INDUCTION OF CEPHALOSPORINASE PRODUCTION BY VARIOUS PENICILLINS IN ENTEROBACTERIACEAE

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The inducer activity of seven penicillins for cephalosporinase (CSase) production and their antibacterial activity against CSase-producing strains were studied using clinical isolates of *Proteus morganii*, *P. rettgeri*, *P. vulgaris*, *Enterobacter cloacae*, and *Serratia marcescens*. Piperacillin, apalcillin, and methicillin showed rather low inducer activity for CSase production in all strains tested. On the other hand, ampicillin, carbenicillin, and sulbenicillin showed high inducer activity for CSase production. Piperacillin, apalcillin, and ampicillin were less stable to CSases than were carbenicillin, sulbenicillin, and methicillin, but much more stable than benzylpenicillin. In the growing culture of CSase-producing strains, piperacillin and apalcillin were rather stable. Against CSase-producing strains, piperacillin and apalcillin were more active than other penicillins tested.

Gram-negative bacteria are generally more resistant to penicillins than are their Gram-positive counterparts. It has been suggested that the production of β-lactamase is responsible for the penicillin resistance of Gram-negative bacteria, particularly, of *Enterobacter cloacae*, indole-positive *Proteus* species, and *Serratia marcescens*[1]. β-Lactamases from these species have been extensively studied by many investigators[2~7]. These β-lactamases are species-specific, chromosomally mediated, and inducible enzymes[1]. Penicillins are generally more stable against these enzymes, cephalosporinases (CSases), than are the cephalosporins. It has been widely reported that some penicillins induce CSase production[8~11] while others fail to do so *e.g.* in *E. cloacae*[8,9]. Piperacillin[12] and apalcillin[13], which have antipseudomonal activity and a broad antibacterial spectrum against Gram-negative bacteria, showed low inducer activity for CSase production in *E. cloacae*[8], *Proteus vulgaris*[9] and *P. rettgeri*[9]. From the results of our previous studies concerning the inducer activity of β-lactam antibiotics for CSase production[8,9], it was speculated that the potent antibacterial activity of piperacillin and apalcillin against CSase-producing Gram-negative bacteria is based on their low inducer activity for CSase production and stability to CSases.

In the present study, we have investigated the correlation between inducer activity for CSase production and the antibacterial activity of seven penicillins, using clinical isolates of *P. morganii*, *P. rettgeri*, *P. vulgaris*, *E. cloacae*, and *S. marcescens*.

Materials and Methods

Bacterial Strains

P. morganii T-211, *P. rettgeri* GN4430, *P. vulgaris* T-178, *E. cloacae* H-27, and *S. marcescens*

W-24 were clinical isolates and stocked in our laboratory. Inducible strains produced only one β-lactamase.

Antibiotics

Piperacillin[12], apalcillin[13,14], ampicillin, benzylpenicillin carbenicillin, sulbenicillin, methicillin, cephaloridine, cephalothin, and cefmetazole were used.

Determination of Minimal Inhibitory Concentration (MIC)

The MICs were determined by an agar dilution technique. An overnight culture of test strains in peptone broth was diluted to final concentrations of 10^6 and 10^8 cells/ml, and one loopful (about 0.005 ml) of each culture was inoculated on heart infusion agar (Eiken, Tokyo) plates using a Microplanter (Sakuma, Tokyo). The peptone broth consisted of 10 g of Polypeptone and 5 g of NaCl in 1,000 ml of distilled water. The MICs were determined after overnight incubation at 37°C.

Partial Purification of Cephalosporinase (CSase)

CSases from each organism used in this study were purified by means of a CM-Sephadex C-50 (Pharmacia, Sweden) column as described in previous papers[3~7]. As an inducer for CSase production, cefmetazole was used with a concentration of 10 μg/ml for *E. cloacae* H-27 and 2.5 μg/ml for other strains.

