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Cefroxadine (CGP-9000), an Orally Active Cephalosporin

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Cefroxadine (CGP-9000; CXD), 7β[D-2-amino-2-(1,4-cyclohexadienyl)-acetamido]-3-methoxy-ceph-3-em-carboxylic acid, is a new orally active cephalosporin derivative. The spectrum of antibacterial activity of CXD is identical with that of cephalexin (CEX), but CXD was twofold more effective than CEX against *Escherichia coli* and *Klebsiella pneumoniae*. CXD was as stable to penicillinase as CEX, but it was hydrolyzed by cephalosporinase, with a relative rate of hydrolysis similar to that of CEX. The affinities of CXD and CEX to penicillin-binding proteins of *E. coli* were estimated; the affinity of CXD to penicillin-binding protein 1Bs was higher than that of CEX. Consistent with this, CXD had more intensive lytic activity than CEX. In vivo antibacterial activities of CXD and CEX were compared using systemic infections of mice with *E. coli* and *K. pneumoniae*; CXD was consistently more active than CEX.

Although there are many cephalosporins available that are suitable for parenteral administration, orally active cephalosporins are less common. Among oral cephalosporins introduced on the market, cephalexin (CEX) is now in widespread use in the treatment of a variety of infectious diseases. Cefroxadine (CXD) is the dihydrophenylglycin derivative of a structurally modified 7-amino-cephalosporanic acid and is structurally related to CEX, but possesses antibacterial properties that distinguish it from CEX. This paper presents the in vitro and in vivo microbiological evaluation of CXD.

MATERIALS AND METHODS

Antibiotics. CXD, 7β[D-2-amino-2-(1,4-cyclohexadienyl)-acetamido]-3-methoxy-ceph-3-em-carboxylic acid, is an orally active cephalosporin derivative (Fig. 1) which was synthesized in the Research Laboratories, Pharmaceuticals Division, CIBA-GEIGY Limited, Basel, Switzerland. Other antibiotics were commercial products.

Test strains. Strains stocked in this laboratory were used as standard stock cultures and are from the Reference Laboratory of Drug-Resistant Bacteria, Gunma University. They were originally isolated from clinical materials.

Media. Heart infusion (HI) agar (Eiken), HI broth (Eiken), and antibiotic medium no. 3 (Difco) were used. Other media were peptone water and medium B. The former consisted of 10 g of polypeptone, 5 g of NaCl, and 1 liter of distilled water; the latter contained 2 g of yeast extract, 10 g of polypeptone, 7 g of $Na_2HPO_4 \cdot 12H_2O$, 2 g of KH_2PO_4, 1.2 g of glucose, and 0.4 g of $MgSO_4 \cdot 7H_2O$ in 1 liter of distilled water.

In vitro antibacterial activity. Minimal inhibitory concentration (MIC) of a drug was determined by an agar dilution method. Overnight culture in peptone water was diluted to 10^6 cells per ml with fresh peptone water. A loopful (about 0.005 ml) of diluted culture was inoculated by Micro-planter (Kubota, Japan) onto agar plates containing a series of serial twofold dilutions of a drug. MIC values were scored after overnight incubation at 37°C.

The effect of inoculum size was determined by means of a twofold serial agar dilution method on HI agar. The inocula were overnight cultures diluted in physiological saline to 10^{-1}, 10^{-3}, and 10^{-5}.

The 50% infective dose. Values for 50% infective dose were determined by the method of Kato et al. (2). A bacterial culture in peptone water was diluted to 2×10^3 to 3×10^3 cells per ml with fresh peptone water. HI agar plates containing various concentrations of a drug were prepared, and a 0.1-ml sample of diluted bacterial suspension was spread on each plate. After overnight incubation at 37°C the number of colonies that had grown on the plate were counted. The mean growth inhibition was calculated from a mean number of colonies on five plates at each drug concentration and on five drug-free agar plates.

Bactericidal activity. An overnight culture of each strain in antibiotic medium no. 3 (Difco) was diluted to a final concentration of about 10^4 cells per ml with antibiotic medium no. 3 containing a series of serial twofold dilutions of a drug. MICs were read after incubation at 37°C for 18 h. One loopful of each culture tube in the MIC test series was spotted onto drug-free HI agar plates, and after incubation at 37°C for 18 h, the minimal bactericidal concentrations of antibiotic were determined as the lower concentration of drug that prevented visible growth on HI agar plates. Another method consisted of counting the number of viable cells at appropriate time intervals after addition of drugs.

