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Penicillin Resistance of *Pseudomonas aeruginosa* Clinical Isolates from Urinary Tract Infections

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ABSTRACT

One hundred and thirteen clinical isolates of *Pseudomonas aeruginosa* from urinary tract infections were tested for their mechanisms of resistance to penicillins. Ninety-eight percent and 100.0% of the strains were resistant (MIC$\geqq$100 μg/ml) to ampicillin and penicillin G, respectively, while only 5.3% were resistant to piperacillin. Low permeability of the outer membrane, and penicillinase production were involved in their resistance mechanisms. Peptidoglycan synthesis in ether-treated cells of two representative strains was inhibited by ampicillin and piperacillin at the concentrations markedly lower than that for penicillin G.

It is well known that antimicrobial chemotherapy is essential for treatment of infectious diseases. Three major problems exist in chemotherapy, e.g. side effects, microbial selection and substitution, and antibiotic resistance. Bacteria with low susceptibility to antibiotics such as *Pseudomonas aeruginosa* and *Serratia marcescens* can survive during chemotherapy and cause opportunistic infections. Although *P. aeruginosa* is susceptible to piperacillin, it is highly resistant to many antibiotics including penicillin G and ampicillin. The mechanism of resistance of *P. aeruginosa* to penicillins has been reported to be due to a barrier effect of the outer membrane, production of penicillinase and low susceptibility of target enzymes to penicillins[1,2,15-17].

P. aeruginosa is often recovered from urinary tract infections[4,6], and it is of importance to study the antibiotic resistance mechanisms of this organism in order to ensure successful chemotherapy of urinary tract infections in the future. In the present study, we investigated the mechanisms of penicillin resistance of *P. aeruginosa* clinical isolates from patients with urinary tract infections.

MATERIALS AND METHODS

Microorganisms

One hundred and thirteen strains of *Pseudomonas aeruginosa* were isolated from patients with urinary tract infections. Bacteria isolated from patients were identified by the Abbott Quantum II-BID System® (Abbott Laboratories, Irving, TX, USA) and Oxi/ferm Tube II® (Roche Japan, Tokyo, Japan) at the Department of Bacteriology, Hiroshima University Hospital.

Antibiotics

The antibiotics used in this study were penicillin G, ampicillin and piperacillin. All antibiotics were purchased commercially.

Minimum inhibitory concentration

Minimum inhibitory concentrations (MICs) of antibiotics were determined by a liquid dilution method. The antibiotics were dissolved in Trypticase soy broth (BBL Microbiology Systems,

Cockeysvill, MD, USA), and diluted two-fold serially with the same broth to give final concentrations varying from 0.2 to 51,200 μg/ml. To serial dilutions of antibiotic, *P. aeruginosa* preculture was inoculated at 10^6 cells/ml, and incubated at 37°C for 24 hr. The MIC was defined as the lowest concentration of antibiotic at which no bacterial growth occurred. MIC_{50} and MIC_{90} represent a drug concentration required to inhibit the growth of 50% and 90% of strains, respectively.

To determine the permeability of the outer membranes of bacteria, MICs in the absence and presence of half MIC of ethylenediaminetetraacetic acid (EDTA) were measured[18]. The permeability of each strain's membrane was expressed as the degree of reduction of MIC caused by EDTA presence (2-fold, 4-fold reduction etc).

Penicillinase assay

Penicillinase activity was determined by a modification of an iodometric technique of Perret[14] as previously described[11]. Enzymes were prepared as follows: Overnight precultures of *P. aeruginosa* were inoculated into fresh broth at a 10% concentration, and incubated in the presence of 1/4 MIC of penicillin G at 37°C for 4 hr[20]. Cells were harvested by centrifugation at 4,500 × *g* at 4°C for 15 min, and washed once with 0.1 M phosphate buffer pH 7.0. Cells were resuspended in the same buffer and disrupted by ultrasound, then centrifuged at 9,000 × *g* at 4°C for 30 min to precipitate the cell debris. The supernatant was frozen at −20°C until used as an enzyme preparation.

