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Affinity of Cefoperazone for Penicillin-Binding Proteins

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Cefoperazone (T-1551, CFP), a new semisynthetic cephalosporin, has a broad spectrum of antibacterial activity. We investigated the affinity of CFP to penicillin-binding proteins (PBPs) and the inhibition of peptidoglycan synthesis by CFP. CFP had high affinities for *Escherichia coli* PBP-3, -1Bs, -2, and -1A, in descending order, and low affinities for PBP-4, -5, and -6. Similarly, CFP showed high affinity for *Pseudomonas aeruginosa* PBP-3, -1A, -1B, -2, and -4, in descending order. It is known that *E. coli* PBP-3 and *P. aeruginosa* PBP-3 participate in cell division. These results are in good agreement with the formation of filamentous cells of *E. coli* and *P. aeruginosa* treated with CFP. CFP had lower inhibitory activities on D-alanine carboxypeptidase IA and IB of *E. coli* than that of penicillin G, but its inhibitory activities on the cross-link formation in peptidoglycan synthesis were the same as those of penicillin G and higher than those of ampicillin.

A number of anti-pseudomonal β-lactam antibiotics have been developed recently, including cefoperazone (T-1551, CFP), a new semisynthetic cephalosporin (3). We studied the mode of action of CFP from the viewpoint of affinity to penicillin-binding proteins (PBPs), inhibition of cross-linking enzymes participating in peptidoglycan synthesis, and morphological changes.

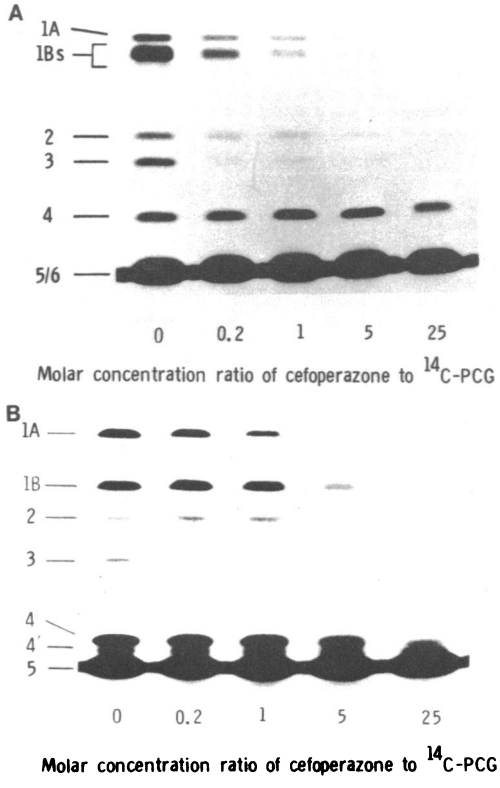


FIG. 1. *Competition of CFP for [^{14}C]penicillin G-binding to PBP. (A) E. coli JE1011; (B) P. aeruginosa NCTC 10490. The concentration of [^{14}C]penicillin G was 82.7 nmol/ml.*

MATERIALS AND METHODS

Antibiotics. CFP and piperacillin were gifts from Toyama Chemical Co., Ltd., Toyama, Japan. Penicillin G potassium salt and ampicillin were commercial products. [^{14}C]penicillin G potassium salt (50.4 mCi/mmol) was purchased from the Radiochemical Centre, Amersham, England.

Organisms. *Escherichia coli* JE1011 (6), a sub-

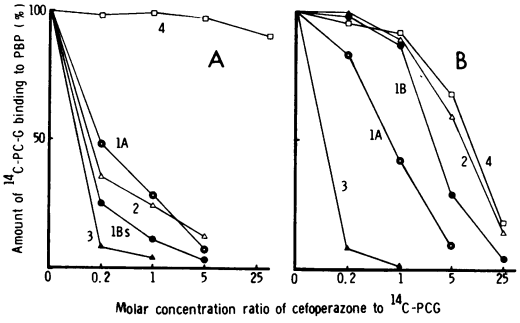


FIG. 2. *Affinity of CFP for PBP. (A) E. coli; (B) P. aeruginosa. Relative amounts of [^{14}C]penicillin G were measured by a densitometer, taking the amount of [^{14}C]penicillin G binding without CFP as 100.*

