551), CFX; cefoxitin, CXM; cefuroxime, CTX; cefotaxime (HR 756), ND; Not determined.

Table 3. Relationship between cephalosporinase activity and levels of resistance to β-lactam antibiotics in cephalosporinase-producing bacteria.

Organisms	Cephalosporinase activity (units/mg dry weight of bacteria)	MIC (μg/ml)												
		Well-known β-lactams						New β-lactams						
		APC	CPC	CET	CER	CEZ	CEX	PIPC	APPC	CPZ	CFX	CXM	CTX	PS-5
E. coli 255	0.72	400	25	800	50	100	1,600	50	100	3.1	100	100	6.3	3.1
E. coli GN206	0.25	200	25	400	25	25	800	12.5	25	0.8	25	25	1.6	3.1
E. coli 255/L-7	0.003	3.1	3.1	1.6	3.1	1.6	6.3	1.6	3.1	≤0.2	3.1	3.1	0.025	3.1
C. freundii GN346	24.2	200	50	800	200	400	3,200	25	25	6.3	50	50	12.5	3.1
C. freundii GN346/16-10	4.0	100	12.5	800	100	200	1,600	25	25	6.3	25	12.5	6.3	1.6
C. freundii GN346/16	0.067	3.1	1.6	12.5	3.1	1.6	12.5	0.8	0.8	≤0.2	1.6	0.8	0.1	1.6
Ent. cloacae 363	24.9	400	100	>3,200	800	1,600	>3,200	12.5	25	6.3	800	200	25	3.1
Ent. cloacae 363/1-3	10.6	400	50	>3,200	400	800	3,200	6.3	25	6.3	800	200	12.5	3.1
Ent. cloacae 363/2	0.05	1.6	1.6	12.5	3.1	1.6	6.3	0.4	0.8	≤0.2	3.1	1.6	0.05	3.1
Ent. cloacae 363/1	<0.01	0.4	1.6	1.6	1.6	0.8	3.1	0.4	0.4	≤0.2	1.6	1.6	0.025	3.1
P. morganii 1510	2.68	400	6.3	1,600	400	200	800	50	200	25	12.5	50	6.3	6.3
P. morganii 1510/3	0.07	50	0.8	400	100	50	100	3.1	12.5	1.6	6.3	3.1	0.2	6.3
P. morganii 1510/9	0.006	1.6	0.8	12.5	6.3	6.3	12.5	≤0.2	0.8	0.4	3.1	0.8	0.025	3.1
P. vulgaris GN76/C-1	2.4	400	25	1,600	800	800	400	12.5	25	25	3.1	800	3.1	6.3
P. vulgaris GN76/C-1/1	1.8	200	12.5	800	800	400	100	1.6	6.3	12.5	3.1	400	0.2	6.3
P. vulgaris GN76/C-1/3	0.03	25	3.1	200	200	50	12.5	1.6	6.3	6.3	3.1	50	0.2	6.3
P. vulgaris GN76/C-1/2	<0.01	1.6	0.4	3.1	3.1	3.1	6.3	≤0.2	0.4	0.4	3.1	1.6	0.025	3.1

tails on the properties of these β-lactamases will be reported subsequently.

Evaluation of β-Lactam Antibiotics by a Determined Set of Strains

Six well-known β-lactam antibiotics and seven new ones were tested for their stability against the four kinds of penicillinases by using a set of strains; the results are shown in Table 2.

Ampicillin and carbenicillin were significantly reduced in their antibacterial activity to *E. coli*, *K. pneumoniae* and *P. mirabilis* by penicillinases. Two anti-pseudomonal penicillins, apalcillin[16] and piperacillin[17], were less sensitive but they were still not effective against the high producers of penicillinase. The activity of cephalosporins and a thienamycin-type β-lactam, PS-5[18], was hardly affective by penicillinases. However, it should be noted that a very large amount of penicillinase in the bacterial cells resulted in a moderate degree of bacterial resistance to some cephalosporins.

The activity of thirteen antibiotics against the five groups of cephalosporinase-producers are shown in Table 3. The usual four cephalosporins, *i.e.*, cephalothin, cephaloridine, cefazolin and cephalexin showed no activity against cephalosporinase-producers. Although penicillins were very stable in their reaction to hydrolysis by cephalosporinases (Fig. 1), their antibacterial activity towards cephalosporinase-producers was reduced. A similar phenomenon was also observed in the case of cefoxitin, cefuroxime and cefotaxime. High levels of cephalosporinase in the bacterial cells resulted in the increase of MIC value of the three new β-lactam antibiotics, although these cephalosporins were known to be poor substrates for many kinds of cephalosporinases[19,20,21]. An exception was cefuroxime, which was effectively hydrolyzed by the cephalosporinase of *P. vulgaris* (our unpublished observation), and this characteristic was well expressed in its antibacterial activity against the *P. vulgaris* strains.

