Microbial Degradation of Cephalothin by Cephalothin-Susceptible *Escherichia coli*

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Cephalothin (CET)-susceptible *Escherichia coli*, which can degrade CET after prolonged incubation in broth containing a concentration of the drug greater than the minimum inhibitory concentration, was found in a clinical specimen. The substrate specificity of the partially purified enzyme to cephalosporin analogs strongly indicated the occurrence of CET-specific degradation. Nuclear magnetic resonance analysis of the degradation reaction demonstrated the appearance of two new signals attributed to deacetyl CET. This suggests the possibility of the presence of acylesterase.

β -Lactamase is involved in the biochemical mechanisms of resistance to β -lactam antibiotics in clinical isolates, and there are a number of such resistant strains (1). Acylesterase (4, 11) is also known to be involved in the biochemical mechanisms of resistance to cephalosporin (CES) by CES-resistant bacteria, and the contribution of CES acylase to the resistance is also reported (3, 13). However, as far as CES-susceptible *Escherichia coli* is concerned, chromosome-mediated β -lactamase is the only known enzyme (9). We have found that cephalothin (CET)-susceptible *E. coli* can grow even in broth containing CET with as much as four times (4 $\times$) the minimum inhibitory concentration (MIC) when the incubation period is prolonged. Furthermore, it is indicated that the strain possesses an enzyme which degrades CET by deacetylation.

MATERIALS AND METHODS

Antibiotics. The antibiotics used were CET, cephaloridine, cefazolin, and cephalixin of the CES group antibiotics and penicillin G, aminobenzylpenicillin, carbenicillin, and sulbenicillin of the penicillin group antibiotics. These drugs were gifts from Shionogi Pharmaceutical Co. Ltd.

Strains. CET-susceptible *E. coli* 437 (EC 437), derived from a urinary tract infection, was mainly used. The controls were CET-resistant *E. coli* 479 (EC 479), derived from a clinical specimen, and an antibiotic-susceptible standard strain, *E. coli* NIHJ-JC2 (EC NIH). *Bacillus subtilis* ATCC 6633 was used as the test strain in the microbioassay to determine the potency of the antibiotics.

Media. Trypticase soy broth (BBL) as a liquid medium and nutrient broth agar (Eiken, Tokyo) as a solid medium were used.

Determination of MIC. An overnight culture of the bacteria was diluted 100-fold with sterile saline solution, and 1 loopful of suspension was inoculated onto nutrient agar plates containing various concentrations of an antibiotic reagent; after incubation for 24 h, the MIC was determined as described in a previous paper (7).

Bacterial growth in broth containing CET. Growth was automatically recorded with a biophotometer (JASCO-Jouan Biolog II) (8). One milliliter of a CET solution, 8.9 ml of Trypticase soy broth, and 0.1 ml of a 10-fold dilution of a Trypticase soy broth overnight culture of the bacteria to be tested were put into a vessel for absorbance determination (the inoculum size being about 10⁶ cells per ml), and the mixture was cultured at 37°C; the growth curve was recorded automatically.

Counting of viable cells in broth containing CET. The bacterial culture was appropriately diluted, and 0.1 ml of the diluent was inoculated onto a nutrient agar plate. After an overnight incubation, the number of viable cells in the bacterial culture was determined from the number of colonies on the plates.

Percentage of CET inactivation in broth containing CET. The remaining CET activity in the CET broth culture was determined by using a cup method. The inactivation of CET was expressed as the percentage of CET degraded.

Protein determination. Protein determination was carried out by the method of Lowry et al. (10), with bovine serum albumin as the standard.

Assay for β -lactamase. The enzyme activity was assayed iodometrically at pH 7.0 with a modification of Perret's method (12, 15).

Preparation of crude enzyme solution. Bacterial cells in the exponential phase of growth were collected by centrifugation and suspended in a small amount of 0.01 M phosphate buffer solution (pH 7.0). After disintegrating cells with a sonic oscillator (20 kcycles; Ohtake, Tokyo), the disrupted cells were cen-

trifuged at $105,000 \times g$ for 2 h at 2°C . The supernatant obtained was dialyzed overnight against distilled water and used as a crude enzyme solution.

Partial purification of crude enzyme solution from EC 437. The crude enzyme solution (14.1 mg of protein per ml) was applied to a Sephadex G-50 column (40 by 2.5 cm) and eluted with 0.01 M phosphate buffer solution (pH 7.0). The fractions which inactivated CET were collected and used as a partially purified enzyme. The enzyme showed 0.764 unit/mg of protein (1 unit is defined as that amount inactivating 1 μmol of CET within 30 min at 37°C in 0.1 M phosphate buffer [pH 7.0]). The specific activity of the enzyme, β -lactamase, was less than 0.026 when penicillin G or CET was used as the substrate.

