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Induction of β-Lactamase by Various β-Lactam Antibiotics in *Enterobacter cloacae*

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The induction of β-lactamase in *Enterobacter cloacae* GN5797 was studied by using 23 β-lactam antibiotics, including newly introduced drugs, as inducers. The β-lactam antibiotics can be classified into three groups on the basis of their inducer activity. Among the tested cephalosporins, cephamycin derivatives such as cefoxitin, cefmetazole, and YMO9330 had high inducer activity even at low drug concentrations. On the other hand, cefoperazone, cefsulodin, piperacillin, and apalcillin showed low inducer activity when compared with the other cephalosporins.

β-Lactamases specified by drug-resistant plasmids have been found to be of the penicillinase type (3). Most of the gram-negative bacteria isolated from clinical materials, however, produce cephalosporinases which are species specific in their substrate profiles (4). The inducible formation of β-lactamase is of special importance in clinical medicine and in the development of new β-lactam antibiotics and has received relatively little attention (1, 5, 6, 11).

This paper deals with the inducer activity of a number of β-lactam antibiotics, including some of the newly developed drugs, for the production of β-lactamase by *Enterobacter cloacae*.

MATERIALS AND METHODS

Bacterial strain. A clinical isolate *E. cloacae* GN5797, a stock culture in this laboratory, was used in this study.

Media. Brain heart infusion broth (Difco Laboratories, Detroit, Mich.) was used for both the growth of *E. cloacae* and the induction of β-lactamase. Heart infusion agar (Eiken, Tokyo, Japan) was used for the determination of the minimal inhibitory concentration of the antibiotics by the agar dilution method.

Antibiotics. Cephaloridine, cefazolin, cephalothin, cephalexin, penicillin G, ampicillin, carbenicillin, cloxacillin, and methicillin were commercially available materials. Cefsulodin and cefotiam (Takeda Pharmaceutical, Osaka, Japan), cefamandole and moxalactam (6059S) (Shionogi Pharmaceutical, Osaka), cefoperazone and piperacillin (Toyama Chemical, Tokyo), cefuroxime (Shinnihon Jitsugyo, Tokyo), ceftizoxime (FK-749) (Fujisawa Yukuhin, Osaka) (12), cefoxitin (Daiichi Pharmaceutical, Tokyo), cefmetazole (Sankyo, Tokyo), YMO9330 (Yamanouchi Pharmaceutical, Tokyo) (13), apalcillin (Sumitomo Chemical, Osaka), and clavulanic acid (Beecham Yakuhin, Tokyo) were the newly introduced drugs.

Determination of the minimal inhibitory concentration. The minimal inhibitory concentration was determined by a serial dilution technique. Overnight cultures of test strains in brain heart infusion broth were diluted to a final concentration of 10^6 cells per ml, and one loopful (0.005 ml) of culture was inoculated onto heart infusion agar plates by use of a Microplanter (Sakuma, Tokyo) inoculator. The minimal inhibitory concentration was determined after overnight incubation at 37°C.

Enzyme assay. β-Lactamase activity was determined either by a spectrophotometric method (10, 14) measuring the decrease in absorbance at an appropriate wavelength of the substrate (100 μM) in a temperature-controlled spectrophotometer (Beckman Model 24) at 30°C or by a modified microiodometric method (7) with penicillins as substrates. The wavelength used for the photometric assay was that which gave a maximum in the difference spectrum when an unhydrolyzed substrate was scanned against a hydrolyzed one (Table 1). The millimolar absorbancy difference (Table 1, column 3) was used to calculate the rate of hydrolysis.

One unit of enzyme activity is defined as the amount of enzyme that hydrolyzed 1 μmol of substrate in 1 min at 30°C in 0.05 M phosphate buffer (pH 7.0).

