Effects of Tazobactam on the Frequency of the Emergence of Resistant Strains from *Enterobacter cloacae*, *Citrobacter freundii*, and *Proteus vulgaris* (β-Lactamase Derepressed Mutants)

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When *Enterobacter cloacae*, *Citrobacter freundii*, and *Proteus vulgaris* were treated with piperacillin (PIPC) in combination with tazobactam (TAZ), the *in vitro* frequency of emergence of resistant strains (β-lactamase producing mutants) was lower than with PIPC or ceftazidime (CAZ) treated bacteria. In a mouse intraperitoneal infection model caused by *E. cloacae*, β-lactamase derepressed mutants were detected following therapy with PIPC or CAZ, although no derepressed mutants were detected after treatment with PIPC in combination with TAZ. This suppression of the selection of derepressed mutants, which produce large amounts of β-lactamases, by the combination of TAZ and PIPC suggests that the combination delays the increase of resistant mutants compared with PIPC alone.

1) Hydrolysis of drugs by β-lactamase produced by the bacteria, 2) changes in the affinity to penicillin-binding proteins, and 3) reduction in the permeability of the bacterial outer membrane to the drugs are considered to be primary mechanisms of resistance of bacteria to β-lactams. Among them, hydrolysis of antibiotics by β-lactamase is considered to be the main cause of resistance to β-lactams, because β-lactamase has been frequently detected from many clinical isolates[1].

β-Lactamases are classified into penicillinases (PCases), cephalosporinases (CEPases), and oxyimino-cephalosporinases (CXases) according to their substrate profile[2]. Most PCases are encoded on transposons and plasmids, primarily inhibit penicillins, which are their substrates, and produce resistance to ampicillin and piperacillin (PIPC). CEPases and CXases are encoded on the chromosome, and are major causes of resistance of Gram-negative rods to cephalosporins, which are their substrates[3]. As countermeasures against resistance to β-lactams, antibiotics which are stable to β-lactamases have been developed, as well as combination of β-lactams with β-lactamase inhibitors to increase their antibacterial activity. Recently, however, bacterial strains resistant even to β-lactams with increased stability against β-lactamases, including third generation cephalosporins, have been reported[4]. The resistance of such bacteria to these β-lactams is considered to be caused partly by an increase of CEPase activity as a result of the change in the manner of CEPase production from inducible to constitutive[5,6]. Such resistant bacteria, which are called derepressed mutants (drd mutants), are easily selected *in vitro* by treatment with β-lactams. Therefore, there exists the possibility to increase the emergence of such resistant bacteria to β-lactam antibiotics in clinical practice[6].

In this study, we examined the frequency of the emergence of resistant mutants after treating bacteria with PIPC alone and in combination with TAZ, a β-lactamase inhibitor, to evaluate the effects of TAZ on the selection of β-lactamase derepressed mutants by the β-lactam antibiotics.

Materials and Methods

Organisms

Clinical isolates maintained in our laboratory were used.

Drugs

PIPC, TAZ, clavulanate (CVA), and sulbactam (SBT) synthesized at our laboratory were used. Cephalotin (CET, Shionogi & Co., Ltd.), ceftazidime (CAZ, Tanabe Seiyaku), and cefoxitin (CFX, Banyu Pharmaceutical Co., Ltd.) were commercially obtained.

Evaluation of Antibiotic Susceptibility

Minimum inhibitory concentrations (MICs) of the test drugs were determined by the agar dilution method[7] with serial dilutions of antibiotics. After incubation in sensitivity test broth (STB, Nissui) at 37°C for 18 hours, the bacterial suspension was diluted with saline and about 10^5 CFU/spot was applied to the drug-containing Sensitivity Disk Agar-N (SDA, Nissui) plates with a multipoint inoculator (Sakuma Seisakusho Co., Ltd.). The MICs were recorded after 18 hours of incubation at 37°C as the lowest concentrations of drug that inhibited visible growth of bacteria.