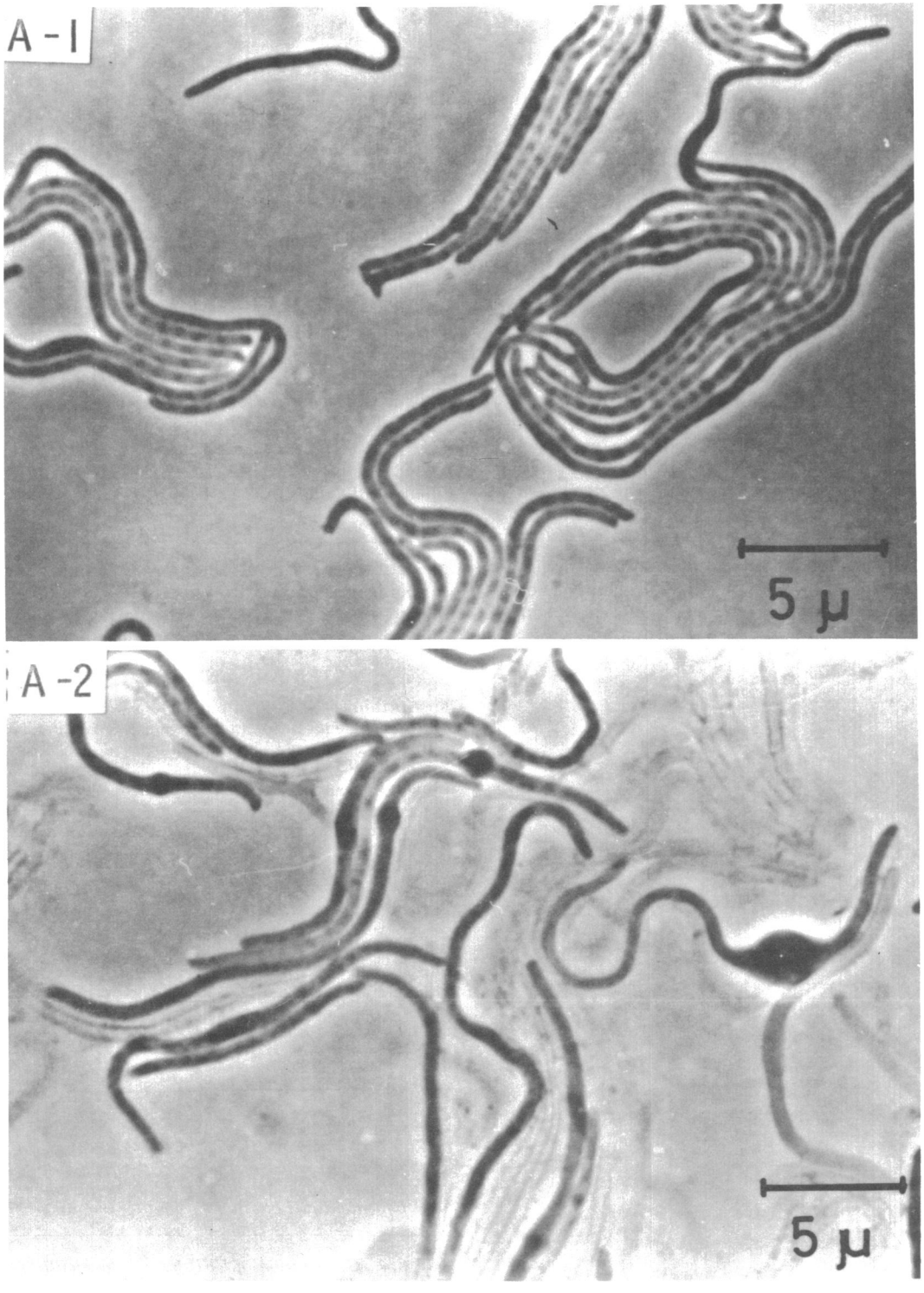