Ampicillin to be used as a substrate was dissolved with 0.05 M phosphate buffer pH 7.0 at a concentration of 2 mM. In a small test tube, 0.1 ml of substrate and 0.8 ml of phosphate buffer were preincubated at 30°C for 5 min. To this tube, 0.1 ml of enzyme preparation was added and incubated at 30°C for 5 min. The reaction was terminated by adding 0.5 ml of 0.15 M sodium tungstate. Iodine-starch solution (1.5 ml) was then added, the mixture was stood at room temperature for 20 min, and the absorbance at 595 nm was measured. To make a blank, sodium tungstate was added before the enzyme preparation. Protein concentration was estimated by a modification of Lowry's method with bovine serum albumin as a standard[8]. Penicillinase activity was expressed as units/ml calculated from the following equation.

$$\text{units/ml} = \frac{\Delta\,\text{OD} \times 0.085}{\text{F}} \times 1/\text{T} \times 1/\text{V}$$

where

Δ OD = OD blank - OD sample

F = iodine consumption factor of degraded substrate

T = incubation time (min)

V = volume of enzyme preparation (ml)

Peptidoglycan synthesis

Peptidoglycan synthesis was assayed using ether-treated cells (ETB cells)[23]. Overnight *P. aeruginosa* precultures were inoculated into 500 ml of fresh trypticase soy broth to a concentration of 5% and incubated at 37°C for 3 hr with shaking. Cells were harvested by centrifugation at 6000 × *g* for 10 min at 4°C. Cells were then washed with basic medium (80 mM KCl, 40 mM Tris-HCl pH 7.5, 7 mM $MgCl_2$, 2 mM ethylene glycol tetraacetic acid, 0.4 mM spermidine, 0.5 M sucrose), and resuspended in 5 ml of the same medium. Five milliliters of ether were added to the bacterial suspension and stirred gently for 1 min. The aqueous phase was removed and centrifuged at 7000 × *g* for 8 min and the concentrated suspension was stored at −20°C before use.

The cross-linking reaction of peptidoglycan biosynthesis was assayed by a modified method[19] of Mirelman et al[9]. In a small test tube, 5 μl UDP[^{14}C]*N*-acetyl glucosamine (New England Nuclear Corp., Boston MA, USA), 20 μl 0.5 mM UDP-*N*-acethyl muramic acid pentapeptide, 20 μl antibiotic, 10 μl 1 M Tris-HCl pH 7.5, 10 μl 1 M NH_4Cl, 5 μl 1 M $MgCl_2$, 5 μl 20 mM 2-mercaptoethanol, 75 μl distilled water, and 50 μl ETB cells (8 mg protein/ml) were mixed and incubated at 37°C for 45 min. The reaction was terminated by the addition of 1 ml 4% sodium dodecyl sulfate (SDS) and boiled for 30 min. The mixture was filtered through a membrane filter (0.45 μm, Millipore Corp., Bedford, MA, USA), and washed with 2 ml of 2% SDS followed by 5 ml of distilled water. The filter was dried, and radioactivity incorporated was measured by liquid scintillation counter.

Statistical analysis

The distributions of MIC, penicillinase activi-

ty and degree of reduction of the MIC by EDTA were not normal. Hence, Kendall's rank correlation coefficient, a non-parametric statistical analysis, was employed.

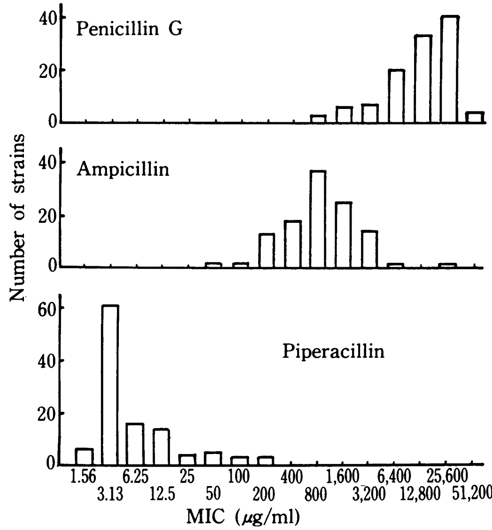

Fig. 1. Distributions of the MICs of penicillin G, ampicillin and piperacillin against clinical isolates of *P. aeruginosa*.

