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Antibacterial Activity of Apalcillin (PC-904) Against Gram-Negative Bacilli, Especially Ampicillin-, Carbenicillin-, and Gentamicin-Resistant Clinical Isolates

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Apalcillin (PC-904) is active against carbenicillin- and ampicillin-resistant strains of gram-negative bacilli. Among *Pseudomonas aeruginosa* strains highly resistant to carbenicillin (≥3,200 μg/ml), half of them were susceptible to PC-904 at a concentration of 50 to 1,600 μg/ml. The minimal inhibitory concentration of PC-904 against *P. aeruginosa* strains resistant to carbenicillin (400 to 1,600 μg/ml) ranged from 3.1 to 25 μg/ml. Ampicillin- and carbenicillin-resistant *Enterobacteriaceae* strains were similarly susceptible to PC-904. However, drug resistance to PC-904 was already apparent among some strains of *P. aeruginosa, Escherichia coli, Klebsiella pneumoniae, Proteus mirabilis, P. vulgaris,* and *P. morganii,* recently isolated in Japan; i.e., 4, 35, 32, 4, 6, and 14% of strains isolated were resistant. PC-904 was more active, on the other hand, than ampicillin and carbenicillin against antibiotic-susceptible *Enterobacteriaceae* and also showed high activity against most species of *Pseudomonadaceae,* especially *P. cepacia* and *P. aeruginosa.* The minimum inhibitory concentrations of PC-904 were greatly affected by inoculum size when the organisms tested were strains producing large amounts of beta-lactamase.

Sodium 6-[D(-)-α-(4-hydroxy-1,5-naphthyridine-3-carboxamido)phenylactamido]-penicillanate or apalcillin (PC-904), a new semisynthetic penicillin, has been shown to have a broad antibacterial spectrum and a potent activity against *Pseudomonas aeruginosa* (9). Recently, ampicillin-, carbenicillin-, and gentamicin-resistant strains of *P. aeruginosa* and *Enterobacteriaceae* have been isolated with increased frequency from clinical materials (1, 4, 11). Furthermore, members of the genus *Pseudomonas* other than *P. aeruginosa,* e.g., *P. cepacia,* and *P. maltophilia,* have appeared as notorious pathogens, because of their insusceptibility to most beta-lactam and aminoglycoside antibiotics (2, 8). This paper is concerned with in vitro activity of PC-904 against many clinical isolates of gram-negative bacilli including *Pseudomonas* species other than *P. aeruginosa,* and against ampicillin-, carbenicillin-, and gentamicin-resistant strains.

MATERIALS AND METHODS

Antibiotics. PC-904, sodium 6-[D(-)-α-(4-hydroxy-1,5-naphthyridine-3-carboxamido)phenylacetamido]-penicillanate, was the product of Sumitomo Chemical Co. Ltd., Osaka, Japan. Ampicillin (ABPC), cefoxitin, gentamicin (GM), and 3',4'-dideoxykanamycin B (DKB) were used. Carbenicillin (CBPC), sulbenicillin, and cefazolin were commercially available materials.

Strains. We used 1,149 strains of gram-negative bacilli obtained from stock cultures in the Reference Laboratory of Drug-Resistant Bacteria, School of Medicine, Gunma University. They were all isolates from clinical materials and consisted of 200 *E. coli* strains, 200 *Klebsiella pneumoniae* strains, 100 *Proteus mirabilis* strains, 50 *P. vulgaris* strains, 50 *P. morganii* strains, 25 *P. rettgeri* strains, 137 *Enterobacter cloacae* strains, 100 *Serratia marcescens* strains, 200 *P. aeruginosa* strains, 53 *P. maltophilia* strains, and 34 *P. cepacia* strains. In addition, 43 CBPC-resistant strains and 56 GM-resistant strains were used to examine the antibacterial activity of PC-904. We also used the type strains of *P. aeruginosa, P. fluorescens, P. putida, P. putrefaciens, P. maltophilia,* and *P. cepacia* to examine the antibacterial spectrum of PC-904 against *Pseudomonadaceae.*

Determination of MICs. Minimal inhibitory concentrations (MICs) were determined by an agar dilution technique. Serial two-fold dilutions of freshly prepared antibiotic solutions were mixed with melted heart infusion agar (Eiken Kagaku Co. Ltd., Tokyo), and the mixture was poured into petri dishes. Plates were inoculated with one loopful of undiluted, 10^{-2}-fold and 10^{-4}-fold diluted overnight culture of organisms in peptone broth (unless specially described, one loopful of 10^{-2}-fold diluted culture was inoculated). The MIC values (μg/ml) were read after 18 h of incubation at 37°C (after 2 days at 30°C when the

antibacterial spectrum of PC-904 against *Pseudomonadaceae* was tested).

