Hiroshima J. Med. Sci.
Vol.40, No.1, 35~40, March, 1991
HIJM 40-6

Mode of Antibacterial Action of Cefprozil, a New Cephalosporin, on *Escherichia coli, Serratia marcescens* and *Morganella morganii*

Kenji NISHIMOTO[1], Tsuguru USUI[1], Yoichiro MIYAKE[2] and Hidekazu SUGINAKA[2]

1) *Department of Urology, Hiroshima University School of Medicine, Kasumi 1-2-3, Minami-ku, Hiroshima 734, Japan*
2) *Department of Microbiology and Oral Bacteriology, Hiroshima University School of Dentistry, Kasumi 1-2-3, Minami-ku, Hiroshima 734, Japan*

ABSTRACT

The mode of antibacterial action of cefprozil (CFPZ, BMY-28100), a newly developed cephalosporin, was investigated using *Escherichia coli* K12, *Serratia marcescens* IFO 12648 and *Morganella morganii* IFO 3848 as test organisms, in comparison with the action of cefaclor (CCL). The minimum inhibitory concentrations (MICs) of CFPZ for these organisms were 1.56, 800 and 25 μg/ml, whereas those of CCL were 1.56, 800 and 100 μg/ml, respectively. The addition of a subinhibitory concentration (1/4 MIC) of ethylenediaminetetraacetic acid (EDTA), which damages the permeability barrier of the outer membrane, markedly reduced the MICs of CFPZ for *E. coli* and *S. marcescens*, compared with those of CCL, whereas the MICs of both antibiotics for *M. morganii* were hardly affected by the presence of EDTA. CFPZ was more stable to β-lactamase activities from these organisms than CCL. The cross-linking reactions of peptidoglycan synthesis catalyzed by the ether-treated cells from these organisms were inhibited by a lower concentration of CFPZ than of CCL.

Key words: *Antibacterial action, Cefprozil*

Gram-negative bacilli, such as *Escherichia coli*, *Serratia marcescens* and *Morganella morganii*, are frequently isolated from patients with urinary tract infections. Since these organisms often show resistance to β-lactam antibiotics, an antibiotic with high antibacterial activity against these organisms is necessary for chemotherapy of urinary tract infections. In general, the antibacterial activity of β-lactam antibiotics to Gram-negative bacilli is influenced by the permeability of the outer membrane[1,36], hydrolysis by β-lactamase[6] and susceptibility of the target enzymes[26].

Cefprozil (CFPZ, BMY-28100) is a newly developed oral cephalosporin, which has broad antibacterial activity against Gram-negative bacteria as well as Gram-positive bacteria[7,9,13]. In the present study, we investigated the antibacterial activity of CFPZ to Gram-negative bacilli, taking into account the three factors mentioned above. Cefaclor (CCL) was used as a control throughout the experiment.

MATERIALS AND METHODS

Antibiotics. CFPZ is 7-[(R)-2-amino-2-(4-hydroxyphenyl) acetamido] -3- [(Z)-propenyl] -3-cephem-4-carboxylic acid monohydrate[13] (Fig. 1) and was provided from Bristol-Myers Research Institute (Tokyo, Japan). CCL from Shionogi Co. & Ltd. (Osaka, Japan) was used as a control antibiotic.

Organisms. The organisms used in this study were *Escherichia coli* K12, *Serratia marcescens* IFO 12648 and *Morganella morganii* IFO 3848. *Staphylococcus aureus* FDA 209P was used as a control. These organisms were cultured in Trypticase soy broth (BBL Microbiology Systems, Cockeysville, MD, USA).

Chemicals. Uridine 5'-diphosphate-N-acetyl-D-[^{14}C] glucosamine, abbreviated UDP-[^{14}C]GlcNAc was obtained from New England Nuclear (Boston, MA, USA). It had a specific activity of 290 mCi/mmol. Uridine 5'-diphosphate-N-acetylmuramyl-L-alanyl-D-glutamyl-meso-diaminopimelyl-D-alanyl-D-alanine (UDP-MurNAc-L-Ala-D-Glu-meso-Dap-D-Ala-D-Ala) was prepared from *Bacillus megaterium* KM cells as described previously[16].