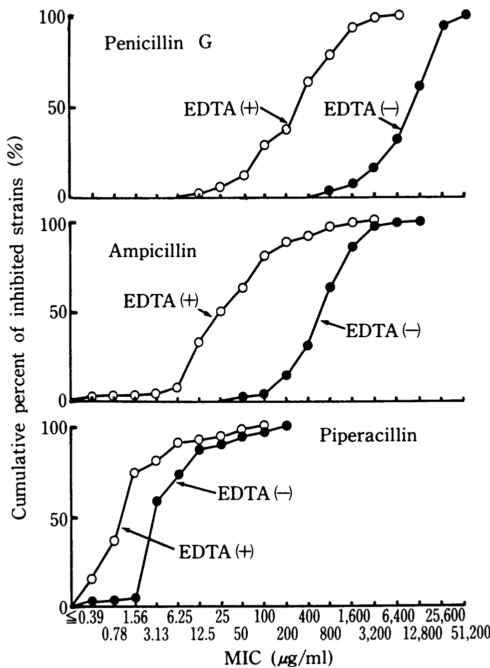

Fig. 2. Cumulative distributions of the MICs of penicillin G, ampicillin and piperacillin in the presence (open circles) and absence (closed circles) of EDTA against clinical isolates of *P. aeruginosa*.

Table 1. MIC50 and MIC90 of penicillin G, ampicillin and piperacillin against *P. aeruginosa* clinical isolates in the presence and absence of EDTA

	MIC50(μg/ml)[a]		MIC90(μg/ml)[b]	
	−EDTA	+EDTA	−EDTA	+EDTA
Penicillin G	12,800	400	25,600	1,600
Ampicillin	800	25	3,200	400
Piperacillin	3.13	1.56	25	6.25

[a]concentration required to inhibit the growth of 50% of bacterial strains.
[b]concentration required to inhibit the growth of 90% of bacterial strains.

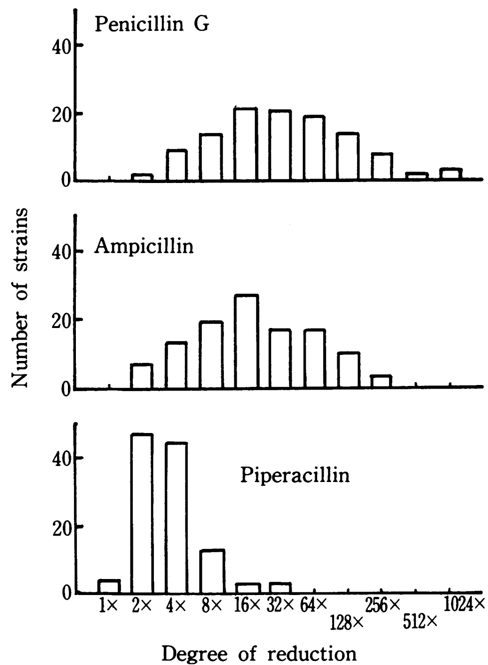

Fig. 3. Distributions of the reduction of MIC by the addition of EDTA

RESULTS

Fig. 1 shows distributions of MICs of penicillin G, ampicillin and piperacillin against *P. aeruginosa* clinical isolates. The mode of MICs for penicillin G was 25,600 μg/ml (range; 800-51,200 μg/ml) and 800 μg/ml (range; 50-25,600 μg/ml) for ampicillin, whereas that of piperacillin was 3.13 μg/ml (range; 1.56-200 μg/ml). The percentage of resistant strains (MIC ≧ 100 μg/ml) were 100.0, 98.2 and 5.3% for penicillin G, ampicillin and piperacillin, respectively. The cumulative distribution of MICs of these three drugs is shown in Fig. 2 (closed circles).