In Vitro Evaluation of the Frequency of the Emergence of Resistant Mutants

After the bacteria were incubated in STB at 37°C overnight, a 5- to 10-fold dilution of the overnight culture was spread on SDA containing 4 to 32 times the MIC of drugs, and colonies were counted after 48-hour incubation at 37°C. Then, colonies were selected randomly and subcultured in drug free-SDA at 37°C, and the antibiotic susceptibility was evaluated.

Measurement of the β-Lactamase Activity

Bacteria were incubated overnight in STB, the culture was diluted 20-fold with fresh STB, and incubated with shaking at 37°C for about 4 hours. Some bacterial strains were incubated with shaking at 37°C for about 2 hours and then CFX was added and incubated for 2 hours to induce β-lactamase. Bacteria were harvested by centrifugation at 1,500 × *g* for 10 minutes, washed twice with 0.05 M phosphate buffer (pH 7), and suspended with the same buffer. This bacterial suspension was sonicated in ice and then centrifuged at 15,000 × *g* for 10 minutes with cooling, and the β-lactamase activity in the supernatant was measured by UV method[8]. CET (100 μM) was used as the substrate.

Evaluation of the Frequency of the Emergence of β-Lactamase Derepressed Mutants in Intraperitoneally Infected Mice

Male ddY mice aged 4 weeks were used in groups of 3. Suspensions of the test bacteria were inoculated intraperitoneally at about 10^8 CFU/mouse, and the drugs were administered subcutaneously at 10 mg/mouse 5 times at intervals of 1 hour. Seven hours after inoculation, the bacteria in the peritoneal cavity were recovered by lavage with saline, and then diluted with saline, spread on nutrient agar (NA, Difco) for a target number of 100～200 colonies, and incubated overnight at 37°C. Iodine-starch agar (3.2 mM I_2, 24 mM KI, 0.4% starch, 0.8% agar; 10 ml) containing 2 mg/ml CET was layered over the agar plates on which the colonies had grown, and colonies that showed decoloration after 30～120 minutes were regarded as those of β-lactamase derepressed mutants. Next, drug sensitivity of these strains was examined to evaluate their resistance.

Results

Effects of β-Lactamase Inhibitors on the Emergence of Resistant Mutants

To study the effects of β-lactamase inhibitors on the emergence of resistant mutants, PIPC-sensitive *E. cloacae* strain 40001 was treated with PIPC alone or in combination with 10 μg/ml of a β-lactamase inhibitor, namely TAZ, CVA, or SBT, and the frequency of the emergence of resistant colonies was compared.

As shown in Fig. 1, the frequency of the emergence of resistant colonies decreased with the increase in the concentration of PIPC, but resistant colonies emerged with a high selection frequency of almost 10^{-6} at 4 times the MIC and were observed even at 16 times the MIC. When TAZ was added, however, no resistant colonies were observed at all PIPC concentrations examined. On the other hand, when PIPC was added with CVA or SBT, the selection frequency of resistant colonies was 10^{-6}～10^{-7} at 4 times the MIC and 10^{-7}～10^{-8} at 8 times the MIC. When CVA was added, resistant colonies were observed even at a PIPC concentration of 16 times the MIC. Resistant colonies thus selected were subcultured in drug free-SDA, and their drug sensitivity was evaluated. MIC of PIPC was over 12.5 μg/ml regardless of the drugs used for their selection, indicating resistance to PIPC; bacteria selected with 16 times the MIC of PIPC included those for which the MIC of PIPC was over 100 μg/ml indicating higher resistance to PIPC.