Assay for MICs with or without EDTA. The minimum inhibitory concentrations (MICs) of each an-

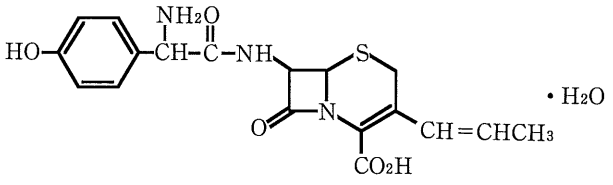

Fig. 1. Chemical structure of CFPZ

tibiotic for the organisms used were determined by a serial two-fold dilution technique in Trypticase soy broth with an inoculum of approximately 10^6 cells per ml. The MIC was defined as the lowest concentration of antibiotic that inhibited visible growth. To determine the permeability of the outer membranes, the effects of ethylenediaminetetraacetic acid (EDTA) were estimated by the same method in the presence of a subinhibitory concentration (1/4 MIC) of EDTA[29]. The MICs of EDTA for *E. coli* K12, *S. marcescens* IFO 12648, *M. morganii* IFO 3848 and *S. aureus* FDA 209P were 3.8, 7.6, 3.8 and 1.9 μmol/ml, respectively.

Induction and preparation of β-lactamase source. Enzymes were prepared as follows[19,31]: Two 500-ml flasks containing 250 ml of Trypticase soy broth, each inoculated with an overnight preculture of the test organism at a 5% concentration, were shaken at 37°C. After 1.5 h of incubation, benzylpenicillin was added as an inducer to give a final concentration of 1/4 MIC for each organism into one culture. The culture was grown for a further 1.5 h together with the control culture, which had received no addition. Bacterial cells from both cultures were harvested by centrifugation at 6,000 × *g* for 10 min at 4°C, washed twice with 10 mM sodium phosphate buffer (pH 7.0), and then resuspended in 8 ml of the same buffer. The suspended cells were disrupted with a Super Sonic Vibrator (UR 150; Tominaga Works Ltd., Tokyo, Japan) for 5 min (20 sec × 15, 5 sec interval) in the cold, and cell debris was removed by centrifugation at 6,000 × *g* for 10 min at 4°C. The supernatant was stored at −20°C until use as an enzyme source.

Assay for β-lactamase activities. β-Lactamase activities were estimated by a spectrophotometric method[25]. The enzyme reaction was carried out at 30°C in 0.1 M phosphate buffer, pH 6.8 containing 0.2 mM substrate. One unit was defined as the amount of activity capable of hydrolyzing 1 μmol of the substrate per min under this condition. Protein was estimated by a modification of Lowry's method[15] with bovine serum albumin as a standard.

Preparation of ether-treated bacterial cells[28,31,33]. Overnight precultures were inoculated into 500 ml of fresh Trypticase soy broth to a concentration of 5% and incubated at 37°C for 3 h with shaking. Bacterial cells at about half-maximal growth were harvested by centrifugation at 6,000 × *g* for 10 min at 4°C. Cells were then thoroughly washed with a basic medium (80 mM KCl, 40 mM Tris-HCl pH 7.5, 7 mM $MgCl_2$, 2 mM ethyleneglycoltetraacetic acid, 0.4 mM spermidine, 0.5 M sucrose), and resuspended in 5 ml of the same medium. Five milliliters of ether was added to the bacterial suspension and stirred gently for 1 min. The aqueous phase was removed and centrifuged at 7,000 × *g* for 8 min and the concentrated cell suspension was stored at −20°C before use as an enzyme source.