Beta-lactamase activity. Exponentially grown cells were harvested and washed once with 0.1 M phosphate buffer (pH 7.0). The cells were disrupted by ultrasonic oscillation. The disrupted cells were spun down by centrifugation, and the supernatant was used as crude enzyme. Beta-lactamase activity was iodometrically determined at pH 7.0 according to the method of Perret (12). One unit of enzyme was defined as the activity that hydrolyzes 1 μmol of the substrate per h at 30°C. Protein was estimated by the method of Lowry et al. (6).

RESULTS

MICs against clinical isolates. The in vitro antibacterial activities of PC-904, ABPC, and CBPC against clinical isolates of gram-negative bacilli are presented in Fig. 1 and Table 1. Table

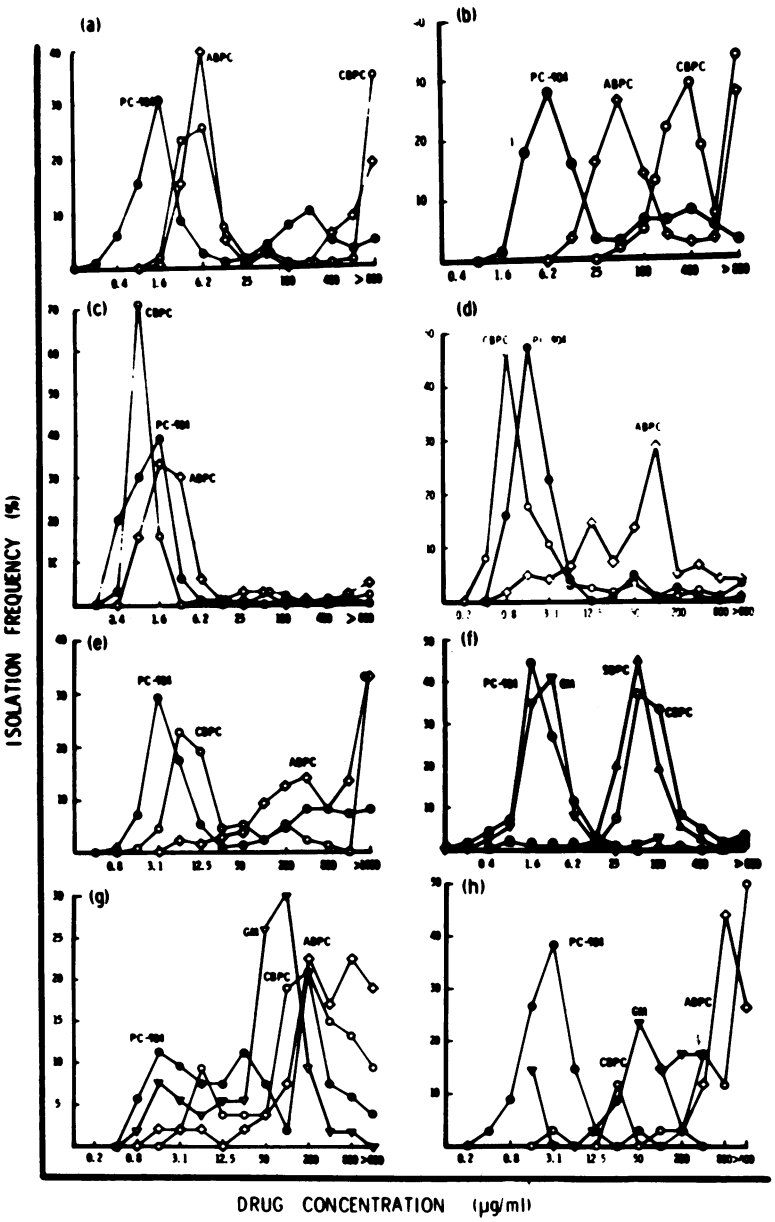

FIG. 1. *Antibacterial activity of PC-904 against gram-negative clinical isolates. (a) E. coli; (b) K. pneumoniae; (c) P. mirabilis; (d) indole-positive Proteus; (e) E. cloacae; (f) P. aeruginosa; (g) P. maltophilia, and (h) P. cepacia.* (●) *PC-904;* (◇) *ABPC;* (○) *CBPC; and* (▽) *GM.*