Induction of β-lactamase. For the determination of the induction of β-lactamase by β-lactam antibiotics, an overnight culture was diluted 20-fold into 10 ml of fresh medium (brain heart infusion broth) and incubated with shaking at 37°C. After 2 h of incubation, inducer was added, and the incubation was continued. After a further 2 h of incubation, the cells were harvested and washed once with 0.1 M phosphate buffer (pH 7.0). The cells were suspended to their original volume in 0.1 M phosphate buffer and disrupted with a UR-150P ultrasonic vibrator (Tomy Seiko Co.) for 2 min at 75 W in an ice-water bath. The broken cells were centrifuged at 13,000 × *g* for 30 min at 4°C, and the resulting supernatant fluid was used as the crude enzyme.

Protein determination. The concentration of protein was determined by the method of Lowry et al. with bovine serum albumin as the standard (2).

RESULTS

Drug resistance of *E. cloacae* GN5797. The minimal inhibitory concentration values of

various β-lactam antibiotics against *E. cloacae* GN5797 are shown in Table 2. *E. cloacae* GN5797 was resistant to most of the β-lactam antibiotics, except for the newly introduced drugs such as cefoperazone, cefotaxime, ceftizoxime, and piperacillin.

TABLE 1. *Differences in extinction between β-lactam antibiotics and corresponding hydrolyzed compounds*[a]

β-Lactam antibiotic	λ (nm)	Δε (1·mm^{-1}·cm^{-1})
Cephaloridine	260	10.2
Cefazolin	263	7.71
Cephalothin	262	7.66
Cephalexin	262.5	6.88
Cefotiam	276	8.82
Cefamandole	274	10.3
Cefoperazone	273	9.00
Cefuroxime	262	8.54
Cefotaxime	264	7.25
Ceftizoxime (FK-749)	250	7.03
Cefoxitin	265	7.38
Cefmetazole	275	8.38
Moxalactam (6059S)	275	7.96
Penicillin G	233	1.14
Ampicillin	235	0.90
Carbenicillin	235	0.83

[a] The cephalosporin (100 μM) or penicillin (1 mM) in 50 mM sodium phosphate buffer, pH 7.0, at 30°C was hydrolyzed by β-lactamase, and the spectra of unhydrolyzed and hydrolyzed compounds were compared in cells with a path length of 1 cm. The second column gives the wavelength (λ) of the maximum in the difference spectrum.

TABLE 2. *Antibacterial activity of various β-lactam antibiotics against E. cloacae GN5797*

Antibiotic	Minimal inhibitory concn (μg/ml)
Cephaloridine	400
Cefazolin	> 800
Cephalothin	> 800
Cephalexin	> 800
Cefotiam	50
Cefamandole	50
Cefoperazone	0.8
Cefsulodin	100
Cefuroxime	100
Cefotaxime	≤ 0.4
Ceftizoxime (FK-749)	≤ 0.4
Cefoxitin	400
Cefmetazole	400
YMO9330	400
Moxalactam (6059S)	12.5
Penicillin G	> 800
Ampicillin	800
Carbenicillin	12.5
Piperacillin	6.3
Apalcillin	12.5
Cloxacillin	> 800
Methicillin	800
Clavulanic acid	100

Formation of β-lactamase in *E. cloacae* GN5797. The kinetics of β-lactamase formation after addition of an inducer were investigated (Fig. 1). Without the addition of an inducer, β-lactamase activity is almost undetectable during any phase of growth. The maximum specific activity was obtained at 2 h after the addition of cefoxitin to cells in mid-log phase. Addition of the drug at the lag phase gave somewhat lower activity. β-Lactamase produced by *E. cloacae* GN5797 was considered to be a typical cephalosporinase according to its substrate profiles (Table 3).

Some properties of β-lactam antibiotics against the β-lactamase from *E. cloacae* GN5797. The β-lactamase from *E. cloacae* GN5797 hydrolyzed cephaloridine, cefazolin, cephalothin, cephalexin, cefotiam, and penicillin G, but the other antibiotics were very stable to this enzyme. Those resistant to hydrolysis by the enzyme, except for cefoperazone, cefsulodin, and clavulanic acid, had an inhibitory effect on enzyme activity at concentrations of 10 μM.