Assay for peptidoglycan biosynthesis[18,28,30,31]. The cross-linking reaction of peptidoglycan biosynthesis was assayed using the ether-treated bacterial cells (ETB cells) by the modification of the method reported by Mirelman et al[18] as described previously. The assay mixture, containing 50 μl of ETB cell suspension (approximately 8 mg of protein per ml), 20 μl of 50 μM UDP-MurNAc-L-Ala-D-Glu-meso-Dap-D-Ala-D-Ala, 5 μl of 0.86 μM UDP-[^{14}C] GlcNAc, 10 μl of 1 M tris (hydroxymethyl) aminomethane-hydrochloride buffer (pH 7.5), 10 μl of 1 M NH_4Cl, 5 μl of 1 M $MgCl_2$, 5 μl of 20 mM 2-mercaptoethanol, 75 μl of H_2O, and 20 μl of each antibiotic solution varying in concentration from 0.1 to 10,000 μg per ml, was incubated at 37°C. After incubation, the reaction mixtures were terminated by addition of 4% sodium dodecylsulfate (SDS) and boiled for 30 min. Insoluble peptidoglycan (cross-linking peptidoglycan) was recovered by filtration on the membrane filter (0.45 μm, Millipore Co., Bedford, MA, USA), and washed with 2 ml of 2% SDS and with 5 ml of distilled water. The filter was dried and then counted in a liquid scintillation counter. The amount of cross-linked peptidoglycan was measured by counting the radioactivity incorporated into the SDS-insoluble fraction.

RESULTS

MICs and effects of EDTA on the MICs. Table 1 shows the MICs of CFPZ and CCL for *E. coli* K12, *S. marcescens* IFO 12648, *M. morganii* IFO 3848 and *S. aureus* FDA 209P with the reduction in the MICs in the presence of a subinhibitory concentration (1/4 MIC) of EDTA. The MICs of CFPZ and CCL for *E. coli* were both 1.56 μg/ml, showing that it is highly sensitive to both antibiotics. The addition of a subinhibitory concentration of EDTA increased the susceptibility of this organism

Table 1. MICs and effects of ethylenediaminetetraacetic acid on the MICs of CFPZ and CCL for *E. coli* K12, *S. marcescens* IFO 12648, *M. morganii* IFO 3848 and *S. aureus* FDA 209P.

Strain	MIC (μg/ml)	
Addition	CFPZ	CCL
E. coli K12		
None	1.56	1.56
EDTA (1/4 MIC)	0.19	0.78
S. marcescens IFO 12648		
None	800	800
EDTA (1/4 MIC)	50	100
M. morganii IFO 3848		
None	25	100
EDTA (1/4 MIC)	25	50
S. aureus FDA 209P		
None	0.19	0.78
EDTA (1/4 MIC)	0.19	0.78

Table 2. Substrate specifities of β-lactamases from *E. coli* K12, *S. marcescens* IFO 12648 and *M. morganii* IFO 3848.

Strain	Enzyme activity (unit/mg of protein)			
	CFPZ		CCL	
	Induce (−)	Induce (+)	Induce (−)	Induce (+)
E. coli K12	0.0001	0.0004	0.0030	0.0045
S. marcescens IFO 12648	0.0004	0.0021	0.0086	0.0840
M. morganii IFO 3848	0.0001	0.0034	0.0059	0.1370

to CFPZ 8 times, whereas its susceptibility to CCL increased only 2 times. On the other hand, the MICs of CFPZ and CCL for *S. marcescens* were both 800 μg/ml, showing that this organism is highly resistant to both antibiotics. The reductions in the MICs of both antibiotics for this organism increased 16 and 8 times, respectively, in the presence of a subinhibitory concentration of EDTA. The MICs of CFPZ and CCL for *M. morganii* were 25 and 100 μg/ml, respectively, which indicates resistance to both antibiotics. No change in the MIC of CFPZ for this organism was observed in the presence of EDTA, whereas its susceptibility to CCL increased only 2 times. The MICs of CFPZ and CCL for *S. aureus*, a Gram-positive organism, used as a control, were not changed by the addition of EDTA (0.19 and 0.78 μg/ml, respectively).