TABLE 1. *Antibacterial activity of PC-904 against gram-negative clinical isolates*

Species	No. of strains	Drug concn (μg/ml)[a]						% Strains inhibited at:					
		MIC_{50}			MIC_{75}			3.1 μg/ml			12.5 μg/ml		
		PC-904	CBPC	ABPC	PC-904	CBPC	ABPC	PC-904	CBPC	ABPC	PC-904	CBPC	ABPC
E. coli	200	1.4	6.0	5.7	77	>800	560	62	25	16	65	58	60
K. pneumoniae	200	6.8	340	60	78	>800	>800	20	0	0	64	0	4
P. mirabilis	100	0.8	0.7	1.6	1.2	0.8	2.8	95	90	79	96	92	86
P. vulgaris	50	1.1	0.8	42	1.5	1.6	100	92	84	15	94	88	35
P. morganii	50	1.4	0.6	62	2.6	1.2	88	80	86	4	86	92	12
P. retigeri	25	1.7	1.8	11	2.6	5.0	25	84	76	11	84	80	60
E. cloacae	137	5.4	25	600	400	>800	>800	37	5	0	60	46	4
S. marcescens	100	150	>800	>800	>800	>800	>800	6	8	0	20	14	8
P. aeruginosa	200	1.4	52	>800	2.6	87	>800	80	1	0	94	3	0
P. maltophilia	53	21	145	280	155	400	660	26	1	4	42	15	6
P. cepacia	34	1.9	800	550	2.9	>800	>800	76	2	0	94	3	0

[a] MIC_{50} and MIC_{75} represent the concentrations required to inhibit the growth of 50 and 75%, respectively, of the total number of strains used.

1 shows the concentrations required to inhibit the growth of 50 and 75% of the total number of strains tested (MIC_{50} and MIC_{75}, respectively), and the percentage of strains belonging to 11 gram-negative species inhibited at 3.1 and 12.5 μg/ml. PC-904 was more active than ABPC and CBPC against *E. coli* strains. The median MIC of PC-904 was located at a concentration of 1.6 μg/ml (Fig. 1a). A total of 62% of strains were inhibited by 3.1 μg or less of PC-904 per ml, but 30 to 40% of strains were resistant to the three penicillins. Antibacterial activity of PC-904 was the highest (its susceptibility peak, 6.2 μg/ml) against *K. pneumoniae* strains, followed by ABPC and CBPC, respectively (Fig. 1b). ABPC and CBPC did not inhibit the growth of any strains of *K. pneumoniae* at a concentration of 12.5 μg/ml. In contrast, PC-904 did inhibit the growth of 64% of strains at the same concentration. However, 30 to 35% of strains of *K. pneumoniae* were resistant to all the penicillins. PC-904 showed the highest activity against *Proteus* species including indole-positive and -negative strains, with an MIC_{50} and MIC_{75} against *Proteus* species of 2 and 3 μg or less per ml, respectively (Fig. 1c and d).

CBPC showed almost the same activity as PC-904 against *P. mirabilis* and indole-positive *Proteus* species. ABPC was less active than PC-904, especially against indole-positive species of *Proteus*. Fifty percent of *E. cloacae* strains were inhibited at concentrations of 5.4, 25, and 600 μg of PC-904, CBPC, and ABPC per ml, respectively. Against *S. marcescens* strains resistant to both ABPC and CBPC, PC-904 was moderately active, and an MIC_{50} of PC-904 was 150 μg/ml. A peak of the MICs of CBPC and sulbenicillin against *P. aeruginosa* was 50 μg/ml. In contrast, PC-904 showed a peak of MIC at 1.6 μg/ml (Fig. 1f); the MIC_{50} and MIC_{90} of this drug were 1.4 and 6.2 μg or less per ml, respectively. Only a small percentage of the strains was resistant to PC-904, and its activity was comparable to that of GM. Furthermore, PC-904 was highly active against *P. cepacia* strains (Fig. 1h), which are progressively isolated from clinical specimens and are insusceptible to all the commercial beta-lactam and aminoglycoside antibiotics (2, 8). PC-904 showed a higher, superior activity to ABPC and CBPC against *P. maltophilia* (Fig. 1g).