Partial purification of crude enzyme solution from EC 479. The crude enzyme solution (6.3 mg of protein per ml) was passed through a Sephadex column and partially purified as described above. The fractions which inactivated CET were then collected. The partially purified enzyme possessed β -lactamase activities of 2.019 units/mg of protein to penicillin G (1 unit is defined as 1 μmol of penicillin G hydrolyzed per h at 37°C ; pH 7.0) and 7.486 units/mg of protein to CET.

Inactivation of antibiotics by partially purified enzyme. A solution of CET (0.05 ml, 500 $\mu\text{g}/\text{ml}$), 0.05 ml of an enzyme solution, and 0.4 ml of 0.1 M phosphate buffer solution (pH 7.0) were mixed and allowed to react at 37°C . After heating in a boiling-water bath for 1 min, the remaining potency of the antibiotic was determined by microbioassay as described above to calculate the inactivation of CET and the inactivation units.

NMR analysis of CET degradation. A 10-mg amount of CET was dissolved in a crude enzyme solution (2.2 mg of protein). After the solution was lyophilized, the dried powder obtained was dissolved in 0.3 ml of D_2O and transferred into a nuclear magnetic resonance (NMR) tube (ID, 5 by 180 mm). NMR spectra were measured at 37°C with a JNM-PS-100 NMR spectrometer (Nihon Denshi) and calibrated against 3-(trimethylsilyl)-propane sulfonic acid sodium salt as an internal standard. When the pH of the reaction mixture reached <6.2 , 1 N NaOH was added to return the pH to 7.0. The reaction mixture was lyophilized and D_2O was added. A time course of NMR spectra was recorded.

RESULTS

Growth of EC 437 in broth containing CET and CET inactivation. MICs of β -lactam antibiotics against EC 437 and EC NIH are shown in Table 1. *E. coli* EC 437 gave MICs

similar to or a little lower than the MICs of any of the tested antibiotics against EC NIH.

Figure 1 shows the growth curves of EC NIH and EC 437 in broth containing CET. Both strains showed a similar growth pattern, with a lag time which prolonged with increasing CET concentrations. EC NIH did not grow in the broth containing more than 25 μg ($1 \times \text{MIC}$) of CET per ml, whereas *E. coli* EC 437 grew even in broth containing more than 12.5 $\mu\text{g}/\text{ml}$ ($1 \times \text{MIC}$). The bacterial growth of EC 437 in broth containing 50 μg ($4 \times \text{MIC}$) of CET per ml was observed after about a 30-h lag time. From the culture in broth containing $4 \times$ the MIC of CET, single colonies were obtained, and the MIC of the isolated bacteria was determined. The MIC was similar to that for the original strain. This finding eliminated the possibility of the growth of a CET-resistant mutant. The minimum bactericidal concentration for EC 437 was determined to be 100 $\mu\text{g}/\text{ml}$ according to the method of Berman et al. (2).

Figure 2 shows the relationship between the variation in the viable cell count in broth containing CET and CET inactivation. With an inoculum size of 10^6 cells per ml, in broth containing $1 \times$ the MIC of CET, the number of viable cells fell to about $10^2/\text{ml}$ and increased thereafter. On the other hand, CET in the medium was rapidly degraded during the incubation period. It is obvious from Fig. 2 that whereas

FIG. 1. Growth curves of *E. coli* strains 437 and NIHJ-JC2 in the presence of increasing amounts of CET.

TABLE 1. Antibiotic susceptibility of strains of *E. coli*

<i>E. coli</i> strain	MIC ($\mu\text{g}/\text{ml}$) ^a					
	CET	CEX	CEZ	ABPC	CBPC	SBPC
437	12.5	12.5	3.1	6.25	6.25	6.25
479	400	400	200	200	12.5	50
NIHJ-JC2	25	12.5	3.1	25	12.5	25

^a CEX, Cephalixin; CEZ, cefazolin; ABPC, aminobenzylpenicillin; CBPC, carbenicillin; SBPC, sulbenicillin.

the number of cells of EC 437 is reduced by the presence of CET, the degrading enzyme from EC 437 inactivates CET. The viable cells of EC 437 begin to grow because of the reduced CET concentration in the broth. In broth containing 4× the MIC of CET with an inoculum size of 10^8 cells per ml, the temporal reduction of the viable cells and the CET inactivation occurred more rapidly.

Mechanism of CET degradation by EC 437. Since the experimental results described above suggested the enzymatic inactivation of CET by EC 437, the CET-inactivating enzyme was extracted from the bacteria and partially purified. The CET-inactivating rate of the enzyme had a positive correlation with the reaction time (Fig. 3). The optimum pH was about 7.5, and the optimum temperature was about 45°C. CET inactivation was not observed in the culture supernatant.