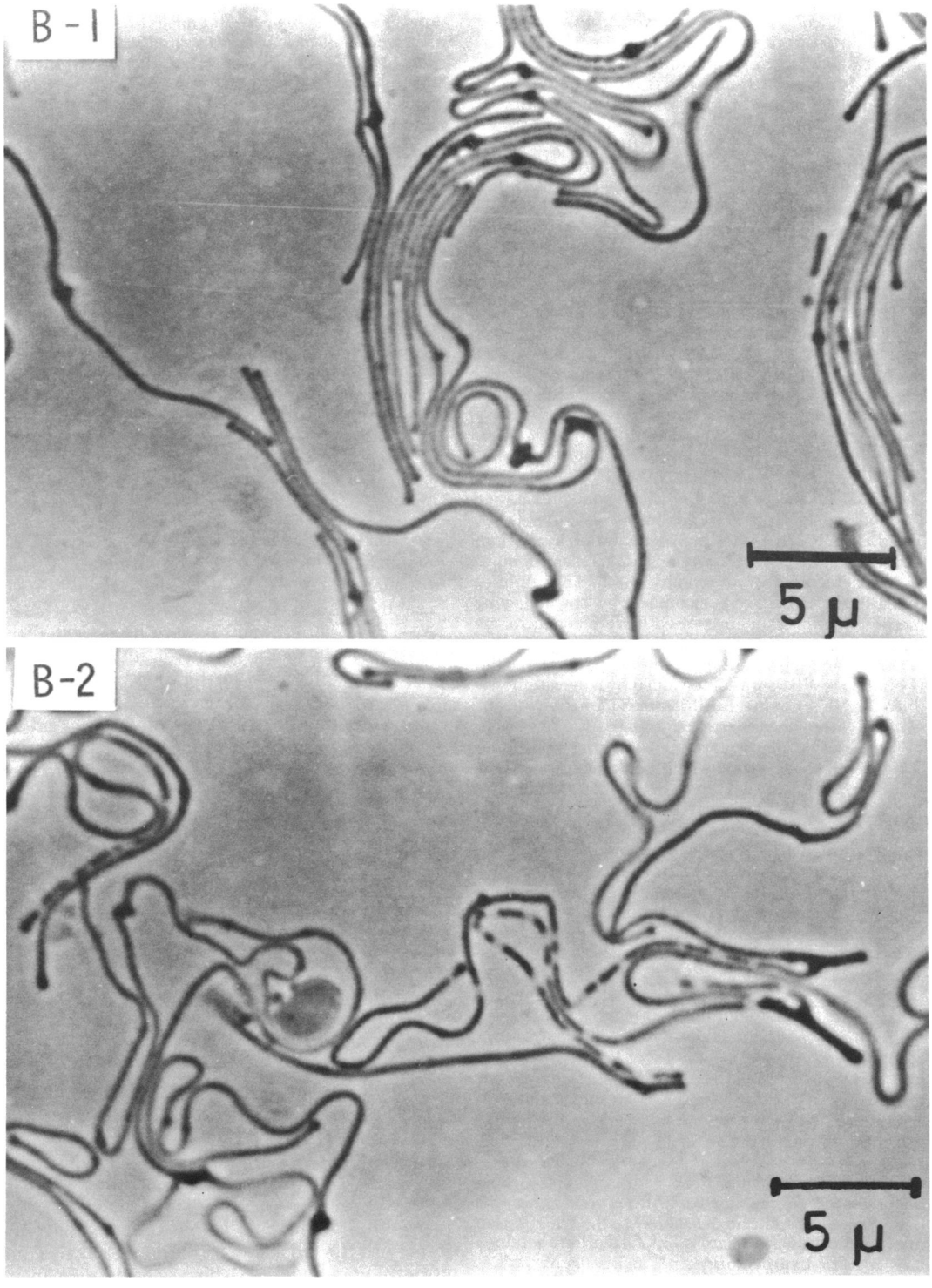