Induction of CSase

For the determination of the CSase induction by penicillins, an overnight culture of each strain was diluted 20-fold into fresh brain heart infusion broth (Difco, U.S.A.) and incubated with shaking at 37°C for 2 hours. The shaken cultures were divided into L-tubes (10 ml) containig various concentrations of penicillins and the incubation was continued for a further 2 hours before the cultures were harvested. Cells were washed once with 5 ml of 0.1 M phosphate buffer (pH 7.0) following which, the cells were resuspended in 10 ml of the same buffer and disrupted sonically for 1 minute at 4°C. The broken cells were centrifuged at $12,000 \times g$ for 30 minutes at 4°C, and the resulting supernatant fluid was used for the enzyme assay and protein determination. Protein determination was performed by the method of LOWRY[15].

β-Lactamase Assay

β-Lactamase activity was determined by either a spectrophotometric method[5,16] or a modified microiodometric method[17]. One unit of enzyme activity was defined as the amount of the enzyme that hydrolyzed one micromole of substrate in one minute at 30°C, in 0.05 M phosphate buffer (pH 7.0).

Measurement of Residual Amount of Penicillins in the Bacterial Cultures

In the experiments of CSase induction described above, one ml of each culture was taken at 2 hours after the addition of each penicillin (final 100 μg/ml) to the culture and diluted twofold with methanol for the purpose of stopping the enzymic reaction. The residual amount of each penicillin in the culture was determined by bioassay.

Antibiotic Assay

Concentrations of penicillins in the culture of CSase-producing strains were determined by the disk-plates diffusion method. For the measurement of piperacillin and apalcillin concentrations, *Micrococcus luteus* ATCC 9341 was used as the test organism, and a locally prepared agar (6 g of Polypeptone, 3 g of yeast extract, 1.5 g of beef extract, and 15 g of agar in 1,000 ml of distilled water) was used as the test medium. For other penicillins, *Bacillus subtilis* ATCC 6633 was used as the test organism and a locally prepared agar (5 g of Polypeptone, 3 g of beef extract, and 15 g of agar in 1,000 ml of distilled water) was used as the test medium. Standard solutions for the measurement of penicillin concentrations in the culture were prepared in brain heart infusion broth, and these were then diluted twofold with methanol. The disks were dried at 37°C for 30 minutes to eliminate methanol before the disks were placed on the agar plates inoculated with the test organism. Plates were incubated at 37°C for 18 hours, and inhibition zones were measured and compared with standards.

Results

Antibacterial Activity of Penicillins against CSase-producing Strains

The MICs of penicillins to CSase-producing strains were determined by an agar dilution technique with about 5×10^3 and 5×10^5 cfu (Table 1). Piperacillin and apalcillin were more active against most of strains tested, and piperacillin showed the most potent activity against *P. vulgaris* T-178, *E. cloacae* H-27, and *S. marcescens* W-24. Ampicillin and benzylpenicillin were inactive to the strains except for *P. rettgeri* GN4430, which was more susceptible to penicillins than other strains. Both carbenicillin and sulbenicillin were inactive to *P. vulgaris* T-178 and *E. cloacae* H-27, and sulbenicillin was also inactive to *S. marcescens* W-24. Methicillin was not active against all strains tested. At larger inoculum (5×10^5 cfu), penicillins always became more inactive to strains tested than at 5×10^3 cfu. But the order of activity of penicillins to CSase-producing strains was similar at both inocula.

Stability of Penicillins to CSases

The CSases were partialy purified from five CSase-producing strains by means of a CM-Sephadex column chromatography. The enzyme production was induced by cefmetazole. The relative rates of hydrolysis of penicillins by five CSases are shown in Table 2. The identity of enzymes which were induced by different inducers was not sufficiently confirmed, but at least substrate profiles of enzymes from each organism which were induced by ampicillin and cefmetazole were very similar to each other (data not shown).

The substrate profiles of CSases from *P. morganii* T-211, *P. rettgeri* GN4430, *E. cloacae* H-27, and *S. marcescens* W-24 were very similar. Piperacillin, apalcillin, and ampicillin showed almost the same stability to these four CSases, and their rates of hydrolysis were about one tenth less than benzylpenicillin. Carbenicillin, sulbenicillin, and methicillin were very stable to these four CSases. The CSase from *P. vulgaris* T-178 had a broader substrate profile than other CSases. Piperacillin, apalcillin, ampicillin, and benzylpenicillin showed the same stability to this CSase and their rates of hydrolysis were about one tenth less than cephaloridine, and even carbenicillin, sulbenicillin, and methicillin, which were very stable to other CSases, showed detectable rates of hydrolysis by this enzyme.