Antibacterial spectrum of PC-904 against *Pseudomonadaceae*. PC-904 was highly active against *P. aeruginosa, P. cepacia,* and *P. maltophilia* strains. Next, we examined the antibacterial activity of PC-904 against other *Pseudomonadaceae* than the above-mentioned species. PC-904 showed high activity against *P. fluorescens, P. putida,* and *P. putrefaciens,* most of which were insusceptible to ABPC and CBPC

TABLE 2. *Antibacterial activity of PC-904 against Pseudomonas species*

Strain	MIC (μg/ml)			
	PC-904	ABPC	CBPC	GM
P. aeruginosa IID5142	1.6	>200	50	3.1
P. aeruginosa IFO3445	3.1	>200	100	3.1
P. aeruginosa IFO3451	0.8	>200	25	1.6
P. aeruginosa NCT10490	0.1	100	0.2	3.1
P. fluorescens IFO3081	3.1	>200	>200	0.8
P. fluorescens IFO12180	1.6	>200	>200	0.2
P. putida IFO3537	3.1	200	>200	0.4
P. putida IFO3538	0.4	6.2	3.1	0.8
P. putrefaciens IFO3908	6.2	25	50	0.8
P. putrefaciens IFO3909	0.8	12.5	12.5	0.2
P. maltophilia GN9085	25	800	800	50
P. maltophilia GN9110	6.2	200	100	100
P. cepacia GN8966	1.6	800	400	25
P. cepacia GN9129	3.1	800	800	200

(Table 2). As a result, PC-904 has a broad antibacterial spectrum and was found to be highly active against most species of *Pseudomonadaceae.*

Antibacterial activity against ABPC- and CBPC-resistant strains. Forty percent of *E. coli* strains isolated in Japan between 1973 and 1976 were resistant to ABPC and CBPC; MICs of these drugs against *E. coli* strains were >50 μg/ml. On the contrary, PC-904 was inactive against 35% of strains, with which MICs equal to more than 25 μg/ml were observed (Fig. 1a). The frequency of isolated strains resistant to these three penicillins was almost the same. However, the MICs of those penicillins varied greatly. Most resistant strains required more than 400 μg of CBPC and ABPC per ml for inhibition. CBPC showed the highest level of resistance (predominantly more than 3,200 μg/ml) against resistant strains, even though the MIC peak of CBPC was located at the same level as that of ABPC against penicillin-susceptible strains (Table 3). As observed in *E. coli,* PC-904 was also moderately active against highly CBPC- and ABPC-resistant strains in other *Enterobacteriaceae* strains (Table 4). Some of the strains resistant to CBPC and ABPC were susceptible to PC-904 (Table 4).

The frequency of isolation of strains resistant to PC-904, ABPC, and CBPC was 32, 35, and 35% in *K. pneumoniae,* 4, 14, and 10% in *P. mirabilis,* 6, 60, and 16% in *P. vulgaris,* and 14, 88, and 8% in *P. morganii,* respectively. In our strains of *P. aeruginosa* isolated in Japan between 1973 and 1976, the frequency of isolation of resistant strains was 4% for PC-904, 9% for CBPC, and 6% for GM. In addition, we collected 43 CBPC- and 56 GM-resistant strains of *P. aeruginosa* and examined the antibacterial activity of PC-904. The in vitro activity of PC-904 against CBPC-resistant strains is shown in Fig. 2. PC-904 was active against these strains, but its activity diminished moderately. A fraction of the strains, the MIC of which was more than 400 μg of CBPC per ml, was susceptible to PC-904 at a concentration of 3.1 to 25 μg/ml. GM did not show any cross-resistance to CBPC in these strains. On the contrary, PC-904 was as active as CBPC and showed an MIC peak of 1.6 μg/ml against GM-resistant strains, 80% of which were also resistant to DKB (Fig. 3).

Effect of inoculum size on MIC. The antistaphylococcal activity of most penicillins is known to be influenced by inoculum size (7, 13). We examined the MIC of PC-904 against gram-negative bacilli by using three different inoculum sizes: one loopful each of 10^4, 10^6, and 10^8 cells per ml. The effects on MICs of varying inoculum sizes for *E. coli* and *P. mirabilis* were small, but those for *K. pneumoniae, P. aeruginosa,* and indole-positive *Proteus* were large. To investigate the factor causing effects related to inoculum size, we divided the clinical isolates into two groups—one penicillin-susceptible and one penicillin-resistant group—and tested the effects of inoculum size of both groups on MICs.