Stability against hydrolysis by β-lactamase. Table 2 shows the activities of β-lactamase from *E. coli* K12, *S. marcescens* IFO 12648 and *M. morganii* IFO 3848 against CFPZ and CCl, compared with β-lactamase activities induced by benzylpenicillin. All organisms used produced only low level β-lactamase activities constitutively. Upon induction, however, activity against CFPZ in *S. marcescens* and *M. morganii* increased about 5 and 34 times, and also that against CCL increased about 10 and 23 times, respectively. By contrast, activity against both antibiotics in *E. coli* was only slightly increased by the induction. As shown in this table, CFPZ was from between 11 and 59 times less rapidly hydrolyzed than CCL by both induced and non-induced β-lactamases from these organisms.

Time course of peptidoglycan biosynthesis by ETB cells. When exogenous peptidoglycan precussors, UDP-MurNAc-L-Ala-D-Glu-meso-Dap-D-Ala-D-Ala and UDP-[^{14}C]GlcNAc were incubated with ETB cells prepared from *E. coli* K12, *S. marcescens* IFO 12648 and *M. morganii* IFO 3848, incorporation of radioactive GlcNAc into the SDS-insoluble fraction was observed as reported previously[5,18,20,27]. The amount of these incorporations increased proportionally with time and reached a maximum at approximately 60 min for *E. coli* and *S. marcescens*, and 90 min for *M. morganii*, followed by a gradual decrease with time in all strains (Fig. 2). These reductions were assumed to be due to hydrolysis of SDS-insoluble peptidoglycan by exogenous autolysin activity (peptidoglycan hydrolase(s)) contained in these ETB

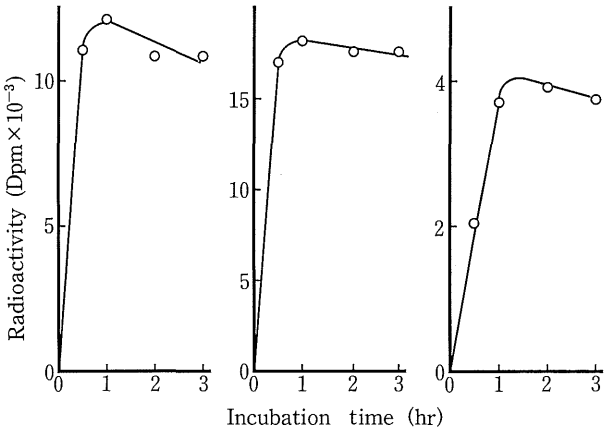

Fig. 2. Time course of cross-linking reaction of peptidoglycan biosynthesis by ETB cells of *E. coli*, *S. marcescens* and *M. morganii*. Assays were carried out as described in the text with ETB cells of *E. coli* K12 (left), *S. marcescens* IFO 12648 (center), and *M. morganii* IFO 3848 (right) at 37°C for the time shown. Data are expressed as Dpm incorporated into each SDS-insoluble fraction.