Stability to β-lactamase. The enzyme samples were prepared as follows. A 1-ml brain heart infusion culture of each strain was diluted 10-fold with medium B and incubated at 37°C. The cells were harvested by centrifugation, washed with 0.05 M phosphate buffer (pH 7.0), and resuspended in the same buffer. The

cells were disrupted in an ultrasonicator. Enzyme activity was photometrically measured (6). All the measurements were made at a substrate concentration of 0.1 mM. The figures in Table 3 are relative values, taking the absolute rate of cephaloridine hydrolysis as 100 for cephalosporinase and that of penicillin G hydrolysis as 100 for penicillinase.

Pseudomonas aeruginosa ML4259 Rms139$^+$ (7) and *Klebsiella pneumoniae* GN49 were used as the standard strains capable of producing the known types of penicillinase, and *Escherichia coli* GN5482, *P. aeruginosa* GN918 (12), *Enterobacter cloacae* GN7471, and *Proteus morganii* GN5407 were used for cephalosporinase.

PBPs. The affinity of CXD and the comparative compound, CEX, to the penicillin-binding proteins (PBPs) was examined. Seven PBPs were detected in *E. coli* JE1011 by the modified method (11) described by Spratt (8). CXD and CEX were used as competitors for ^{14}C-labeled penicillin G binding to *E. coli* PBPs. The concentrations of competitor added to the reaction mixture were 1-, 5-, and 25-fold greater than the concentration of ^{14}C-labeled penicillin G (34 μg/ml). The binding reaction was terminated, and the proteins of the inner membrane were selectively solubilized and fractionated on a sodium dodecyl sulfate-polyacrylamide slab gel. The PBPs were detected by fluorography with X-ray film (Fuji Rxs). The level of [^{14}C] penicillin G binding to 1A and 1Bs at each competitor concentration was quantitated by densitometry of the X-ray film.

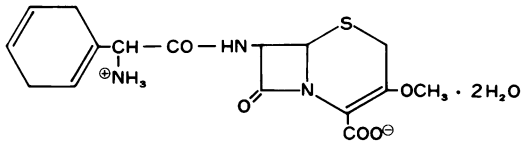

FIG. 1. *Structure of CXD (CGP-9000).*

In vivo antibacterial activity. The in vivo antibacterial activity of CXD was determined by experimental infection of mice with gram-negative bacteria. Twenty male mice (ICR strain) weighing 18 to 22 g were used for each dose level. The mice were challenged intraperitoneally with sufficient microorganisms to kill all nontreated mice within 48 h. The microorganisms were grown on an HI agar plate and suspended in physiological saline solution. Mice infected with *E. coli* ML4707 and *K. pneumoniae* GN6445 were treated orally immediately after infection and 3 h later. The total number of surviving mice was recorded, usually 1 week after infection, and the amount of a single dose (milligrams per kilogram) that gave protection to 50% of the infected mice was estimated by means of a log-probit plot (3).

RESULTS

Antibacterial spectrum. The spectrum of antibacterial activity of CXD against gram-positive and gram-negative bacteria is shown in Table 1. CXD was active against both gram-positive and gram-negative organisms susceptible to CEX. Both antibiotics exhibited almost similar effectiveness against gram-positive organisms, but CXD was more active against CEX-susceptible gram-negative organisms.

TABLE 1. *Antibacterial activity of CXD against standard strains of bacteria*

Test organism[a]	MIC (μg/ml)[b]			
	CXD		CEX	
	10^6	10^8	10^6	10^8
Staphylococcus aureus FDA209PJC-1	1.56	3.13	3.13	6.25
S. aureus E-46	3.13	6.25	6.25	12.5
S. aureus Terajima	1.56	3.13	3.13	6.25
Escherichia coli NIHJ-JC-2	3.13	6.25	12.5	12.5
Salmonella typhi 901	1.56	3.13	3.13	6.25
S. paratyphi 1015	1.56	3.13	3.13	6.25
S. schottmuelleri 8006	1.56	3.13	3.13	6.25
Klebsiella pneumoniae PCI-602	1.56	3.13	3.13	6.25
Serratia marcescens IAMI 184	>100	>100	>100	>100
Proteus vulgaris OX-19	>100	>100	>100	>100
P. rettgeri IFO3850	>100	>100	>100	>100
P. mirabilis IFO3849	6.25	12.5	50	100
Pseudomonas aeruginosa IF03445	>100	>100	>100	>100

[a] The tested strains are the standard strains stocked at the Laboratory of Bacterial Resistance, School of Medicine, Gunma University.