Inducibility with β-lactam antibiotics. To determine inducibility with β-lactam antibiotics, various concentrations of the drugs were added to the culture at the mid-log phase (about 10^9

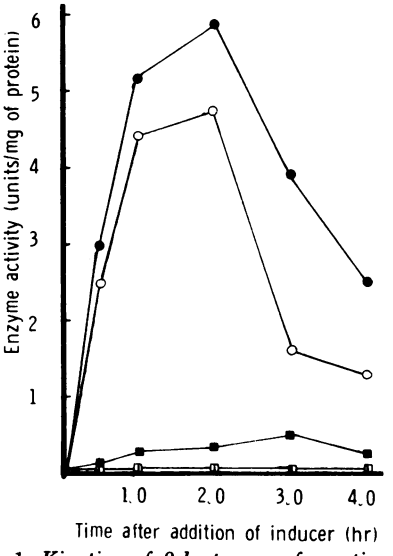

FIG. 1. *Kinetics of β-lactamase formation in E. cloacae GN5797. An overnight culture of E. cloacae GN5797 was diluted 20-fold with brain heart infusion broth and incubated with shaking at 37°C. Cefoxitin was added to a final concentration of 10 μg/ml as an inducer for β-lactamase formation at the start of the incubation (lag phase, ○), 2 h after incubation (mid-log phase, ●), and 4 h after incubation (stationary phase, ■). The induced cells were harvested by centrifugation at 0.5, 1, 2, 3, and 4 h after the addition of cefoxitin. □, β-Lactamase formation without an inducer.*

TABLE 3. *Induction of β-lactamase by various β-lactam antibiotics in E. cloacae GN5797 and some properties of β-lactamase produced*[a]

Antibiotic	Enzyme induction at drug concn (μg/ml)[b]: 1	10	100	1,000	Relative rate of hydrolysis (%)[c]	Inhibitory effect on enzyme activity (%)[d]
Group A						
Cephaloridine	0.3	0.4	11.2	22.8	100	—[e]
Cefazolin	1.4	5.4	16.4	18.1	56	—
Cephalothin	0.6	0.8	1.5	13.7	440	—
Cephalexin	0.5	0.7	3.3	10.3	44	—
Cefotiam	0.6	3.1	16.1	22.8	63	—
Cefamandole	0.6	2.6	10.2	9.0	1	55
Penicillin G	0.2	0.4	3.7	8.9	15	—
Cefoxitin	2.4	8.8	24.4	6.6	<1	93
Cefmetazole	2.1	9.9	23.2	22.8	<1	93
YM09330	2.1	9.5	20.4	7.8	<1	95
Moxalactam	4.3	17.0	0.3	0.1	<1	97
Ampicillin	0.9	5.9	28.1	13.4	<1	42
Clavulanic acid	0.2	10.2	9.1	1.4	—	0
Group B						
Carbenicillin	0.5	5.9	2.8	0.7	<1	100
Cefuroxime	0.2	2.4	6.8	4.6	<1	100
Cefotaxime	0.4	2.5	0.8	0.1	<1	92
Ceftizoxime	0.7	1.3	7.8	4.6	<1	97
Group C						
Cefoperazone	0.1	0.1	1.1	3.1	<1	8
Cefsulodin	0.1	0.1	2.0	2.0	<1	0
Piperacillin	0.1	0.1	0.5	7.8	<1	38
Apalcillin	0.1	0.1	3.0	3.2	<1	30
Methicillin	0.1	0.1	0.1	0.1	<1	92
Cloxacillin	0.1	0.1	0.1	0.5	<1	100

[a] *E. cloacae* was grown in brain heart infusion broth containing various concentrations of β-lactam antibiotics as shown in the table.