Affinity of Penicillins for CSases

The *Ki* values of penicillins for the purified CSases were determined using cephalothin as a substrate (Table 3). The *Ki* values of piperacillin, apalcillin, and ampicillin for all CSases tested were

Table 1. Antibacterial activity of penicillins against CSase-producing strains.

Organism	MIC (μg/ml)[a]						
	Piperacillin	Ampicillin	Apalcillin	Benzylpenicillin	Carbenicillin	Sulbenicillin	Methicillin
P. morganii T-211	0.4 (3.1)	100 (400)	1.6 (6.3)	>400 (>400)	0.8 (6.3)	6.3 (50)	200 (400)
P. rettgeri GN4430	0.4 (0.8)	1.6 (25)	1.6 (1.6)	12.5 (100)	0.4 (1.6)	0.4 (0.8)	50 (50)
P. vulgaris T-178	3.1 (12.5)	>400 (>400)	6.3 (12.5)	>400 (>400)	100 (>400)	100 (200)	>400 (>400)
E. cloacae H-27	12.5 (50)	>400 (>400)	50 (200)	>400 (>400)	100 (200)	200 (400)	>400 (>400)
S. marcescens W-24	1.6 (6.3)	200 (400)	6.3 (50)	>400 (>400)	12.5 (25)	50 (50)	>400 (>400)

[a] MICs were determined by an agar dilution technique. Values in the parentheses are MICs at 5×10^5 cfu.

Table 2. Relative rates of hydrolysis of penicillins by partially purified CSase.

Organism	Relative rate of hydrolysis of penicillins (%)[a]							
	Piperacillin	Ampicillin	Apalcillin	Benzylpenicillin	Carbenicillin	Sulbenicillin	Methicillin	Cephaloridine
P. morganii T-211	2	4	4	43	<0.1	0.1	<0.1	100 (2.0)[b]
P. rettgeri GN4430	0.1	0.3	0.1	4	0.1	<0.1	<0.1	100 (2.5)
P. vulgaris T-178	22	24	17	16	1.4	0.4	0.4	100 (10.0)
E. cloacae H-27	0.2	0.6	0.3	4	<0.1	0.1	0.1	100 (20.0)
S. marcescens W-24	0.1	0.1	0.1	13	<0.1	<0.1	0.1	100 (7.0)

[a] Relative rates of hydrolysis are expressed in the percentage of hydrolysis of cephaloridine. Enzyme activities were determined by microiodometric method and the concentration of each substrate was 100 μM.

[b] Values in the parentheses are the specific activity (unit/mg of protein) of the enzymes used for the determination of substrate profiles.

Table 3. Affinity of penicillins for the purified CSases.

Sources of CSase	*Ki* values of penicillins (μM)[a]						
	Piperacillin	Ampicillin	Apalcillin	Benzylpenicillin	Carbenicillin	Sulbenicillin	Methicillin
P. morganii T-211	0.8	0.9	1.2	6.8	0.05	0.08	0.03
P. rettgeri GN4430	1.2	0.2	1.2	1.3	5.0	5.4	34.0
P. vulgaris T-178	11.0	13.0	7.7	6.1	8.2	1.6	1.4
E. cloacae H-27	1.0	0.6	0.8	1.3	0.2	0.3	0.06
S. marcescens W-24	0.03	0.03	0.05	1.3	0.4	0.2	0.6

[a] *Ki* values were determined using cephalothin as a substrate, and were calculated from Lineweaver-Burk plots.