[b] Overnight HI broth culture was diluted with physiological saline, and one loopful (0.005 ml) of 10^6 or 10^8 cells per ml was inoculated.

Both CXD and CEX acted equally poorly against *Serratia marcescens*, *Proteus* species, and *P. aeruginosa*.

Antibacterial activity. The antibacterial activity of CXD against gram-positive and gram-negative bacteria was compared in about 50 to 300 clinical isolates of each species of bacteria including *Staphylococcus aureus*, *E. coli*, *K. pneumoniae*, and *Proteus mirabilis* (Fig. 2). The percentage of isolates of gram-negative bacteria inhibited by CXD was somewhat larger than that of CEX. In the case of *S. aureus*, the percentage of isolates inhibited by CXD was almost identical to that for CEX. The concentration of CXD required to inhibit the growth of 50% of the total number of tested *E. coli* strains (MIC_{50}) was 3.13 to 6.25 μg/ml, whereas that of CEX was 6.25 to 12.5 μg/ml. The MIC_{50} of CXD against *K. pneumoniae* strains was 3.13 to 6.25 μg/ml, and that of CEX was 6.25 to 12.5 μg/ml. Therefore, CXD was approximately twofold more active than CEX against *E. coli* and *K. pneumoniae* strains. CXD and CEX exhibited almost identical MIC_{50} values against *P. mirabilis* strains.

The determination of 50% infective dose with CXD and CEX was carried out against *E. coli* ML4707 and *K. pneumoniae* GN6445 (Fig. 3).

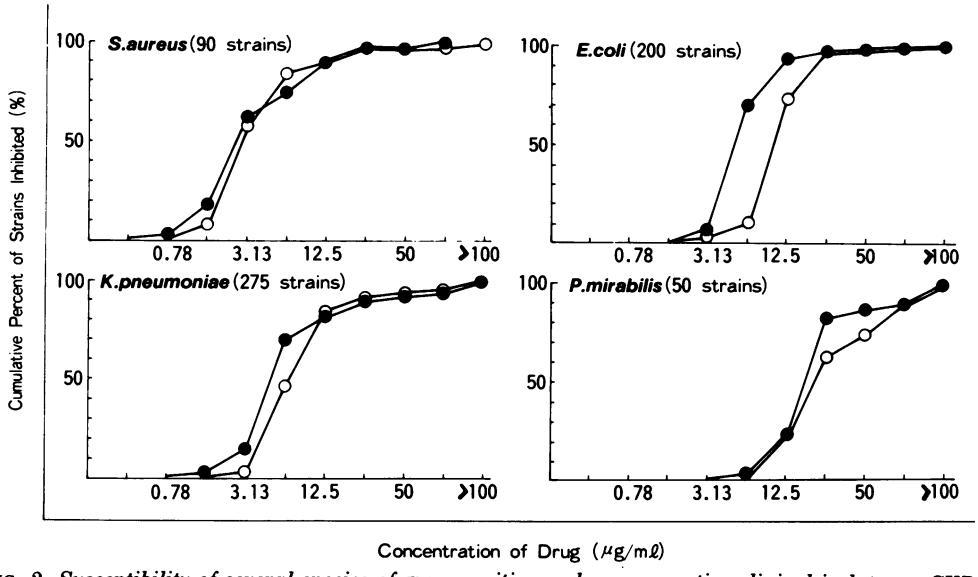

FIG. 2. *Susceptibility of several species of gram-positive and gram-negative clinical isolates to CXD (●) and CEX (○). Inoculum size, one loopful of 10^6 cells per ml.*