In Vitro Evaluation of the Frequency of the Emergence of Resistant Bacteria

Tables 1～3 show the frequency of the emergence of resistant colonies of CEPase-producing *E. cloacae*, *Citrobacter freundii*, and CXase-producing *Proteus vulgaris* after incubation with 4 times to 32 times the MIC of TAZ/PIPC (1:4 combination drug), PIPC+TAZ (10 μg/ml), PIPC, and CAZ. In *E. cloacae* 40001 and 40029, resistant colonies emerged at a frequency of about 10^{-8} with TAZ/PIPC and PIPC+TAZ at 4 times the MIC but no resistant colonies emerged at concentrations of more than 8 times the MIC. With PIPC alone, resistant colonies emerged at a frequency of 10^{-8}～10^{-9} at 8 times the MIC or over, and with CAZ, resistant colonies were observed at a frequency of 10^{-7} even at 32 times the MIC in 40001 strain. In *E. cloacae* 40012, few resistant colonies were noted after treatment with TAZ/PIPC, PIPC+TAZ, or PIPC alone, but resistant colonies were observed after treatment with CAZ even at 32 times the MIC as with strain 40001. Many resistant

Fig. 1. Selection frequencies of β-lactamase derepressed colonies from *Enterobacter cloacae* 40001 in the presence of times the MIC of PIPC alone and added with TAZ, CVA and SBT.

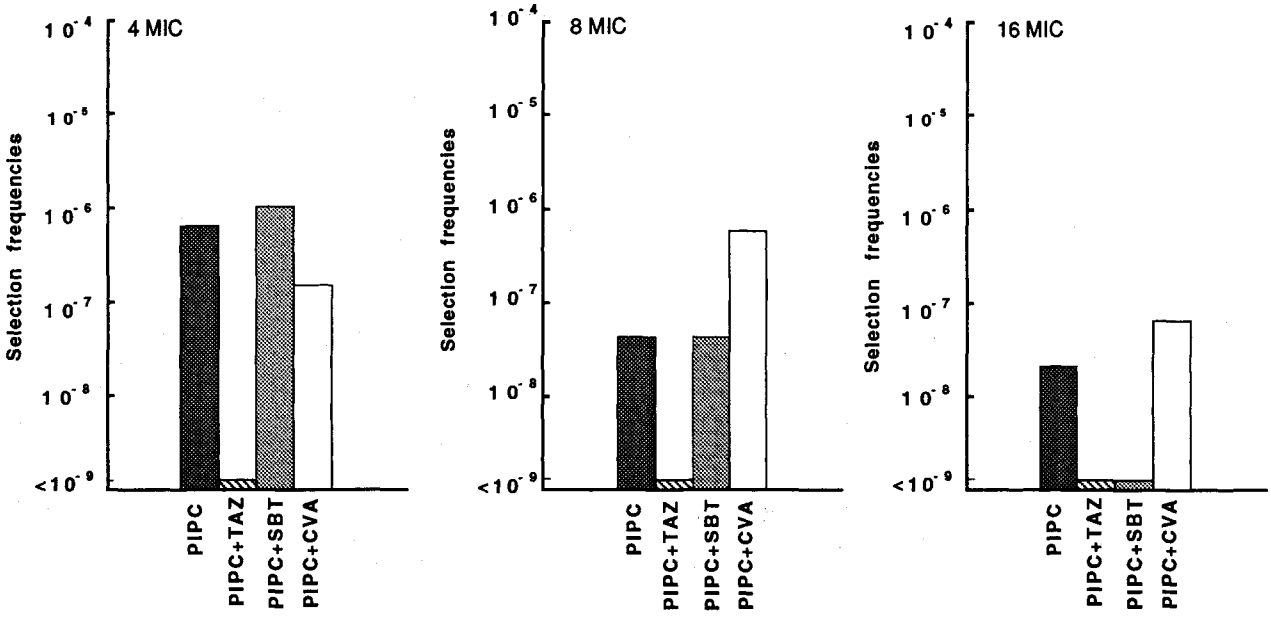

Table 1. Selection frequencies of β-lactamase derepressed mutants from *Enterobacter cloacae*.