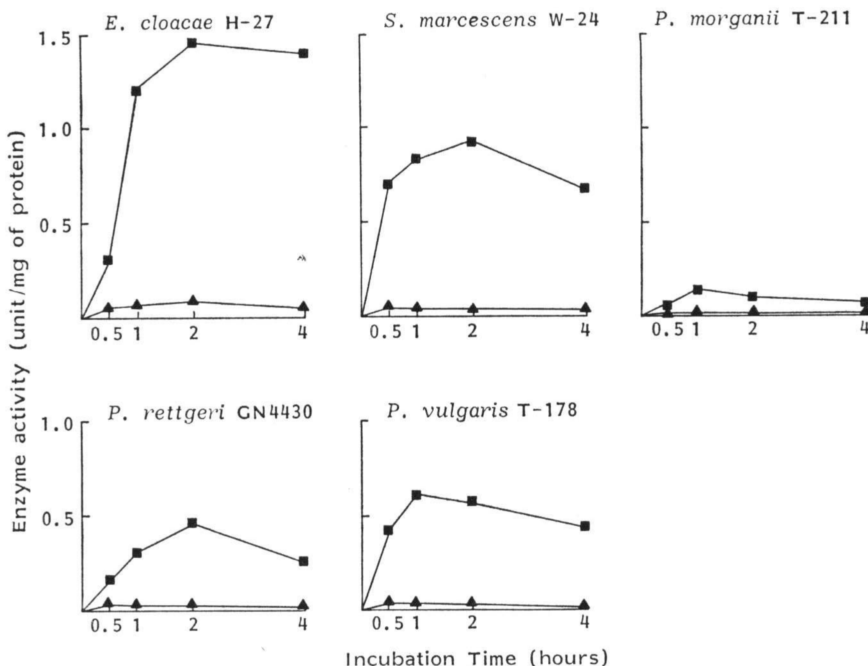
very similar to each other. These three penicillins showed the highest affinity for *S. marcescens* CSase among the CSases tested and rather low affinity for *P. vulgaris* CSase. Carbenicillin and sulbenicillin also showed the very similar affinity for CSases to each other. They had the highest affinity for *P. morganii* CSase among the CSases tested and rather low affinity for *P. rettgeri* and *P. vulgaris* CSases. Affinity of benzylpenicillin for CSases was relatively lower than other penicillins'. Methicillin showed rather high affinity for CSases except for *P. rettgeri* CSase whose *Ki* value with methicillin was large (34 μM) while *Ki* values of other drugs for this enzyme were rather small.

Kinetics of CSase Production

Ampicillin was added at 50 μg/ml to the mid-log phase of CSase-producing strains, and the induced cells were centrifuged at various incubation times. The enzyme activity (unit/mg of protein) of sonic extracts was determined using cephaloridine as a substrate (Fig. 1). The enzyme activities of all strains became maximum after about 2 hours of incubation with ampicillin and gradually decreased after that. Therefore, the comparison of the inducer activity of penicillins was done by measurement of enzyme activity of cells which were treated with drugs for 2 hours.

Fig. 1. Kinetics of CSase production in CSase-producing strains.

Sonic extracts prepared from induced cells were used in the spectrophotometric assay with cephaloridine (100 μM) as a substrate. The enzyme activity of induced cells with 50 μg/ml of ampicillin (■) were compared with those of control cells (▲).



Inducer Activity of Penicillins for CSase Production

The cells in mid-log phase were treated with various concentrations of penicillins for 2 hours. The specific activities of CSases extracted from penicillin-treated cells were determined by the spectrophotometric method using cephaloridine (100 μM) as a substrate.

The strains, except *E. cloacae* H-27, did not produce detectable amounts of CSase without inducers. *E. cloacae* H-27 produced a small amount of CSase even without inducers. CSase activity of cells induced by penicillins are shown in Table 4. Piperacillin, apalcillin, and methicillin showed lower inducer activity for CSase production in all strains tested. At 100 μg/ml of piperacillin, apalcillin, and methicillin, the CSase activity was slightly larger than observed at 1 or 10 μg/ml, but these values were smaller than those of other penicillins. Ampicillin, carbenicillin, sulbenicillin, and benzylpenicillin induced well in all strains tested. Ampicillin was a good inducer for CSase production in all strains even at low drug concentrations.