FIG. 3. *Morphological changes caused by CFP. A-1 and A-2 show the cells of E. coli JE1011 2 and 4 h, respectively, after treatment with CFP at a final concentration of 0.2 μg/ml (2MIC). B-1 and B-2 show the cells of P. aeruginosa NCTC 10490 3 and 8 h, respectively, after treatment with CFP at a final concentration of 12.5 μg/ml (2MIC).*


B-1
5 μ
B-2
5 μ

strain of *E. coli* K-12, and *Pseudomonas aeruginosa* NCTC 10490 were used in the experiment on PBPs. *E. coli* JE10012 (*dacB* mutant [5]), JE11191 (*dacA* mutant [4]), and 3 (*dacA dacB* double mutant [4]) were kindly provided by M. Matsuhashi, Institute of Applied Microbiology, University of Tokyo, and were used for enzyme assay. *E. coli* JE1011 and *P. aeruginosa* NCTC 10490 that were stock cultures of this laboratory were used in the morphological experiment.

Determination of MICs. Minimum inhibitory concentrations (MICs) were determined by the serial dilution technique. Overnight cultures of test strains in peptone broth were diluted to a final concentration of about 10^6 cells per ml, and one loopful (0.005 ml) of each culture was inoculated on heart infusion agar plates by use of an inoculator, that is, a microplanter (Sakuma, Tokyo, Japan). MICs were determined after overnight incubation at 37°C. Peptone broth for the preculture of *P. aeruginosa* strains contained 0.3% KNO_3 to yield a homogeneous culture.

Detection of PBPs. *E. coli* JE1011 and *P. aeruginosa* NCTC 10490 were cultured in Penassay broth (antibiotic medium no. 3, Difco) at 37°C. Cells were harvested at the exponential phase, and membrane fractions were prepared as previously described (7, 10, 11). ^{14}C-PBP complexes were separated by sodium dodecyl sulfate-polyacrylamide slab gel electrophoresis and detected by fluorography (7, 12). The affinities of CFP for PBPs were examined by the following method. Various amounts of CFP were added to the reaction mixture containing 84 nmol of [^{14}C]penicillin G per ml, and the extent of inhibition of [^{14}C]penicillin G binding to PBPs was determined by a densitometer.

Assay of enzyme inhibition. The activity of D-alanine carboxypeptidases IA and IB was measured by substitution of the terminal D-alanine of uridine diphosphate-*N*-acetylmuramyl pentapeptide (UDP-MurNAc-pentapeptide) to [^{14}C]glycine. Membrane fractions with activities of D-alanine carboxypeptidases IA and IB were prepared from *dacB* and *dacA* mutants which are defective in D-alanine carboxypeptidase IB and IA, respectively. The activity of the cross-linking peptidoglycan and the concomitant release of D-alanine were measured as described previously (2, 5) with membrane fractions prepared from the *dacA dacB* double mutant, which lacks both D-alanine carboxypeptidases IA and IB. The reaction mixture for measuring the activity of D-alanine carboxypeptidase IA contained 0.1 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride, (pH 9.0), 1.7 mM 2-mercaptoethanol, 0.12% Triton X-100, 3.5 mM UDP-MurNAc-L-Ala-D-Glu-*meso*-diaminopimelic acid-D-Ala-D-Ala, 30 μM [^{14}C]glycine, membrane fraction (ca. 90 μg of protein), and various amounts of β-lactam antibiotics in a final volume of 30 μl. The reaction mixture for the assay of D-alanine carboxypeptidase IB was similar to that for D-alanine carboxypeptidase IA except for 0.07 M Tris-hydrochloride, pH 8.5, and the addition of 0.03 M $MgCl_2$. The reaction mixture for the assay of cross-link formation contained 0.07 M Tris-hydrochloride, pH 8.5, 0.03 M $MgCl_2$, 0.002 mM 2-mercaptoethanol, UDP-MurNAc-pentapeptide labeled at *meso*-[^{14}C]diaminopimelic acid (22,000 cpm), 0.3 mM UDP-*N*-acetylglucosamine, the membrane fraction (ca. 100 μg), and an appropriate amount of β-lactam antibiotics, and the reaction mixture for assay of the concomitant release of D-alanine with cross-link formation was similar to that for the assay of cross-link formation except for 0.2 mM UDP-*N*-acetylglycosamine and UDP-MurNAc-pentapeptide labeled at terminal 2-D-alanine instead of the substrate labeled at *meso*-diaminopimelic acid. The reaction of D-alanine carboxypeptidase IA, cross-link formation, and the concomitant release of D-alanine were carried out at 37°C, and the reaction of D-alanine carboxypeptidase IB was performed at 30°C; they were stopped by boiling the mixture at 100°C for 1 min. The products of the reaction were separated by paper chromatography and radioactivity on the paper was counted by a liquid scintillation spectrometer.

Morphological changes in CFP-treated bacteria. *E. coli* JE1011 and *P. aeruginosa* NCTC 10490 were precultured in tryptosoy broth for 3 to 4 h, and the cultures (10^6 cells per ml) were inoculated on heart infusion agar plates containing various amounts of CFP. Then, the cell shapes of these strains after treatment with CFP were observed by a phase-contrast microscope.