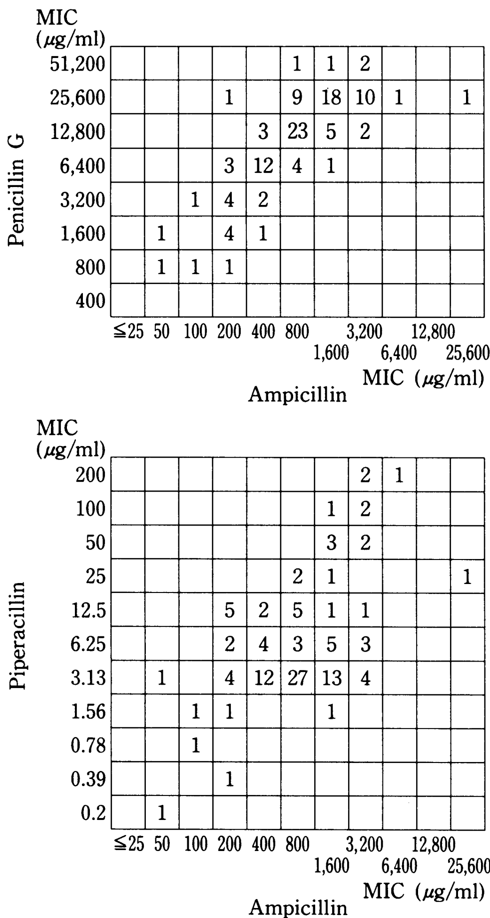

Fig. 4. Correlations of MICs of ampicillin with those of penicillin G and piperacillin against clinical isolates of *P. aeruginosa*. Relationships between the MICs of ampicillin and penicillin G ($\tau=0.71$, $p \leqq 0.01$) and those between ampicillin and piperacillin ($\tau=0.42$, $p \leqq 0.01$) were significant. Numbers in the squares represent numbers of strains.

MIC_{50}'s and MIC_{90}'s of these drugs are listed in Table 1.

Fig. 2 also shows the cumulative distributions of the MICs of three drugs in the presence of EDTA (open circles). The addition of EDTA considerably reduced the MICs of ampicillin and penicillin G, whereas limited reduction of the MICs of piperacillin was observed. Comparison of MIC_{50} and MIC_{90} in the presence and absence of EDTA is shown in Table 1. The modes of reductions of the MICs by the addition of EDTA are 16× (range; 2-256×) and 32× (range; 2-1024×) for ampicillin and penicillin G, respectively, and between 2 × and 4 × (range; 1-32 ×) for piperacillin (Fig. 3).

The relationships between the MIC of ampicillin, penicillin G and piperacillin are shown in Fig. 4. The MICs of ampicillin correlated highly with those of penicillin G (correlation coefficient $\tau=0.71$, $p \leqq 0.01$) and piperacillin ($\tau=0.42$, $p \leqq 0.01$). From these results, further experiments were performed with ampicillin.

The MICs for ampicillin were plotted against cell strain penicillinase activities in Fig. 5. A linear relationship was observed between MICs and penicillinase activities ($\tau=0.41$, $p \leqq 0.01$).

Ampicillin MIC's were then plotted against the degree of EDTA-induced MIC reduction (Fig. 6). There was a high degree of correlation, indicating that the MIC is determined by the outer membrane permeability ($\tau=0.12$, $0.01 \leqq p \leqq 0.05$).

Table 2 lists the MICs in the presence and absence of EDTA, permeability of the outer membrane and penicillinase activities of three representative ampicillin- and penicillin G-resistant strains (18D, 26C, 46C). Strain 18D demonstrated high permeability of the outer membrane and high penicillinase activity. In contrast, strains 26C and 46C produced little penicillinase and their outer membrane permeabilities were lower than that of 18D. Of these two strains, 26C demonstrated higher MIC and lower permeability of the outer membrane to penicillin G and ampicillin than did 46C.

Finally, susceptibility of the cross-linking enzyme, responsible for peptidoglycan biosynthesis, to antibiotics was examined. Fig. 7 shows the time course of the cross-linking reaction of peptidoglycan biosynthesis by ETB cells of strains 26C and 46C. When exogenous peptidoglycan precursors, UDP-MurNAc-pentapeptide and UDP-[^{14}C]GlcNAc were incubated with ETB cells prepared from both strains, incorporation of radioactive GlcNAc into the SDS-insoluble fraction (cross-linked peptidoglycan) was similar to that reported for a laboratory strain of *P. aeruginosa*[19]. The rate of incorporation was time dependent and reached a maximum after approximately 2 hours incubation, followed by a gradual decrease with time (Fig. 7). This reduction was assumed to be due to hydrolysis of SDS-insoluble peptidoglycan by endogenous autolysin activity (peptidoglycan

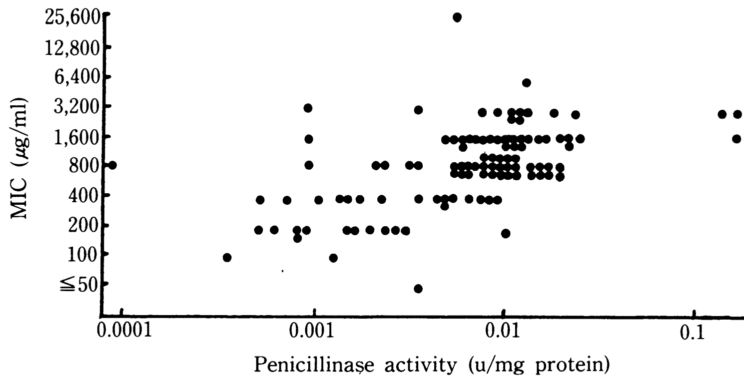

Fig. 5. Correlation between the MICs of ampicillin, and penicillinase activity of clinical isolates of *P. aeruginosa*. The MIC was highly correlated to penicillinase activity ($\tau=0.41$, $p\leqq0.01$).