[b] β-Lactamase production is expressed as the specific activity of the induced enzyme (units per milligam of protein) with cephalothin as a substrate.

[c] Relative rate of hydrolysis of various β-lactam antibiotics by the crude enzyme from *E. cloacae* GN5797 is expressed in percent of the activity with cephaloridine. The method for enzyme assay was described in the text.

[d] Each drug (10 μM) was added to the reaction mixture containing enzyme and cephalothin as a substrate, and enzyme activity was immediately assayed by the direct photometric method.

[e] —, Not done.

cells per ml), and the culture was incubated with shaking at 37°C for a further 2 h before the organisms were harvested. Under these conditions most of the drugs showed little or no effect on the growth of *E. cloacae* GN5797 when their concentrations were less than 100 μg/ml. Many drugs repressed the growth of the culture or lysed the cells at 1,000 μg/ml, but methicillin, cloxacillin, penicillin G, cephaloridine, cefazolin, cephalothin, cephalexin, and cefotiam did not show any effect on growth of the culture even at 1,000 μg/ml, probably due to the low penetrability of methicillin and cloxacillin and due to the rapid hydrolysis of cephaloridine, cefazolin, cephalothin, cephalexin, cefotiam, and penicillin G by the enzyme.

The inducibility of β-lactamase by various β-lactam antibiotics in *E. cloacae* GN5797 is shown in Table 3. Based on inducibility, the β-lactam antibiotics can be classified into three groups, i.e., with high (A), intermediate (B), or low (C) inducer activity. Those agents which showed very high enzyme induction at the tested concentrations were classified as group A. Those which showed very low enzyme induction at concentrations of 1 and 10 μg/ml were classified as group C. Those difficult to classify into either A or C were placed in group B. Ampicillin and cephamycin derivatives such as cefoxitin, cefmetazole, YM09330, and moxalactam showed high inducer activity at lower concentrations than was the case for cephaloridine, cefazolin, cephalothin, cephalexin, cefotiam, and penicillin G, which are in the same group and are easily hydrolyzed by the enzyme. Cefuroxime-type cephalosporins and carbenicillin belong to group

B. Cefoperazone, cefsulodin, piperacillin, and apalcillin, which are newly introduced drugs, showed a low inducibility (group C). Methicillin and cloxacillin showed the lowest inducibility among the antibiotics, possibly due to the low penetrability of bacterial cells by these drugs.

DISCUSSION

Among the 23 β-lactam antibiotics, 11 belong to group A based on their inducer activity for β-lactamase in *E. cloacae* GN5797. Cephaloridine, cefazolin, etc., are easily hydrolyzed by the enzyme, and higher concentrations of these drugs were required for induction of the enzyme than were required of ampicillin and cephamycin derivatives which are resistant to hydrolysis by the enzyme. In gram-positive bacteria (8) methicillin and cloxacillin are good inducers, but they were inactive for the production of β-lactamase in *E. cloacae* GN5797. It seems very likely that these drugs cannot penetrate through the cell envelope of *E. cloacae* GN5797, resulting in ineffectiveness against the organisms. Cefoperazone, cefsulodin, piperacillin, and apalcillin have low inducer activity for enzyme production despite the fact that they have a low inhibitory effect on enzyme activity, i.e., low affinity for the enzyme. It should be noted that cephamycin derivatives have high inducer activity for β-lactamase formation in *E. cloacae* GN5797 and have strong inhibitory activity against the enzyme.

β-Lactamase has been considered to play a significant role in bacterial resistance against the β-lactam antibiotics (4, 9). Therefore, much attention has been paid to the stability of the drug to β-lactamase, and various drugs resistant to particular β-lactamases have been developed. Little attention has been paid to the inducer activity for β-lactamase formation by various β-lactam antibiotics, and it would appear that more attention should be given to this property in developing newer antibiotics and in clinical medicine.

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