Species and strain No.	Drug	MIC* (μg/ml)	Selection frequencies of derepressed mutants: Time the MIC of drug			
			4MIC	8MIC	16MIC	32MIC
Enterobacter cloacae 40001	TAZ/PIPC	3.13	7.1×10^{-9}	N.D.	N.D.	N.D.
	PIPC+TAZ(10)	3.13	1.4×10^{-8}	N.D.	N.D.	N.D.
	PIPC	3.13	9.1×10^{-7}	1.4×10^{-8}	7.1×10^{-9}	N.D.
	CAZ	0.39	2.8×10^{-6}	1.8×10^{-6}	1.2×10^{-6}	2.3×10^{-7}
40008	TAZ/PIPC	3.13	N.D.	N.D.	N.D.	N.D.
	PIPC+TAZ(10)	3.13	N.D.	N.D.	N.D.	N.D.
	PIPC	3.13	1.8×10^{-8}	1.8×10^{-8}	N.D.	N.D.
	CAZ	3.13	1.8×10^{-8}	N.D.	N.D.	N.D.
40012	TAZ/PIPC	3.13	1.8×10^{-7}	N.D.	N.D.	N.D.
	PIPC+TAZ(10)	3.13	2.7×10^{-8}	N.D.	N.D.	N.D.
	PIPC	3.13	1.7×10^{-7}	N.D.	N.D.	N.D.
	CAZ	0.39	4.4×10^{-6}	1.3×10^{-6}	3.7×10^{-7}	1.3×10^{-7}
40029	TAZ/PIPC	1.56	4.2×10^{-6}	5.0×10^{-7}	1.3×10^{-8}	N.D.
	PIPC+TAZ(10)	1.56	2.6×10^{-6}	6.8×10^{-8}	N.D.	N.D.
	PIPC	1.56	2.7×10^{-6}	2.5×10^{-6}	2.1×10^{-6}	2.0×10^{-7}
	CAZ	0.2	4.2×10^{-6}	3.6×10^{-8}	3.6×10^{-6}	3.8×10^{-6}

* Inoculum size ; 10^5 CFU/spot.
N. D. = Not detectable with the method employed.

colonies were observed in *E. cloacae* 40029 with all drugs, but the frequency of their emergence was lower after treatment with PIPC+TAZ and TAZ/PIPC than after treatment with PIPC alone. CAZ treatment resulted in the greatest number of resistant colonies.

In *C. freundii*, fewer resistant colonies were observed than in *E. cloacae* with all drugs, and few resistant colonies were observed in most strains after treatment

Table 2. Selection frequencies of β-lactamase derepressed mutants from *Citrobacter freundii*.

Species and strain No.	Drug	MIC* (μg/ml)	Selection frequencies of derepressed mutants			
			Time the MIC of drug			
			4MIC	8MIC	16MIC	32MIC
Citrobacter freundii No13	TAZ/PIPC	3.13	N.D.	N.D.	N.D.	N.D.
	PIPC+TAZ(10)	3.13	N.D.	N.D.	N.D.	N.D.
	PIPC	3.13	N.D.	N.D.	N.D.	N.D.
	CAZ	0.39	1.1×10^{-7}	N.D.	N.D.	N.D.
No14	TAZ/PIPC	3.13	N.D.	N.D.	N.D.	N.D.
	PIPC+TAZ(10)	3.13	N.D.	N.D.	N.D.	N.D.
	PIPC	3.13	1.7×10^{-7}	N.D.	N.D.	N.D.
	CAZ	0.2	4.0×10^{-7}	2.3×10^{-8}	N.D.	N.D.
No15	TAZ/PIPC	0.78	5.1×10^{-8}	N.D.	N.D.	N.D.
	PIPC+TAZ(10)	0.78	N.D.	N.D.	N.D.	N.D.
	PIPC	1.56	7.7×10^{-8}	N.D.	N.D.	N.D.
	CAZ	0.2	2.6×10^{-7}	1.3×10^{-7}	7.7×10^{-8}	1.0×10^{-7}
No26	TAZ/PIPC	3.13	N.D.	N.D.	N.D.	N.D.
	PIPC+TAZ(10)	3.13	N.D.	N.D.	N.D.	N.D.
	PIPC	3.13	1.6×10^{-8}	N.D.	N.D.	N.D.
	CAZ	0.39	1.1×10^{-7}	4.9×10^{-8}	4.9×10^{-8}	N.D.
No36	TAZ/PIPC	3.13	N.D.	N.D.	N.D.	N.D.
	PIPC+TAZ(10)	3.13	N.D.	N.D.	N.D.	N.D.
	PIPC	3.13	N.D.	N.D.	N.D.	N.D.
	CAZ	0.39	3.4×10^{-8}	N.D.	N.D.	N.D.