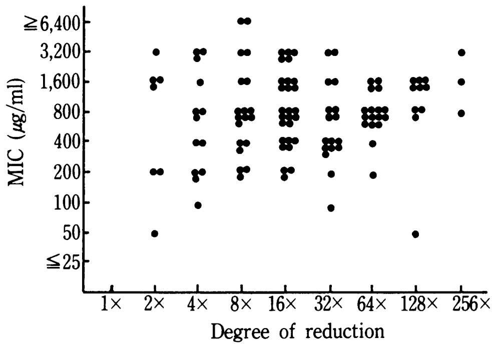

Fig. 6. Correlation between the MICs of ampicillin against *P. aeruginosa* and degree of reduction of MIC by the addition of EDTA. Correlation between them was significant ($\tau=0.12$, $0.01\leqq p\leqq0.05$).

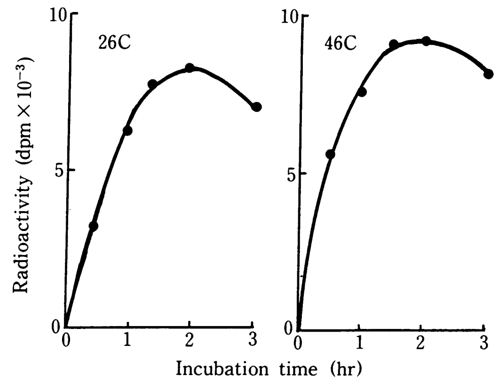

Fig. 7. Time course of cross-linking reaction of peptidoglycan biosynthesis by ETB of *P. aeruginosa* strains 26C and 46C.

Table 2. MIC, penicillinase activity and degree of MIC reduction by the addition of EDTA of three representative strains

Strain	Addition	MIC (μg/ml)			Penicillinase activity (u/mg protein)
		PCG	ABPC	PIPC	
18D	None	12,800	3,200	200	0.1916
	EDTA(1/2 MIC)	3,200	1,600	100	
		(4x)[a]	(2x)	(2x)	
26C	None	12,800	800	3.13	0.0006
	EDTA(1/2 MIC)	100	12.5	0.78	
		(128x)	(64x)	(4x)	
46C	None	800	100	0.78	0.0005
	EDTA(1/2 MIC)	25	3.13	0.39	
		(32x)	(32x)	(2x)	

[a]the number in the parenthesis is the degree of reduction of MIC by the addition of EDTA
PCG, penicillin G; ABPC, ampicillin; PIPC, piperacillin
2x, 4x etc = 2-fold, 4-fold etc