RESULTS

Affinity of cefoperazone for PBPs of *E. coli* and *P. aeruginosa*. The affinities of CFP for seven *E. coli* PBPs (PBP-1a, -1Bs, -2, -3, -4, -5, and -6) and six *P. aeruginosa* PBPs (PBP-1A, -1B, -2, -3, -4, and -5) were investigated by measuring the competition of unlabeled CFP with [^{14}C]penicillin G for binding to PBPs. The patterns of competition between CFP and penicillin G for binding to PBPs of *E. coli* and *P. aeruginosa* in sodium dodecyl sulfate-polyacrylamide slab gel electrophoresis are shown in Fig. 1, and Fig. 2 shows these patterns quantitatively. CFP had extremely high affinities for PBP-3 in both *E. coli* and *P. aeruginosa*, which participate in septum formation (10), and also had high affinities for PBP-1Bs, -2, and -1A in *E. coli*, and PBP-1A in *P. aeruginosa*, respectively. This antibiotic showed low affinities for PBP-4, -5, and -6 in *E. coli* and PBP-2, -4, and -5 in *P. aeruginosa*.

Inhibition of cross-linking reaction in peptidoglycan synthesis in vitro. The inhibitory effects of CFP on peptidoglycan cross-linking and the D-alanine carboxypeptidase reaction in *E. coli* are shown in Table 1. CFP inhibited the cross-linking reaction at the same concentration as that of penicillin G, and this concentration was lower than those of piperacillin and ampicillin. But the same concentration of CFP as those of piperacillin and ampicillin was needed to inhibit the D-alanine release concomitant with the cross-link formation. CFP also did not inhibit D-alanine carboxypeptidase IA (corresponding to PBP- 5/6 in *E. coli*) (4, 7) and D-alanine carboxypeptidase IB (corresponding to PBP-4 in *E. coli*) (1, 5), which is a sensitive enzyme to penicillins, as strongly as penicillin G

TABLE 1. *Inhibition of peptidoglycan cross-linking and D-alanine carboxypeptidase reaction in vitro*[a]

Antibiotic	MIC (μg/ml)	Concn (μg/ml) of drug required for 50% inhibition			
		Cross-link formation	D-Alanine release concomitant with cross-link formation	D-Alanine carboxypeptidase 1A	D-Alanine carboxypeptidase 1B
CFP	0.2	1	10	1	1
Piperacillin	1.56	3	10	0.1	10
Ampicillin	6.25	10	10	3	0.03
Penicillin G	50	1	3	1	0.01

[a] The enzyme source was *E. coli* JE1011.

did. This is in good agreement with the low affinities of CFP for these PBPs.

Morphological changes in *E. coli* and *P. aeruginosa* treated with CFP. Cells of *E. coli* and *P. aeruginosa* treated with CFP at a concentration twofold higher than the MIC are shown in Fig. 3. These cells showed the shape of the filament and had no septa. This phenomenon is compatible with the high affinity of CFP for PBP-3 in *E. coli* and *P. aeruginosa*. The formation of bulges in the cells of these strains was observed 2 h after treatment with CFP, and the cells of *E. coli* and *P. aeruginosa* had begun to lyse 3 and 8 h after treatment with CFP, respectively.

DISCUSSION

CFP (3), a new broad-spectrum cephalosporin, altered the rod-shaped cells of *E. coli* and *P. aeruginosa* to filamentous cells and lysed these cells after further incubation with this antibiotic. CFP showed extremely high affinity for PBP-3 of *E. coli* and *P. aeruginosa* in comparison with that of penicillin G, and also had high affinities for PBP-1Bs, -2, and -1A of *E. coli* and PBP-1A of *P. aeruginosa*. From these results, it is found that CFP behaves like a typical cephalosporin in its affinity for *E. coli* PBPs. PBP-1Bs of *E. coli* are known to participate in cell elongation (9, 11), PBP-2 participates in the maintenance of rod shape (9, 11), and PBP-3 participates in septum formation (10); these proteins are supposed to correspond to PBP-1A, -2, and -3 of *P. aeruginosa*, respectively (8). CFP had somewhat lower affinities for PBPs of *P. aeruginosa* than for PBPs of *E. coli* except for PBP-3, indicating the requirement of a longer incubation time for cell lysis in *P. aeruginosa* than in *E. coli*. CFP had only low affinities for PBP-4 and -5/6 of *E. coli*, which correspond to D-alanine carboxypeptidase IB and IA, respectively, and failed to inhibit the activities of these enzymes so strongly. However, CFP inhibited the cross-link formation in peptidoglycan synthesis in *E. coli*, in which PBP-1Bs are presumed to be concerned, as same as penicillin G. It is supposed that inhibitory activity causes the high antibacterial activity against gram-negative bacteria.

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