With all penicillins and cephalosporins tested, the effect of inoculum size on MICs was small with susceptible strains of *E. coli.* With resistant strains, those effects were large for PC-904, ABPC, CBPC, and cefazolin, but small for cefoxitin (Table 4). A similar phenomenon was

TABLE 3. *Effect of inoculum size on MIC against ABPC-susceptible and -resistant strains of E. coli*

Drug	No. of cells/ml[a]	No. of ABPC-susceptible strains (25 strains) at MIC (μg/ml)						No. of APBC-resistant strains (25 strains) at MIC (μg/ml)											
		0.4	0.8	1.6	3.1	6.2	12.5	1.6	3.1	6.2	12.5	25	50	100	200	400	800	1,600	3,200	>3,200
APBC	10^4		1	5	16	3								1	12	3	1	7		1
	10^6				16	9									3	2	7	4	7	2
	10^8				15	10														25
PC-904	10^4	2	16	7							9	6	2	4	2	2				
	10^6		15	10								2		9	4	1	2	5	1	1
	10^8		2	12	11												1		1	23
CBPC	10^4		3	2	16	3	1									2		8	5	10
	10^6			1	13	10	1											3	9	13
	10^8				10	14	1												1	24
CEZ	10^4			21	4			6	9	7	1				1			1		
	10^6			18	7				13	4	4	2					1	1		
	10^8				13	10	2			1	3	2	7	5	5			2		
CFX	10^4			2	14	9		4	11	7	2			1						
	10^6			1	9	14	1	1	11	10	2				1					
	10^8				7	15	3	1	5	13	4	1			1					

[a] One loopful of each (10^4, 10^6, and 10^8 cells/ml) was inoculated.

TABLE 4. *Antibacterial activity of PC-904 against ABPC- and CBPC-resistant Enterobacteriaceae*

Strain	MIC (μg/ml)		
	PC-904	CBPC	ABPC
E. coli GN6385	1.6	25	50
E. coli GN3620	1.6	50	6.2
E. coli GN6390	3.1	100	25
E. coli GN6834	6.2	400	50
E. coli GN6313	12.5	>800	400
E. coli GN6284	25	>800	800
E. coli GN6399	200	>800	>800
K. pneumoniae GN5618	6.2	800	800
K. pneumoniae GN5606	6.2	800	100
K. pneumoniae GN5581	12.5	>800	200
K. pneumoniae GN5621	25	>800	400
K. pneumoniae GN5593	50	>800	>800
P. mirabilis GN7406	0.4	100	800
P. mirabilis GN5353	3.1	50	>800
P. mirabilis GN5222	25	1.6	200
P. vulgaris GN5253	1.6	50	>800
P. vulgaris GN7009	25	>800	400
P. vulgaris GN7919	100	400	>800
P. morganii GN4392	6.2	50	100
P. morganii GN4382	6.2	1.6	800
P. morganii GN5307	50	800	>800
P. rettgeri GN7776	3.1	50	>800
P. rettgeri GN4424	200	800	>800

observed with *P. aeruginosa*. Generally, these effects were small against CBPC-susceptible strains, but large against CBPC-resistant strains (Table 5). However, the antibacterial activity of PC-904 was diminished even against susceptible strains when one loopful of 10^8 cells per ml was inoculated. On the other hand, the inoculum effect on the MIC of CBPC against the susceptible group was small, but CBPC showed a high level of resistance against the resistant group; the MIC of CBPC was >3,200 μg/ml.

We tested the relationship between inoculum size effects on MIC and specific activity of beta-lactamase produced by using clinical isolates of *P. aeruginosa* (Fig. 4). In strains that produced beta-lactamase in large amounts (specific activity for PC-904, more than 10 U/mg of protein), the antibacterial activity of PC-904 was greatly affected by inoculum size. In contrast, the effect of inoculum size on MIC was small against strains producing beta-lactamase in small amounts, most of which were susceptible to PC-904 and CBPC. We found a strong relationship between the effects of inoculum size on MIC and specific beta-lactamase activity. The MIC values of PC-904 against the strains capable of producing higher levels of beta-lactamase were greatly affected by strain inoculum size.