* Inoculum size ; 10^5 CFU/spot.
N. D. = Not detectable with the method employed.

Table 3. Selection frequencies of β-lactamase derepressed mutants from *Proteus vulgaris*.

Species and strain No.	Drug	MIC* (μg/ml)	Selection frequencies of derepressed mutants			
			Time the MIC of drug			
			4MIC	8MIC	16MIC	32MIC
Proteus vulgaris 35004	TAZ/PIPC	1.56	N.D.	N.D.	N.D.	N.D.
	PIPC+TAZ(10)	1.56	N.D.	N.D.	N.D.	N.D.
	PIPC	1.56	6.1×10^{-8}	2.3×10^{-8}	N.D.	N.D.
	CAZ	0.1	N.D.	N.D.	N.D.	N.D.
35005	TAZ/PIPC	1.56	N.D.	N.D.	N.D.	N.D.
	PIPC+TAZ(10)	1.56	N.D.	N.D.	N.D.	N.D.
	PIPC	1.56	6.8×10^{-8}	3.0×10^{-8}	N.D.	N.D.
	CAZ	0.1	N.D.	N.D.	N.D.	N.D.
35016	TAZ/PIPC	1.56	N.D.	N.D.	N.D.	N.D.
	PIPC+TAZ(10)	1.56	N.D.	N.D.	N.D.	N.D.
	PIPC	1.56	9.2×10^{-8}	3.8×10^{-8}	N.D.	N.D.
	CAZ	0.1	2.3×10^{-8}	N.D.	N.D.	N.D.

* Inoculum size ; 10^5 CFU/spot.
N. D. = Not detectable with the method employed.

Table 4. Characterization of parent strain and derepressed-resistant mutant.

Strain	Selection Drug	MIC (μ g/ml)				β-lactamase activity[a]	
		TAZ/PIPC	PIPC +TAZ*	PIPC	CAZ	control	induced[b]
Enterobacter cloacae 40001	none	3.13	3.13	3.13	0.39	0.0085	0.054
	TAZ/PIPC	50	12.5	50	100	21.56	22.75
	PIPC+TAZ	50	12.5	100	50	24.01	26.20
	PIPC	50	25	100	100	22.97	29.01
	CAZ	25	12.5	100	50	25.31	23.07
E. cloacae 40029	none	3.13	3.13	3.13	0.39	0.012	0.151
	TAZ/PIPC	50	12.5	200	100	7.624	9.493
	PIPC+TAZ	50	12.5	200	100	16.55	22.63
	PIPC	50	25	200	100	21.60	21.25
	CAZ	25	12.5	100	100	20.06	23.41
Proteus vulgaris 35016	none	0.78	0.78	1.56	0.05	0.016	0.508
	PIPC	3.13	1.56	25	0.39	3.22	7.67
	CAZ	1.56	0.78	25	0.39	2.48	1.24

[a] Units/mg protein (substrate; 100 μM cephalotin).
[b] Cefoxitin concentration (10 μg/ml for, *E. cloacae* 1 μg/ml for *C. freundii*).
* Tazobactam was used at fixed concentration of 10 μg/ml.

with TAZ/PIPC or PIPC+TAZ. However, resistant colonies appeared at $10^{-7} \sim 10^{-8}$ after the treatment with CAZ.

With *P. vulgaris*, no resistant colonies emerged after treatment with TAZ/PIPC or PIPC+TAZ at all concentrations, but resistant colonies were noted in some strains at a frequency of 10^{-8} after treatment with PIPC or CAZ at 4 or 8 times the MIC.