The Residual Activity of Penicillins and the Viable Cells in the Cultures of CSase-producing Strains

The residual activity of penicillins and the viable cells of CSase-producing strains after 2 hours of incubation with 100 μg/ml of penicillins were determined in order to make sure whether the low enzyme activity of cells which were induced by some penicillins was based on the rapid inactivation of drugs by the induced enzymes or the significant decrease of viable cells. The residual activity of penicillins and the viable cells are shown in Tables 5 and 6, respectively. The conditions employed in these experiments were the same as those for induction of CSase production at 100 μg/ml of drug con-

Table 4. Induction of CSase production by penicillins in CSase-producing strains.

Organism	Without drug	Enzyme activity (unit/mg of protein, $\times 10^{-2}$) at drug concentration (μg/ml)																				
		Piperacillin			Ampicillin			Apalcillin			Benzyl-penicillin			Carbenicillin			Sulbenicillin			Methicillin		
		1	10	100	1	10	100	1	10	100	1	10	100	1	10	100	1	10	100	1	10	100
P. morganii T-211	<1	<1	<1	2	2	22	18	<1	<1	3	2	17	27	<1	19	9	<1	14	13	<1	<1	<1
P. rettgeri GN4430	<1	3	6	27	21	89	109	3	9	76	9	39	80	10	93	79	13	114	68	4	6	22
P. vulgaris T-178	<1	2	2	16	5	38	119	1	2	19	7	40	100	7	57	104	3	92	134	1	2	24
E. cloacae H-27	2	2	2	4	3	15	182	2	2	3	2	2	130	2	7	265	2	9	195	2	2	4
S. marcescens W-24	<1	2	2	14	4	25	134	1	2	16	1	2	13	2	18	81	2	11	77	1	2	2

Enzyme activity was determined by the spectrophotometric method using cephaloridine (100 μM) as a substrate.

Table 5. Residual activity of penicillins in the cultures of CSase-producing strains.

Organism	Residual amount of penicillins (%)[a]						
	Piperacillin	Ampicillin	Apalcillin	Benzylpenicillin	Carbenicillin	Sulbenicillin	Methicillin
P. morganii T-211	59	3.7	23	<0.3	84	93	100
P. rettgeri GN4430	100	26	81	3.9	39	100	95
P. vulgaris T-178	30	<0.5	10	<0.3	<1.4	<3.3	81
E. cloacae H-27	100	<0.5	96	<0.3	30	81	100
S. marcescens W-24	100	18	92	<0.3	6.4	86	92

[a] Penicillins were added to each culture of CSase-producing strain at mid-log phase. After 2 hours of incubation, the residual amounts of penicillins were determined. The data are expressed as percentages of the initial concentrations of penicillins added.

Table 6. The viable cells in the cultures of CSase-producing strains after 2 hours of incubation with 100 μg/ml of penicillins.

Organisms	Log of viable cells/ml after treatment of following penicillins							
	Without drug	Piperacillin	Ampicillin	Penicillin	Benzyl-Penicillin	Carbenicillin	Sulbenicillin	Methicillin
P. morganii T-211	9.9 (9.0)[a]	8.6	9.0	8.6	9.0	7.8	7.0	9.9
P. rettgeri GN4430	9.8 (9.0)	8.9	8.7	9.0	9.0	8.5	7.8	9.0
P. vulgaris T-178	9.8 (9.0)	8.9	9.8	8.9	9.8	9.6	9.0	9.8
E. cloacae H-27	9.8 (9.0)	9.3	9.8	9.3	9.8	9.0	9.0	9.7
S. marcescens W-24	9.6 (8.9)	7.8	8.9	8.4	9.0	6.9	7.0	9.6

[a] Values in the parentheses are log of viable cells before the addition of penicillins.

centration. Methicillin was the most stable in the cultures of all strains among penicillins tested. On the other hand, benzylpenicillin was the least stable drug among penicillins tested, followed by ampicillin. In the cultures of *P. rettgeri* GN4430, *E. cloacae* H-27, and *S. marcescens* W-24, piperacillin, apalcillin, and sulbenicillin maintained their activity after 2 hours of incubation, while ampicillin, carbenicillin, and benzylpenicillin lost their activity in the cultures of these strains. In the culture of *P. vulgaris* T-178, all penicillins, except for methicillin, easily lost their activity after 2 hours of incubation. Even in this culture, however, piperacillin and apalcillin maintained 30% and 10% activity, respectively, after 2 hours of incubation.