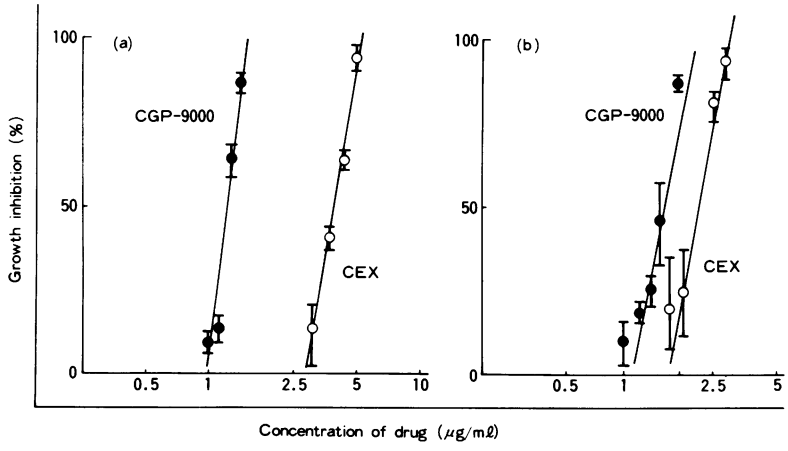

FIG. 3. *Antibacterial activity of CXD and CEX against E. coli ML4707 and K. pneumoniae GN6445. Each point indicates an arithmetic mean of five determinations. Each bar indicates a standard deviation. (a) E. coli ML4707; (b) K. pneumoniae GN6445.*

Linearity was observed over a drug concentration range that inhibited from 5 to 95% of the bacterial growth. The 50% infective doses for CXD and CEX against *E. coli* ML4707 were 1.2 and 3.9 μg/ml, respectively. Against *K. pneumoniae* GN6445, the 50% infective doses of CXD and CEX were 1.45 and 2.15 μg/ml, respectively. Therefore, CXD is more effective in vitro against *E. coli* ML4707 and *K. pneumoniae* GN6445 than is CEX.

The effect of inoculum size on antibacterial activity of CXD and CEX against clinical isolates of *E. coli* is shown in Table 2. The size of the inoculum in the test had a significant effect on MIC_{50} and MIC_{75}: the in vitro activity of CXD increased when the size of the inoculum was decreased, as compared to only a slight increase for that with CEX.

Bactericidal activity. The MICs and minimal bactericidal concentrations obtained with CXD and CEX against each of 25 clinical isolates of *E. coli* and *K. pneumoniae* are illustrated in Fig. 4. Ninety-eight percent of the *E. coli* strains were inhibited by CXD at a concentration of 6.25 μg/ml, as compared to 50% of the strains inhibited by CEX at the same antibiotic concentration. The bactericidal effectiveness of CXD was also more pronounced than that of CEX; CXD affected 80% of the strains at the same concentration, as compared to only 20% affected by CEX. In the case of *K. pneumoniae*, the percentage of isolates inhibited by CXD was twofold larger than that by CEX, and the percentage of isolates killed by CXD was also about twofold larger than that by CEX.

Bactericidal activity of CXD against *E. coli* ML4707 was examined by counting viable cells (Fig. 5). When the culture reached a density of approximately 10^4 cells per ml, from one-fourth- to fourfold the MICs of CXD and CEX were added, and incubation was continued. After 4 h of incubation, both drugs reduced the number of viable cells to 10^2 at concentrations of one- to fourfold the MIC. CXD had more bactericidal activity than CEX against *E. coli* ML4707 (Fig. 5).

Susceptibility to β-lactamases. The relative rate of hydrolysis of five cephalosporins and

TABLE 2. *Effect of inoculum size on antibacterial activity of CXD and CEX against 100 E. coli strains*

Inoculum (cells per ml)[a]	Drug concn (μg/ml)			
	MIC_{50}		MIC_{75}	
	CXD	CEX	CXD	CEX
10^8	11.5	16.5	20	23
10^6	5.4	10.5	9	15.5
10^4	4.8	9.8	6	13

[a] Overnight culture was diluted with physiological saline. One loopful of the diluted culture was inoculated.

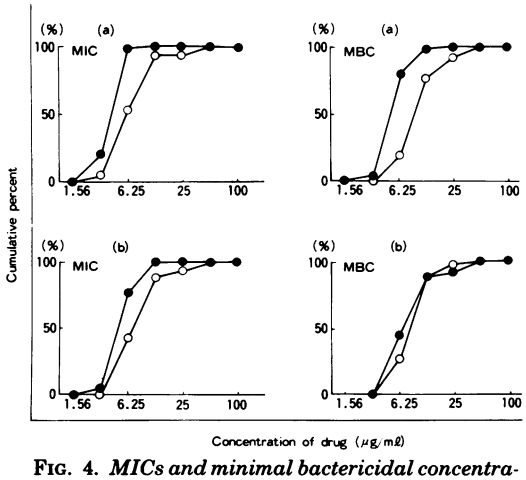

FIG. 4. *MICs and minimal bactericidal concentrations of CXD (●) and CEX (○). Twenty-five strains of E. coli and K. pneumoniae were used. (a) E. coli; (b) K. pneumoniae.*