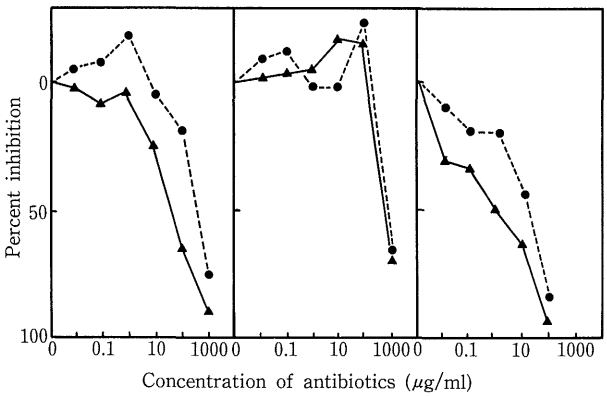

Fig. 3. Inhibition of cross-linking reaction of peptidoglycan biosynthesis in *E. coli*, *S. marcescens* and *M. morganii* by CFPZ and CCL. Assays were carried out as described in the text in the presence of the indicated concentrations of CFPZ (▲) or CCL (●) with ETB cells of *E. coli* K12 (left), *S. marcescens* IFO 12648 (center) and *M. morganii* IFO 3848 (right) at 37°C for 30, 30 and 45 min, respectively. Results are expressed as percentage inhibition of incorporation into the SDS-insoluble fraction (cross-linking peptidoglycan) by each antibiotic.

cells[11]. The incorporation of [^{14}C]GlcNAc into SDS-insoluble peptidoglycan was linear for more than 45 min for *E. coli* and *S. marcescens*, and for 60 min for *M. morganii*. Thus, the following experiments on the effects of antibiotics were carried out using a 30-min incubation period for *E. coli* and *S. marcescens*, and a 45-min incubation period for *M. morganii*.

Effects of addition of CFPZ on peptidoglycan biosynthesis. The incorporation of [^{14}C]GlcNAc into the SDS-insoluble peptidoglycan by ETB cells of *E. coli* K12 and *M. morganii* IFO 3848 was inhibited almost completely by 1,000 and 100 μg/ml of CFPZ, respectively. The concentration of CFPZ giving 50% inhibition was 70 μg/ml for *E. coli* and 2 μg/ml for *M. morganii*. By contrast, CCL was less effective than CFPZ in the inhibition of the cross-linking reaction in both organisms and the concentrations giving 50% inhibition for *E. coli* and *M. morganii* were 600 and 30 μg/ml, respectively. On the other hand, SDS-insoluble peptidoglycan synthesis in *S. marcescens* IFO 12648 was not completely inhibited by 1,000 μg/ml of both antibiotics, and the concentrations of CFPZ and CCL giving 50% inhibition were 700 and 800 μg/ml, respectively (Fig. 3).

DISCUSSION

Primary urinary tract infections are usually caused by strains of *E. coli* that are sensitive to many antibacterial agents. On the other hand, *S. marcescens* and *M. morganii* are identified as a cause of recurrent and chronic urinary tract infections. These species emerge particularly among patients with urinary tract calculi, indwelling catheter, anatomical anomalies, and after urologic surgery, and often involve multiresistant organisms. In contrast to *E. coli*, most antibacterial agents have weak activity against *S. marcescens* and *M. morganii*. A variety of new agents have been introduced recently for treatment of these urinary tract infections. CFPZ is a newly developed cephalosporin, and had strong activity against *E. coli* K12. On the other hand, it did not inhibit *S. marcescens* IFO 12648 and had poor activity against *M. morganii* IFO 3848. At present, three factors are known to affect the antibacterial activity of β-lactam antibiotics against Gram-negative organisms: 1) permeability of the outer membrane, 2) stability against hydrolysis by β-lactamase localized in the periplasm, and 3) affinity to target enzymes located on the cytoplasmic membrane (inner membrane)[12,17,26,32,34]. In the present study, we examined the influence of these three factors on the antibacterial activity of CFPZ against the above organisms.

The activity of EDTA is attributed to the chelation of the divalent metals which are required for the structural integrity of the cell envelope[2,8,14,21,22]. The addition of EDTA has been reported to reverse the resistance of Gram-negative organisms, which is brought about by the increase in permeability of the outer membrane[10,24,35]. The reduction of the MIC by the addition of EDTA indicates the existence of a permeability barrier in the outer membrane for the antibiotic[1,3,4,35,36]. In our present study, the addition of EDTA markedly reduced the MICs of CFPZ for *E. coli* and *S. marcescens*, which suggests the disturbance of permeability through the outer membrane for CFPZ, compared with that for CCL. By contrast, the reduction of the MIC of CFPZ for *M. morganii* was not recognized, which indicates a higher permeability of CFPZ than that of CCL.