In Vitro Evaluation of Antibiotic Susceptibility and β-Lactamase Production of Resistant Mutants

Table 4 shows the results of measurement of antibiotic susceptibility and β-lactamase activity of resistant colonies of *E. cloacae* 40001, 40029, and *P. vulgaris* 35016 after subculturing in a drug free medium.

In *E. cloacae*, antibiotic susceptibility was reduced in strains which emerged after drug treatment regardless of the antibiotic used for their selection. MICs of TAZ/PIPC and PIPC increased 8~64 times, and that of CAZ increased 128~256 times, compared with the parent strains. The β-lactamase activity of some of these strains reached 20 units/mg protein at non-induced conditions, increasing more than a thousand times compared with about 0.01 units/mg protein of the parent strain. Also, while β-lactamase production was induced by the addition of CFX in parent strains, little induction was observed in the resistant strains. In *P. vulgaris* 35016, the antibiotic susceptibility of resistant strains selected with PIPC and CAZ to TAZ/PIPC, PIPC, and CAZ was reduced 2~4, 16, and 8 times, respectively, compared with that of parent strains. The β-lactamase activity of resistant strains also increased compared with the parent strains, though less remarkably than in *E. cloacae*, and there was only slight induction of enzyme production by the addition of CFX. From these findings, we conclude that drd mutants were selected in the above process.

Table 5. Selection of *in vivo* derepressed mutants from *Enterobacter cloacae* 40001.

Drug	No. of tested colonies	No. of derepressed mutants
TAZ/PIPC	18580	0
PIPC	3209	10
CAZ	11746	4

Mice; ddY-strain, 4 weeks old, 3/group. Infection; intraperitoneal challenge. Therapy; subcutaneous injection, 10 mg/mouse × 5.

In Vivo Evaluation of the Emergence of Resistant Mutants

Table 5 shows resistant bacteria observed in a mouse intraperitoneal infection model with *E. cloacae* 40001 after treatment with TAZ/PIPC, PIPC, and CAZ.

No bacterial strains that produced high levels of β-lactamase were observed in the 18,580 colonies recovered from the TAZ/PIPC group. Ten colonies of β-lactamase derepressed strains were detected in the 3,209 colonies recovered (selection frequency was about 10^{-3})

Table 6. Susceptibilities and β-lactamase activities of *in vivo* derepressed mutants from *Enterobacter cloacae* 40001.

Strains		MIC (μg/ml)*			β-lactamase activity** (units /mg protein)
		TAZ/PIPC	PIPC	CAZ	
parent strain		3.13	3.13	0.39	0.0085
PIPC treaed mutants	P1	50	100	100	8.56
	P2	50	100	100	8.77
	P3	50	100	100	7.55
	P4	50	100	100	4.93
	P5	100	100	100	7.06
	P6	50	100	100	7.23
	P7	50	100	100	7.43
	P8	50	>100	100	7.03
	P9	50	100	100	6.94
	P10	100	100	>100	7.05
CAZ treated mutants	C1	50	>100	>100	2.09
	C2	100	>100	>100	2.63
	C3	50	>100	>100	2.24
	C4	50	100	100	6.09

* Inoculum size 10^5 CFU/spot.
** No induction.

in the PIPC group, and 4 of the 11,746 colonies (about 10^{-4}) recovered in the CAZ group were β-lactamase derepressed mutants. Susceptibility and β-lactamase activity were measured in bacterial strains selected in the PIPC and CAZ groups. MIC of TAZ/PIPC was 50～100 μg/ml, and those of PIPC and CAZ were 100 μg/ml or over, indicating that these strains were more resistant than their parent strains. β-Lactamase production was also increased (Table 6).