The most stable β-lactam antibiotic, judged from the tests with our set of strains, was PS-5.

Antibacterial Activity of Twelve β-Lactam Antibiotics against the Bacteria Producing both Penicillinase and Cephalosporinase

The groups of strains mentioned above produce only one kind of β-lactamase. However, it is quite common for clinical isolates in Gram-negative bacteria to produce two kinds of β-lactamases[12], *i.e.*, a species-specific β-lactamase and a penicillinase mediated by R plasmid. In order to evaluate the antibacterial activity of β-lactam antibiotics to these types of resistant bacteria, we prepared the cephalosporinase-producing bacteria harboring R plasmid RGN823. These strains produced both the species-specific cephalosporinase and the type I penicillinase. Their susceptibility to twelve β-lactam antibiotics are presented in Table 4. The MICs of cephalosporins were not modified by the additive production of the penicillinase, but the MIC of penicillins were markedly reduced.

Table 4. Antibacterial activity of twelve β-lactam antibiotics against the bacterial strains producing both cephalosporinase and penicillinase.

β-Lactams	MIC (μg/ml)		
	E. coli 255 RGN823	*C. freundii* GN346 RGN823	*P. morganii* 1510 RGN823
APC	>3,200	>3,200	1,600
CPC	>3,200	1,600	>3,200
CET	800	1,600	1,600
CER	100	200	400
CEZ	100	800	200
CEX	1,600	3,200	800
PIPC	1,600	400	100
APPC	800	400	400
CPZ	25	25	25
CFX	100	100	6.3
CXM	100	50	50
CTX	6.3	6.3	6.3

Discussion

β-Lactamase-stability of the β-lactam antibiotic is often measured by the relative hydrolytic rate of the β-lactam bond by the enzyme *in vitro*. This assay is usually performed at saturation concentration of the substrate by using a soluble extracellular enzyme. However, the characteristics of a β-lactam antibiotic assumed from these *in vitro* conditions are not always a reflection of its antibacterial activity on β-lactamase-producing bacteria.

This phenomenon is thought to be attributed to the affinity of a β-lactamase to a substrate as in the case of type I penicillinase to cephaloridine. The phenomenon may be also attributed to difference observed in the conditions between *in vitro* and the bacterial cell. When the bacterial cells were exposed to physiological concentrations of the antibiotic, the achieved concentration of the drug in the periplasm, a space around the targets of the antibiotic, is significantly lower than that for the β-lactamase assay *in vitro*[22]. On the other hand, the β-lactamase concentration in the periplasm was of the order of 10^2 to 10^3 μM in the high resistant strains, calculated on the basis of the periplasmic volume assayed by STOCK *et al.*[23]. These enzyme concentrations are higher than 10^5 times that of the *in vitro* β-lactamase assay.

The method described in this paper offers more practical information about the stability of a given β-lactam antibiotic to nine kinds of β-lactamases, representing the principal ones produced by Gram-negative bacteria.