β-lactamase produced from Gram-negative organisms was also considered responsible for the resistance activity against various β-lactam antibiotics[23]. However, the three organisms used had an extremely low level of β-lactamase activity constitutively. Hence, we used the disrupted cells with the induction by a subinhibitory concentration of benzylpenicillin as an enzyme source. CFPZ was reported to be stable to various β-lactamases[7]. Our present study also proved it to be very stable against hydrolysis by β-lactamase produced by the three organisms used with or without benzylpenicillin. In contrast to CFPZ, CCL was predominantly hydrolyzed at a high rate by β-lactamase produced by each organism.

Table 3. Relationships between the antibacterial activities of CFPZ and CCL for *E. coli* K12, *S. marcescens* IFO 12648, *M. morganii* IFO 3848 and three β-lactam-resistant factors.

	E. coli[a]		*S. marcescens*[b]		*M. morganii*[c]	
	CFPZ	CCL	CFPZ	CCL	CFPZ	CCL
MIC	=		=		>	
Outer membrane[d]	<		<		>	
β-Lactamase[e]	>		>		>	
Target enzymes[f]	>		>		>	

[a] *E. coli* K12
[b] *S. marcescens* IFO 12648
[c] *M. morganii* IFO 3848
[d] Permeability of the outer membrane
[e] Stability to hydrolysis by β-lactamase
[f] Sensitivity to the target enzymes

The next study revealed that the peptidoglycan biosynthesis reported by Mirelman et al[18] occurred in ETB cells prepared from these three organisms. Both antibiotics inhibited the incorporation of GlcNAc into the SDS-insoluble fraction in the three organisms used. The biosynthetic activities of *E. coli* and *M. morganii* were inhibited by moderately low concentrations of CFPZ, as the SDS-insoluble fraction was supposed to be a cross-linked peptidoglycan, and the inhibitory concentrations of CCL were relatively higher than those of CFPZ. In *S. marcescens*, the inhibitory concentration of CCL was only a little higher than that of CFPZ.

From the above results, CFPZ showed poor permeability to the outer membrane against *E. coli* and *S. marcescens*. However, the high resistance against hydrolysis by β-lactamase and the high affinity for the target enzymes conpensated for the poor permeability. Hence, CFPZ and CCL were equally active against these two organisms. The higher antibacterial activity of CFPZ against *M. morgagnii* was revealed to be brought about by the higher permeability of the outer membrane, the better stability to hydrolysis by β-lactamase and the higher sensitivity to the target enzymes. A combination of these factors can be considered for the susceptibility of *M. morganii* to CFPZ (Table 3).

(Received November 30, 1990)
(Accepted January 31, 1991)