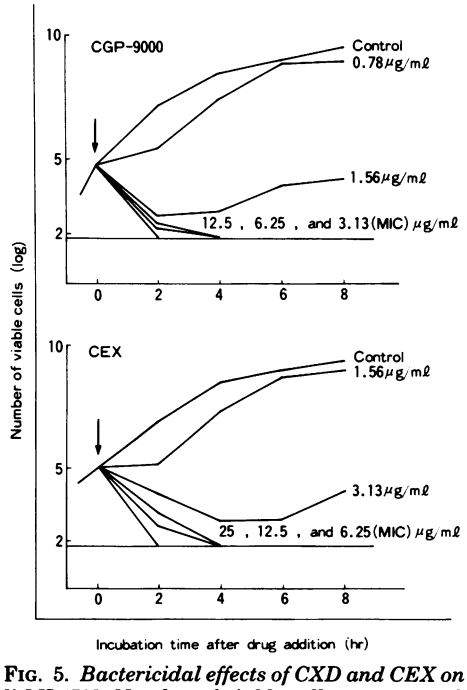

FIG. 5. *Bactericidal effects of CXD and CEX on E. coli ML4707. Number of viable cells was counted on drug-free agar plates at 2-h intervals after addition of drugs (arrow).*

TABLE 3. *Substrate profiles of various β-lactamases*

Enzyme source	Type of β-lactamase	Sp act (U/mg of protein)	Relative rate of hydrolysis[a]					
			CER	CXD	CEX	CEZ	CET	PC-G
E. coli GN5482	Cephalosporinase	0.24	100	33	41	135	691	29
P. aeruginosa GN918	Cephalosporinase	0.24	100	43	32	160	480	25
E. cloacae GN7471	Cephalosporinase	3.58	100	62	56	50	402	83
P. morganii GN5407	Cephalosporinase	5.22	100	58	31	74	242	100
P. aeruginosa ML4259 Rms139$^+$	Penicillinase IV	0.05	9	1	1	1	1	100
K. pneumoniae GN69	Penicillinase	0.11	15	1	1	3	3	100

[a] CER, cephaloridine; CEZ, cefazolin; CET, cephalothin; PC-G, penicillin G.

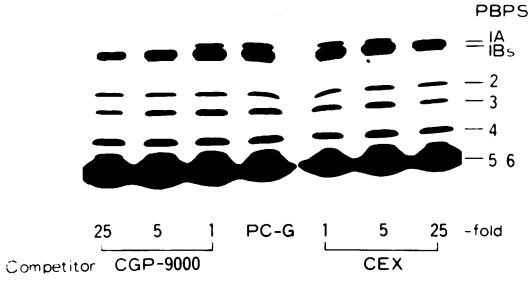

FIG. 6. *Competitive inhibition of CXD (CGP-9000) and CEX for ^{14}C-labeled penicillin G binding to E. coli PBPs. Abscissa indicates the concentration of CXD added to the reaction mixture: 1-, 5-, and 25-fold the concentration of ^{14}C-labeled penicillin G (34 μg/ml). Ordinate indicates the E. coli PBPs.*

penicillin G by R plasmid-mediated penicillinase and cephalosporinase is shown in Table 3. CXD, CEX, and other cephalosporins were stable against a type IV penicillinase produced by a *P. aeruginosa* strain carrying an R plasmid and against the penicillinase produced by *K. pneumoniae* GN69. On the other hand, CXD, CEX, and the other cephalosporins tested were susceptible to four cephalosporinases. CXD was more stable than other cephalosporins except for CEX and was slightly less stable than CEX against hydrolysis by cephalosporinases from *P. aeruginosa* GN918, *E. cloacae* GN7471, and *P. morganii* GN5407, but slightly more stable than CEX against hydrolysis by the cephalosporinase from *E. coli* GN5482.