References

1) SAWAI, T.; K. TAKAHASHI, S. YAMAGISHI & S. MITSUHASHI: Variant of penicillinase mediated by an R factor in *Escherichia coli*. J. Bacteriol. 104: 620~629, 1960
2) MATTHEW, M. & R. W. HEDGES: Analytical isoelectric focusing of R factor-determined β-lactamases: Correlation with plasmid compatibility. J. Bacteriol. 125: 713~718, 1976
3) YAMAGISHI, S.; K. O'HARA, T. SAWAI & S. MITSUHASHI: The purification and properties of penicillin β-lactamases mediated by transmissible R factors in *Escherichia coli*. J. Biochem. 66: 11~20, 1969
4) YAMAGISHI, S.; T. YAMAMOTO & T. SAWAI: Anion effect on oxacillin-hydrolyzing penicillinase of an R plasmid. *In* S. MITSUHASHI, L. ROSIVAL & V. KRČMÉRY (*ed*), Plasmid, Medical and Theoretical Aspects. p. 331~338, Avicenum, Czechoslovak Medical Press, Prague, 1977
5) PERRET, C. J.: Iodometric assay of penicillinase. Nature (London) 174: 1012~1013, 1954
6) SAWAI, T.; I. TAKAHASHI & S. YAMAGISHI: Iodometric assay method for beta-lactamase with various beta-lactam antibiotics as substrates. Antimicr. Agents Chemoth. 13: 910~913, 1978
7) SAWAI, T.; S. YAMAGISHI & S. MITSUHASHI: Penicillinases of *Klebsiella pneumoniae* and their phylogenetic relationship to penicillinases mediated by R factors. J. Bacteriol. 115: 1045~1054, 1973
8) FUJII-KURIYAMA, Y.; M. YAMAMOTO & S. SUGAWARA: Purification and properties of beta-lactamase from *Proteus morganii*. J. Bacteriol. 131: 726~734, 1977
9) EGAWA, R.; T. SAWAI & S. MITSUHASHI: Drug resistance of enteric bacteria. XII. Unique substrate specificity of penicillinase produced by R factor. Japan. J. Microbiol. 11: 173~178, 1967
10) 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. Japan. J. Microbiol. 12: 423~434, 1968
11) MATSUMOTO, H.; T. SAWAI, T. TAZAKI, S. YAMAGISHI & S. MITSUHASHI: Characterization of the chromosomally mediated penicillinase in *Klebsiella pneumoniae*. Japan. J. Microbiol. 16: 169~176, 1972
12) SAWAI, T.; I. ANDO-KURODA, T. MURATA & S. YAMAGISHI: Characterization of cephalosporin resistance in clinical isolates of *Escherichia coli*. J. Pharm. Dyn. 3: 364~366, 1980
13) TAKAHASHI, I.; T. SAWAI, T. ANDO & S. YAMAGISHI: Cefoxitin resistance by a chromosomal cephalosporinase in *Escherichia coli*. J. Antibiotics 33: 1037~1042, 1980
14) SAWAI, T.; S. NAKAJIMA, T. MOROHOSHI & S. YAMAGISHI: Thermolabile repression of cephalosporinase synthesis in *Citrobacter freundii*. Microbiol. Immunol. 21: 631~638, 1977
15) LINDSTRÖM, E. B.; H. G. BOMAN & B. B. STEELE: Resistance of *Escherichia coli* to penicillins. VI. Purification and characterization of the chromosomally mediated penicillinase present in *amp* A-containing strains. J. Bacteriol. 101: 218~231, 1970
16) NOGUCHI, H.; Y. EDA, H. TOBIKI, T. NAKAGOME & T. KOMATSU: PC-904, a novel broad-spectrum semisyn-

thetic penicillin with marked antipseudomonal activity: Microbiological evaluation. Antimicr. Agents Chemoth. 9: 262~273, 1976

17) UEO, K.; Y. FUKUOKA, T. HAYASHI, T. YASUDA, H. TAKI, T. TAI, Y. WATANABE, I. SAIKAWA & S. MITSUHASHI: *In vitro* and *in vivo* antibacterial activity of T-1220, a new semisynthetic penicillin. Antimicr. Agents Chemoth. 12: 455~460, 1977
18) OKAMURA, K.; S. HIROTA, Y. OKUMURA, Y. FUKAGAWA, Y. SHIMAUCHI, K. KOUNO & T. ISHIKURA: PS-5, a new β-lactam antibiotic from *Streptomyces*. J. Antibiotics 31: 480~482, 1978
19) ONISHI, H. R.; D. R. DAOUST, S. B. ZIMMERMAN, D. HENDLIN & E. O. STAPLEY: Cefoxitin, a semisynthetic cephamycin antibiotic: resistant to beta-lactamase inactivation. Antimicr. Agents Chemoth. 5: 38~43, 1974
20) O'CALLAGHAN, C. H.; R. B. SYKES, D. M. RYAN, R. D. FOORD & P. W. MUGGLETON: Cefuroxime—A new cephalosporin antibiotic. J. Antibiotics 29: 29~37, 1976
21) FU, K. P. & H. C. NEU: Beta-lactamase stability of HR 756, a novel cephalosporin, compared to that of cefuroxime and cefoxitin. Antimicr. Agents Chemoth. 14: 322~326, 1978
22) SAWAI, T.; K. MATSUBA, A. TAMURA & S. YAMAGISHI: The bacterial outermembrane permeability of β-lactam antibiotics. J. Antibiotics 32: 59~65, 1979
23) STOCK, J. B.; B. RAUCH & S. ROSEMAN: Periplasmic space in *Salmonella typhimurium* and *Escherichia coli*. J. Biol. Chem. 252: 7850~7861, 1977