Discussion

Many antibiotics have been developed and used clinically for the treatment of infections. Among them, β-lactams are used most frequently. Especially, a number of β-lactams effective against *E. cloacae*, *C. freundii*, *Proteus* spp. and *Pseudomonas* spp., which are known as third generation cephalosporins, have been developed since about 15 years ago. During this period, these third generation cephalosporins showed excellent effects on Gram-negative bacteria and have achieved remarkable results in the treatment of infections. However, there appeared a few novel problems. The first is the unusual increase in the incidence of methicillin-resistant *Staphylococcus aureus* (MRSA) and the increase in the frequency of isolation of streptococci[9]. These problems are considered to be results of bacterial selection through the increased use of third generation cephalosporins, which have no great effects on Gram-positive bacteria. The second is the appearance of Gram-negative bacteria which are resistant to third generation cephalosporins[4]. The cause of this resistance is frequently β-lactamase production. Changes in the substrate specificity due to point mutation of TEM type and SHV type PCase genes and widening of the substrate profile (extended-spectrum β-lactamase) have been reported[10]. On the other hand, resistance to β-lactams due to changes in the manner of enzyme production has been reported in *E. cloacae*, *C. freundii*, and *Pseudomonas aeruginosa*, which produce chromosomally mediated β-lactamase[5,6]. The original inducible β-lactamase production of these bacteria is changed to constitutive production, and results in a large amount of β-lactamase being produced, with resistance to a wide spectrum of antibiotics including third generation cephalosporins, which used to be considered stable. These bacteria are so-called derepressed mutants. This resistance often results from overproduction of ampC β-lactamase, which can be caused by mutations in the ampD gene[11]. Such mutants can be easily isolated at a high frequency of about 10^{-6}～10^{-8} by treating bacteria with drugs *in vitro*[6]. This high mutation rate from bacteria that produce CEPase inducibly to those that produce it constitutively is considered to be a cause of the increase in resistance to third generation cephalosporins. Therefore, the risk of selection of bacteria highly resistant to cephalosporins is suggested to be greater in such drugs that have low CEPase inducibility and are stable against CEPase as are third generation cephalosporins[12,13]. On the basis of these observations, PIPC, which does not easily induce CEPase and is relatively stable against CEPase as has been reported[14,15], is considered to resemble third generation cephalosporins with regard to antibacterial activity.

We, therefore, compared the frequency of the emergence of resistant bacteria after treatment with PIPC or CAZ alone and PIPC in combination with TAZ to

evaluate whether the addition of the β-lactamase inhibitor affects the selection of resistant mutants. Resistant colonies selected *in vitro* by the treatment of PIPC in combination with TAZ were fewer than those selected by the treatment of PIPC or CAZ alone. In an *in vivo* experiment using a mouse intraperitoneal infection model by *E. cloacae*, resistant mutants were detected in the PIPC group and the CAZ group but not in the TAZ/PIPC group similar to the *in vitro* results. Thus, the use of TAZ and PIPC in combination may prevent the selection of resistant bacteria called derepressed mutants and delay the increase in such resistant mutants.

The basis for this emerging resistance seems to be that β-lactamase producing populations always contain a few derepressed mutants. Usually these mutants occur at a frequency of $10^{-6} \sim 10^{-7}$, but they may be more frequent in some Gram-negative bacterial strains. When these heterogenous populations are challenged with TAZ/PIPC, even the mutant cell fails to manufacture β-lactamase with TAZ and are killed by the PIPC. The derepressed variants, however, produced the high amount of enzyme, and so can protect themself from PIPC.

As observed above, resistance to β-lactams is closely related to β-lactamase, but its cause is being diversified as exemplified by the advent of extended-spectrum β-lactamase and derepressed mutants. β-Lactamase inhibitors are considered to be useful, because they inhibit extended-spectrum β-lactamases and, as suggested by this study, may delay the increase in resistant strains called derepressed mutants. Moreover, next generation cephalosporins that are effective against a wider spectrum of bacteria than third generation cephalosporins and are stable against β-lactamase have been developed and recently put into clinical use[16,17]. However, as experienced with third generation cephalosporins, the use of these drugs is expected to cause further diversification of β-lactam resistance and the development of new resistant bacteria in increased numbers. Therefore, from the laboratory viewpoint, greater attention to the appropriate use of antibacterial agents is considered to be all the more important.

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