Affinity of CXD to PBPs of *E. coli*. The affinities of CXD and the comparative compound, CEX, to PBPs were estimated by measuring the competition of unlabeled CXD and CEX with [^{14}C]penicillin G for binding to PBPs. The PBPs separated from *E. coli* by sodium dodecyl sulfate-acrylamide slab gel electrophoresis were detected by fluorography on X-ray film (11). The pattern of competition of unlabeled CXD and CEX with [^{14}C]penicillin G for binding to PBPs of *E. coli* is shown in Fig. 6,

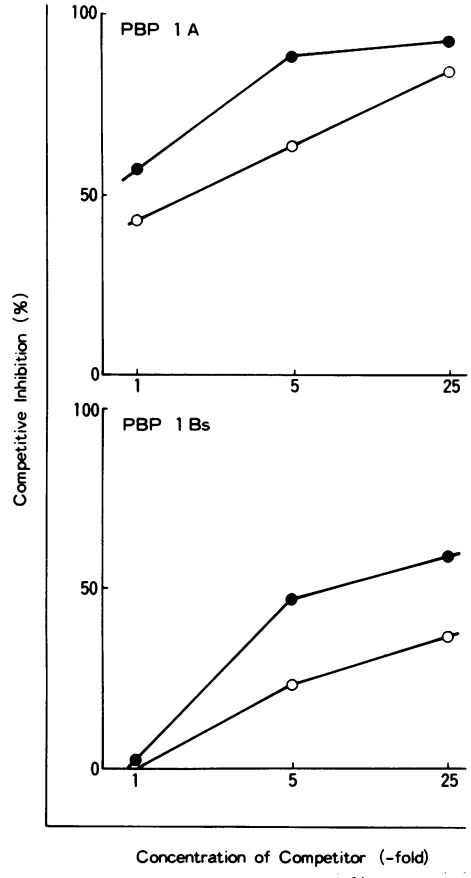

FIG. 7. *Competitive inhibition of ^{14}C-labeled penicillin G bound to PBP-1A and -1Bs at each competitor concentration. Competitor was added to the reaction mixture at 1-, 5-, and 25-fold the concentration of ^{14}C-labeled penicillin G (34 μg/ml). CXD (●); CEX (○).*

and the quantitation of the remaining radioactivity of [^{14}C]penicillin G is shown in Fig. 7. Both CXD and CEX compete for the binding to PBP-1A and PBP-1Bs. The affinity of CXD to PBP-1A and -1Bs was higher than that of CEX. There

TABLE 4. *In vivo antibacterial activity of CXD against systemic infection*[a]

Challenge organism	Challenge dose	Drug	MIC (μg/ml)	ED_{50} (95% confidence limit) (mg/kg)
E. coli ML4707	3×10^7 cells ($60 \times LD_{50}$) in saline	CXD	3.13	21.4 (16.0–29.7)[b]
		CEX	6.25	35.1 (26.1–47.5)[b]
K. pneumoniae GN6445	3×10^7 cells ($30 \times LD_{50}$) in saline	CXD	3.13	10.9 (8.2–14.2)[c]
		CEX	3.13	45.1 (27.8–125.8)[c]

[a] Drug was administered orally at 0 and 3 h after infection. Challenge was by intraperitoneal injection with a saline suspension of each organism. ED_{50}, 50% effective dose; LD_{50}, 50% lethal dose.
[b,c] $P < 0.05$.

was no difference in the affinities to PBP-2, -3, -4, and -5/6 between CXD and CEX.

In vivo antibacterial activity. Chemotherapeutic effects of CXD on experimental infections of mice with *E. coli* ML4707 and *K. pneumoniae* GN6445 are shown in Table 4. With both strains, CXD was more effective. Although CXD had MICs almost equal to those of CEX, the protective activity of CXD in experimental infection in mice was greater than that of CEX.

DISCUSSION

PBP-1Bs of *E. coli* is reported to be involved in the cross-linking reaction of cell wall peptidoglycan (11). The lack of PBP-1A alone in *E. coli* does not affect cell growth, because this protein is supposed to be a detour enzyme-carrying function compensating for the lack of PBP-1Bs (11). PBP-2 is supposed to be involved in maintaining the cell shape (8, 9), and PBP-3 is supposed to be concerned in septum formation (8). PBP-4 is identical to D-alanine carboxypeptidase IB (1, 5), and PBP-5/6 corresponds to D-alanine carboxypeptidase IA (4, 10). Therefore, in view of the affinity of CXD to PBP-1Bs, it seems likely that CXD has more intensive lytic activity than CEX.

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