REFERENCES

1. **Angus, B.L., Carey, A.M., Caron, D.A., Kropinski, A.M.B. and Hancock, R.E.W.** 1982. Outer membrane permeability in *Pseudomonas aeruginosa*: comparison of a wild-type with an antibiotic supersusceptible mutant. Antimicrob. Agents Chemother. **21**: 299–309.
2. **Asbell, M.A. and Eagon, R.G.** 1966. Role of multivalent cations in the organization, structure, and assembly of the cell wall of *Pseudomonas aeruginosa*. J. Bacteriol. **92**: 380–387.
3. **Barret, E. and Asscher, A.W.** 1972. Action of ethylenediaminetetraacetic acid (EDTA) on carbenicillin-resistant strains of *Pseudomonas aeruginosa*. J. Med. Microbiol. **5**: 355–359.
4. **Benz, R. and Bauer, K.** 1988. Permeation of hydrophilic molecules through the outer membrane of gram-negative bacteria. Eur. J. Biochem. **176**: 1–19.
5. **Brown, C.A. and Perkins, H.R.** 1979. In vitro synthesis of peptidoglycan by β-lactam-sensitive and -resistant strains of *Neisseria gonorrhoeae*: effects of β-lactam and other antibiotics. Antimicrob. Agents Chemother. **16**: 28–36.
6. **Bush, K.** 1989. Characterization of β-lactamases. Antimicrob. Agents Chemother. **33**: 259–263.
7. **Chin, N. and Neu, H.C.** 1987. Comparative antibacterial activity of a new oral cephalospirin, BMY-28100. Antimicrob. Agents Chemother. **31**: 480–483.
8. **Eagon, R.G. and Carson, K.J.** 1965. Lysis of cell walls and intact cells of *Pseudomonas aeruginosa* by ethylenediaminetetraacetic acid and lysozyme. Can. J. Microbiol. **11**: 193–201.
9. **Eliopoulos, G.M., Reiszner, E., Wennersten, C. and Moellering, R.C.Jr.** 1987. In vitro activity of BMY-28100, a new oral cephalosporin. Antimicrob. Agents Chemother. **31**: 653–656.
10. **Hamilton-Miller, J.M.T.** 1965. Effect of EDTA upon bacterial permeability to benzylpenicillin. Biochem. Biophys. Res. Commun. **20**: 668–691.
11. **Hartmann, R., Holtje, J.V. and Schwarz, U.** 1972. Targets of penicillin action in *Escherichia coli*. Nature (London) **235**: 426–429.
12. **Izaki, K., Matsuhashi, M. and Strominger, J.L.** 1968. Biosynthesis of the peptidoglycan of bacterial cell walls. XIII Peptidoglycan transpeptidase and D-alanine carboxypeptidase: penicillin-sensitive enzymatic reaction in strains of *Escherichia coli*. J. Biol. Chem. **243**: 3180–3192.
13. **Leitner, F., Pursiano, T.A., Buck, R.E., Tsai, Y.H., Chisholm, D.R., Misiek, M., Desiderio, J.V. and Kessler, R.** 1987. BMY 28100, a new oral cephalosporin. Antimicrob. Agents Chemother. **31**: 238–243.
14. **Leive, L.** 1974. The barrier function of the gram-negative envelope. Ann. N.Y. Acad. Sci. **235**: 109–129.
15. **Lowry, O.H., Rosenbrough, N.J., Farr, A.L. and Randall, R.J.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**: 265–275.
16. **Lugtenberg, E.J.J., Van Schijndel-Van Dam, A. and Van Bellegem, T.H.M.** 1971. In vivo and in vitro action of new antibiotics interfering with the utilization of N-acetyl-glucosamine-N-acetyl-muramyl-pentapeptide. J. Bacteriol. **108**: 20–29.
17. **Mirelman, D., Nuchamowitz, Y. and Rubinstein, E.** 1981. Insensitivity of peptidoglycan biosynthetic reaction to β-lactam antibiotics in a clinical isolate of *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **19**: 687–695.
18. **Mirelman, D., Yashouv-Gan, Y. and Schwarz, U.** 1976. Peptidoglycan biosynthesis in a thermosensitive division mutant of *Escherichia coli*. Biochemistry **15**: 1781–1790.
19. **Mizoguchi, J., Suginaka, H. and Kotani, S.** 1979. Mechanism of synergistic action of a combination of ampicillin and dicloxacillin against a β-lactamase-producing strain of *Citrobacter freundii*. Antimicrob. Agents Chemother. **16**: 439–443.
20. **Morre, B.A., Jevons, S. and Brammer, K.W.** 1979. Peptidoglycan transpeptidase inhibition in *Pseudomonas aeruginosa* and *Escherichia coli* by penicillins and cephalosporins. Antimicrob. Agents Chemother. **15**: 513–517.
21. **Nicas, T.I. and Hancock, R.E.W.** 1983. Alteration of susceptibility to EDTA, polymyxin B and gentamicin in *Pseudomonas aeruginosa* by divalent cation regulation of outer membrane protein H1. J. Gen. Microbiol. **129**: 509–517.
22. **Nikaido, H. and Nakae, T.** 1979. The outer membrane of gram-negative bacteria. Adv. Microb. Physiol. **20**: 163–250.
23. **Richmond, M.H. and Sykes, R.B.** 1973. The β-lactamases of gram-negative bacteria and their possible physiological role. Adv. Microbiol. Physiol. **9**:

31–88.