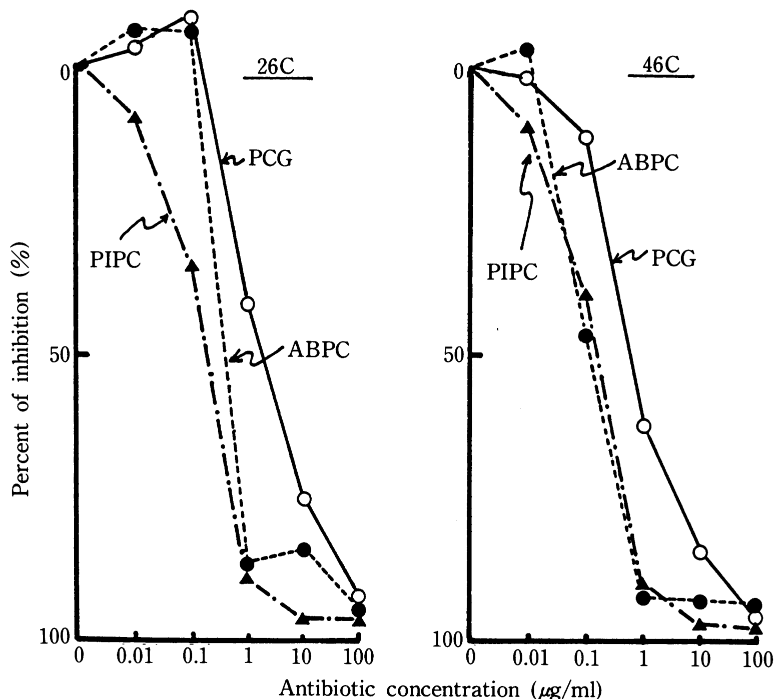

Fig. 8. Inhibition of the cross-linking reaction of peptidoglycan biosynthesis in *P. aeruginosa* strains 26C and 46C by penicillin G, ampicillin and piperacillin. PCG, penicillin G; ABPC, ampicillin; PIPC, piperacillin.

hydrolase(s))[5], contained in both strins of ETB cells. From the kinetic data of the cross-linking reaction, it was clear that an incubation time of 30 min gave a linear rate of ^{14}C incorporation in both strains; thus this incubation time was employed in subsequent experiments. High penicillinase activity of strain 18D hampered the biosynthesis assay for this strain. When penicillins (penicillin G, ampicillin, piperacillin) were added to the assay system, all of these drugs suppressed the cross-linking of peptidoglycan biosynthesis, dose dependently (Fig. 8). The inhibition of the enzyme in strains 26C and 46C by penicillin G was weaker than the inhibition exerted by ampicillin and piperacillin. In strains 26C and 46C there was no apparent difference in the dose dependence of antibiotic-induced inhibition of peptidoglycan cross-linking.

DISCUSSION

P. aeruginosa is frequently isolated from patients with urinary tract infections, and most of which are resistant to many antibiotics[4,6]. The resistance of this organism to β-lactams, the most common antibiotic, has been studied by several investigators. Resistance is mostly achieved by either impermeability of the outer membrane and/or by β-lactamase production[1]. To determine which mechanism participates in penicillin resistance, we processed the data obtained in this study by non-parametric statistical analysis.

The outer membrane of Gram-negative bacteria is a barrier which selectively admits substances, including antibiotics, into the cell[24]. Thus the permeability of the outer membrane must affect the susceptibility of microoganisms to antibiotics. EDTA changes the structure of the outer membrane by chelating divalent cations, resulting in an increase in permeability of Gram-negative bacteria to a variety of unrelated molecules[7,12,13]. Hence the reduction of the MIC in the presence of EDTA demonstrates the role of the outer membrane as a permeability barrier. The ability of antibiotics to pass through the outer membrane is also limited by this phenomenon[10]. In general, reduction of the MIC to a low level by EDTA, was caused by an increase in permeability of the outer membrane. Godfrey and Bryan[3] reported that the permeability of the outer membrane correlated well with the MIC of piperacillin, which is resistant

to penicillinase. However, no correlation was observed for piperacillin in the present study (data not shown). The MICs of ampicillin were highly correlated with the degree of MIC reduction. This finding indicates that the impermeability of the outer membrane is the main factor influencing ampicillin resistance. This result agreed well with results obtained from experiments with a standard strain[16].

Hydrolysis of β-lactams is the second resistance mechanism. In our experiments, a higher penicillinase activity resulted in a higher ampicillin MIC. This indicates that hydrolysis of penicillin is also involved in the resistance mechanism. Some strains produced very high penicillinase activities, which may be episomally determined. Thus, this ability can be transmitted to other bacteria.

Although relationships were obtained by statistical analysis of the MIC and size of MIC reduction by EDTA or penicillinase activity, penicillin resistance may be the result of a combination of the outer membrane barrier, penicillinase production and other factor(s).

In the strains with high penicillinase activity, it is very difficult to assess the susceptibility of the cross-linking enzyme to penicillins. Hence, we chose two strains with only trace penicillinase activities, and with a high and a low MIC for the cross-linking reaction assay. Although the MIC of ampicillin to strain 26C was higher than that to strain 46C, the susceptibilities of the cross-linking enzyme to ampicillin in both strains were almost the same. This finding with strains that produce very weak penicillinase activities, suggests that penicillin resistance is due to the permeability barrier of the outer membrane rather than to the susceptibility of the target enzyme.

Needless to say, we can not exclude the possibility that factor(s) other than the three we have noted here participate in the resistance of *P. aeruginosa* to penicillins.

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