Table 2 shows the substrate specificities of the enzyme obtained from EC 437 against antibiotics

together with the substrate specificities of β -lactamase obtained from EC 479 (Table 1). The enzyme from EC 437 inactivated cephaloridine to a slight degree, but did not inactivate cephalixin, penicillin G, aminobenzylpenicillin, and carbenicillin. The enzyme inactivated CET spe-

FIG. 3. Kinetics of CET inactivation by partially purified enzyme. A 0.05-ml amount of CET (500 μ g/ml), 0.05 ml of an enzyme solution, and 0.4 ml of 0.1 M phosphate buffer solution (pH 7.0) were mixed and allowed to react for 30 min at 37°C. The remaining potency of the antibiotic was determined by microbioassay.

TABLE 2. Substrate specificity of partially purified enzymes from *E. coli* 437 and 479

Enzyme from:	Rate of inactivation (%) ^a					
	CET	CER	CEX	PC-G	ABPC	CBPC
<i>E. coli</i> 437	100	11.9	0	0	0	0
<i>E. coli</i> 479	100	62.5	50.0	57.5	18.8	0

^a Expressed as percentage of inactivation of CET (50 μ g/ml). CER, cephaloridine; CEX, cephalixin; PC-G, penicillin G; ABPC, aminobenzylpenicillin; CBPC, carbenicillin.

FIG. 2. CET inactivation and bacterial growth in broth containing CET. (A) Inoculum size, 10^6 cells per ml; concentration of CET, 12.5 μ g/ml (1× MIC). (B) Inoculum size, 10^8 cells per ml; concentration of CET, 50 μ g/ml (4× MIC)

FIG. 4. NMR spectra during degradation of CET. A 10-mg amount of CET was dissolved in a crude enzyme solution, and, after the solution was lyophilized, the dried powder obtained was dissolved in 0.3 ml of D_2O . A time course of NMR spectra was recorded at 37°C.

cifically, and this inactivation occurred rapidly. The substrate specificity of the enzyme from EC 437 was obviously different from that of the enzyme obtained from EC 479 (β -lactamase). Taking the chemical structures of the substrates into consideration, it is possible that the enzyme from EC 437 breaks the acetoxy group at the C10 position in the side chain of CET.

CET was added to the EC 437 enzyme solution in an NMR tube to study the structural change of CET. The result is shown in Fig. 4. Methyl proton (2.11 ppm) in the acetoxy group of CET shifted upfield, showing a new strong signal at 1.92 ppm. When sodium acetate was added to the NMR tube, an identical peak was observed at 1.92 ppm. Accordingly, it was concluded that the acetate was liberated from the C10 acetoxy group of the CET molecule. In addition, deacetyl CET was assigned by the appearance of the peak at 4.27 ppm, which corresponded with the peak of C10 proton of deacetyl CET.

DISCUSSION

Demain et al., (4) and Nishida et al. (11) reported on the inactivation enzyme (acylsterase) of CES-resistant *E. coli*. The present paper is the first report on a CET acylsterase by a susceptible *E. coli* isolate (EC 437). Inactivation of an antibiotic by a susceptible bacterium may become one of the factors leading to unsuccessful antibiotic chemotherapy, even when the effectiveness has been established by the MIC test. In the case of EC 437, bacterial growth depends on the mutual relationship between the antibacterial effect of CET and the production of an inactivating enzyme by the bacteria. When the inoculum size is small and the quantity of CET is large, bacterial growth due to CET inactivation is not seen in vitro. These findings suggest that the degradation of CET has to be taken into account to determine adequate dosage and interval for the clinical use of CET.

NMR analysis of CET degradation is discussed in the report by Hamilton-Miller et al. (5). These authors reported on changes in NMR spectra during enzymatic hydrolysis of CESs, and they pointed out that the analysis could be performed only in the 4 τ to 5 τ (6- to 5-ppm) region because of the noise derived from protein. In our experiment, the NMR spectrum could be observed in all the regions even when CET was used. This method may be applicable for the kinetic study of a hydrolyzing enzyme. The antibacterial activity of deacetyl CET is known to be about 20% of that of CET (6, 14). The enzymatic degradation of CET by EC 437 described in this paper is a first-order reaction and pro-

ceeds through deacetylation of the CET molecule.

Nineteen percent of CET-susceptible *E. coli* (162 strains) isolated from patients with urinary tract infections demonstrated the same property as EC 437 in terms of the growth pattern in broth containing 4 $\times$ the MIC of CET. Although enzymatic degradation of CET by EC 437 could be explained in part by the presence of acylsterase, the possibility of another degradation process (e.g., unusually slow-acting β -lactamase, etc.) of CET cannot be ruled out. A study on this question is now in progress in our laboratory.

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