24. **Rogers, S.W., Gilleland, H.E.Jr. and Eagon, R.G.** 1969. Characterization of a protein-lipopolysaccharide complex released from cell walls of *Pseudomonas aeruginosa* by ethylenediaminetetraacetic acid. Can. J. Microbiol. **15**: 743–748.
25. **Ross, G.W., Chanter, K.V., Harris, A.M., Kibby, S.M., Marshll, M.J. and O'callaphan, C.H.** 1973. Comparison of assay techniques for β-lactamase activity. Anal. Biochem. **54**: 9–16.
26. **Strominger, J.L., Blumberg, P.M., Suginaka, H., Umbreit, J. and Wickus, G.G.** 1971. How penicillin kills bacteria: Progress and problems. Proc. R. Soc. Lond. B. **179**: 369–383.
27. **Suginaka, H., Ichikawa, A. and Kotani, S.** 1974. Penicillin-resistant mechanisms in *Pseudomonas aeruginosa*: Effects of penicillin G and carbenicillin on transpeptidase and D-alanine carboxypeptidase activities. Antimicrob. Agents Chemother. **6**: 672–675.
28. **Suginaka, H., Kotani, S., Takata, N. and Ogawa, M.** 1980. Effect of cefotaxime (HR-756) on biosynthesis of cell wall peptidoglycan in *Pseudomonas aeruginosa* KM 338 and *Escherichia coli* K 12. FEMS Microbiol. Lett. 8: 79–82.
29. **Suginaka, H., Shimatani, M., Kotani, S., Ogawa, M., Hama, M. and Kosaki, G.** 1979. Antibacterial mechanisms of cefsulodin against *Pseudomonas aeruginosa* and *Escherichia coli*. FEMS Microbiol. Lett. **5**: 177–179.
30. **Takata, N., Ogawa, M., Kosaki, G. and Suginaka, H.** 1981. Effect of 6059-S, a novel oxacephem, on cross-linking reaction of peptidoglycan biosynthesis in *Escherichia coli*, *Pseudomonas aeruginosa* and *Serratia marcescens*. Arch. Microbiol. **130**: 90–91.
31. **Takata, N., Suginaka, H., Kotani, S., Ogawa, M. and Kosaki, G.** 1981. β-lactam resistance in *Serratia marcescens*: Comparison of action of benzylpenicillin, apalcillin, cefazolin, and ceftizoxime. Antimicrob. Agents Chemother. **19**: 397–401.
32. **Tomasz, A.** 1979. The mechanism of the irreversible antimicrobial effects of penicillins: How the beta-lactam antibiotics kill and lyse bacteria. Ann. Rev. Microbiol. **33**: 113–137.
33. **Vosrberg, H.P. and Hoffman-Berling, H.** 1971. DNA synthesis in nucleotide permeable *Escherichia coli*. J. Mol. Biol. **58**: 739–753.
34. **Waxman, D.J. and Strominger, J.L.** 1983. Penicillin-binding-proteins and the mechanism of action of β-lactam antibiotics. Ann. Rev. Biochem. **52**: 825–869.
35. **Weiser, R., Asscher, A.W. and Wimpenny, J.** 1968. In vitro reversal of antibiotic resistance by ethylenediamine tetraacetic acid. Nature (London) **219**: 1365–1366.
36. **Yoshimura, F. and Nikaidou, H.** 1982. Permeability of *Pseudomonas aeruginosa* outer membrane to hydrophilic solutes. J. Bacteriol. **152**: 636–642.