The viable cells of all strains reached $4 \sim 8 \times 10^9$ cells/ml after 2 hours of incubation without drugs. Carbenicillin and sulbenicillin significantly decreased viable cells of *P. morganii* T-211, *P. rettgeri* GN4430, and *S. marcescens* W-24. Other penicillins depressed more or less the growth of all strains but they did not so significantly decrease the viable cells as carbenicillin and sulbenicillin did.

Penicillins with low inducer activity at 100 μg/ml were not always unstable in the cultures of CSase-producing strains and had not always the high bectericidal activity.

Discussion

In many cases, the enzyme activity of cells induced by piperacillin, apalcillin, and methicillin was rather low though the activity of these drugs remained in the cultures of CSase-producing strains and cells were not so significantly killed by these drugs. This suggests that the low enzyme activity of cells induced by piperacillin, apalcillin, and methicillin was based on neither the rapid inactivation of these drugs by the induced enzyme nor the significant decrease of viable cells. Therefore, the low inducer activity of piperacillin, apalcillin, and methicillin should be considered a unique and important property over other penicillins. On the other hand, the inducer activity of ampicillin for CSase production is thought to be high because this drug increased enzyme activity even at lower concentrations (*i.e.* at 1 or 10 μg/ml), and in addition, this drug could induce the high level of enzyme at 100 μg/ml in spite of its considerable inactivation by the induced enzyme. The inducer activity of carbenicillin and sulbenicillin for CSase production is thought to be as high as that of ampicillin, and much higher than those of piperacillin, apalcillin, and methicillin. Benzylpenicillin is probably also a good inducer as well as ampicillin though the observed values of enzyme activity of cells induced by benzylpenicillin were smaller than those by ampicillin. The rapid inactivation of benzylpenicillin might result in its low inducer activity observed.

Penicillins are much more stable to CSases than cephaloridine, cefazolin, *etc*[2,6]. Our results also confirmed this. In addition, our results show that there is difference in the stability of penicillins to CSases though their rates of hydrolysis by CSases were very low. To typical CSases, benzylpenicillin was least stable, followed by piperacillin, apalcillin, and ampicillin. Carbenicillin, sulbenicillin, and methicillin were most stable to all CSases tested. The hydrolysis rates of piperacillin, apalcillin, and ampicillin were very similar to each other, and those of carbenicillin and sulbenicillin were also similar. The same feature was observed in affinity of penicillins for CSases. The *Ki* values of piperacillin, apalcillin, and ampicillin were very similar to each other, and those of carbenicillin and sulbenicillin were also similar.

The behavior of piperacillin, apalcillin, and ampicillin to CSases were very similar. Piperacillin and apalcillin, however, have the advantage of a low inducer activity for CSase production that inactivates drugs or serves as a barrier to target proteins[19]. This low inducer activity might bring piperacillin and apalcillin superior activity against CSase-producing strains over ampicillin. Carbenicillin and sulbenicillin were more stable to CSases than piperacillin, apalcillin, and ampicillin but their inducer activity for CSase production was as high as that of ampicillin. Hence they were more active to CSase-producing strains than ampicillin, but less or slightly less active than piperacillin and apalcillin. Methicillin showed unique action on CSase-producing strains. That is, this drug had rather weak antibacterial activity to CSase-producing strains though it did not induce CSase production and was very stable in the culture of CSase-producing strains as well as to CSases. It is well known, furthermore, that this drug has high antibacterial activity against Gram-positive bacteria and is a good inducer for penicillinase synthesis in Gram-positive bacteria[20,21]. These properties might derive from its low penetrability through cell envelope of Gram-negative bacteria. These facts suggest that the permeability barrier is one of critical factors which define the antibacterial activity of penicillins against CSase-producing strains and affect the inducer activity of penicillins for CSase production.

CSase production actually plays an important role in antibacterial activity of penicillins against CSase-producing bacteria, but the significance of CSase production is not uniform. All penicillins tested were active against *P. rettgeri* GN4430 though this strain produced an inducible CSase in the same manner as other strains did. This problem will be resolved, for example, by the study using a mutant which devoid of ability of CSase production or inducibility for CSase production.

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