DISCUSSION

PC-904, a new semisynthetic penicillin antibiotic, has been reported to have a broad antibacterial spectrum against gram-positive cocci and gram-negative bacilli and, in addition, a potent activity against *P. aeruginosa* (9).

This paper confirmed the high activity of PC-904 against *P. aeruginosa* and clarified that this agent showed high activity against several species of *Pseudomonadaceae* other than *P. aeruginosa*, e.g., *P. cepacia*, *P. maltophilia*, *P. fluorescens*, and *P. putida*. *P. cepacia* and *P. maltophilia* have been important pathogens in opportunistic infections even though there is a low frequency of isolation at this time; these species are highly insusceptible to tetracycline, sulfonamide, streptomycin, kanamycin, ABPC, cephaloridine, and agents active against *P.*

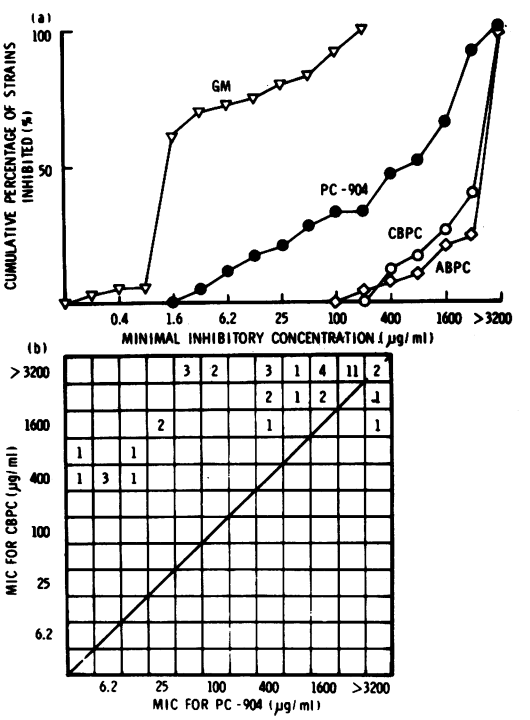

FIG. 2. *Antibacterial activity of PC-904 against CBPC-resistant P. aeruginosa. (a) Cumulative percentage of susceptible strains. (b) Correlogram of MICs between PC-904 and CBPC. Symbols:* (●) *PC-904;* (○) *CBPC;* (◇) *ABPC; and* (▽) *GM.*

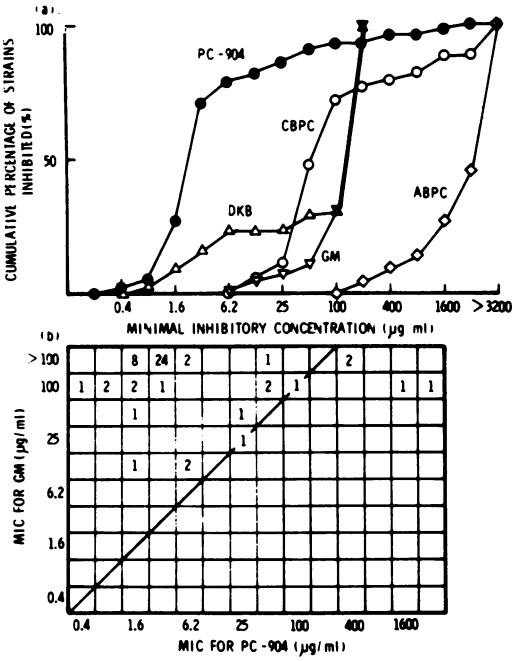

FIG. 3. *Antibacterial activity of PC-904 against GM-resistant P. aeruginosa. (a) Cumulative percentage of susceptible strains. Symbols: (●) PC-904; (○) CBPC; (◇) ABPC; (▽) GM; and (△) DKB. The upper limited concentrations of GM and DKB were 100 μg/ml for the susceptibility test. The points of 200 μg/ml in the figure show the strains not inhibited by 100 μg of GM and DKB per ml. (b) Correlogram of MICs between PC-904 and CBPC.*

aeruginosa such as CBPC, GM, and colistin. Furthermore, CBPC- and GM-resistant strains of *P. aeruginosa* have been increasingly isolated (1, 3, 4, 5).

Our experiments showed that CBPC- and GM-resistant strains were isolated in Japan between 1973 and 1976 at a frequency of 9 and 6%, respectively. Recently, the isolation frequency of GM-resistant strains has increased to 30 to 40% in some parts of Japan (unpublished data). PC-904 as well as CBPC did not show cross-resistance to GM. However, about 18% of GM-resistant strains in *P. aeruginosa* showed resistance to multiple drugs, including beta-lactam antibiotics. *P. aeruginosa* strains resistant to CBPC at medium level (MIC, 400 to 1,600 μg/ml) were susceptible to PC-904. The MICs of PC-904 against the strains highly resistant to CBPC (MIC, ≥ 3,200 μg/ml) were found to be 50 to 1,600 μg/ml. PC-904 was hydrolyzed by beta-lactamase at almost the same rate as CBPC and ABPC, except for carbenicillinase, which hydrolyzed CBPC at a predominantly high rate. PC-904 was moderately stable to carbenicillinase

TABLE 5. *Effect of inoculum size on MIC against CBPC-susceptible and -resistant strains of P. aeruginosa*

Drugs	No. of cells/ml[a]	No. of CBPC-susceptible strains (10 strains) at MIC (μg/ml)											No. of CBPC-resistant strains (12 strains) at MIC (μg/ml)										
		0.8	1.6	3.1	6.2	12.5	25	50	100	200	400	800	1,600	3,200	>3,200	12.5	25	50	100	200	400	800	1,600	3,200	>3,200
CBPC	10^4						2	5	2	1													1		11
	10^6						1	6	2	1														1	11
	10^8							2	2	6														1	11
PC-904	10^4	4	2	3	1											1	1	3	5	2					
	10^6	1	4	4	1												1	2		4			4	1	
	10^8			3	2			1	2	2									2	1			1		8
ABPC	10^4												7	1	2									2	10
	10^6												5	2	3									1	11
	10^8													3	7										12

[a] One loopful of each (10^4, 10^6, and 10^8 cells/ml) was inoculated.

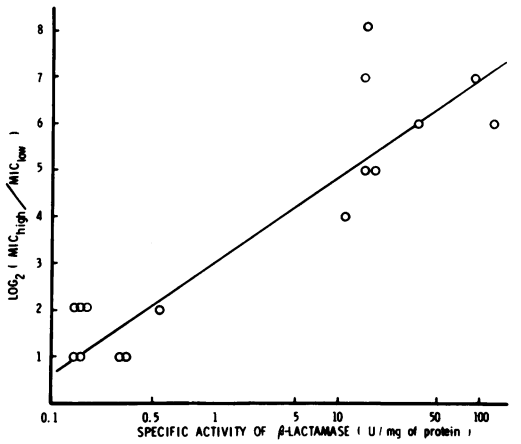

FIG. 4. *Relationship between effect of inoculum size on MICs and specific beta-lactamase activity against PC-904. Abscissa indicates the ratio of MIC_{high} to MIC_{low} (log_2). MIC_{high}, MIC when one inoculation loopful was 10^6 cells/ml; MIC_{low}, MIC when one inoculation loopful was 10^4 cells/ml.*

(unpublished data). However, its stability to beta-lactamase does not explain sufficiently the high antibacterial activity of PC-904 against gram-negative bacilli, including ABPC- and CBPC-susceptible and -resistant strains. On the contrary, PC-904 showed high accessibility to target site(s) through the cell envelope and high affinity to penicillin-binding proteins (14), presumably including penicillin-susceptible enzyme(s) involved in crosslinking the cell wall (unpublished data). In general, the effect of inoculum size on MICs was large for ABPC- and CBPC-resistant strains, but small for strains susceptible to both drugs. Most ABPC- and CBPC-resistant strains harbored plasmid-encoding penicillinase production. The MICs were greatly affected by inoculum size against organisms with higher penicillinase production. The MIC of CBPC for inhibition of susceptible strains was not greatly affected by inoculum size, compared with that of ampicillin derivatives, but the MIC level for inhibition of resistant strains was the highest among beta-lactam antibiotics used. The effect of inoculum size was the lowest on cefoxitin's MIC against both susceptible and resistant strains. CBPC has a high affinity for chromosome-mediated beta-lactamase (cephalosporinase) derived from *P. aeruginosa* and is a good inhibitor against this enzyme (unpublished data).

Yamamoto et al. (15) reported that the antibacterial activity of CBPC is reduced by an unknown barrier directed by beta-lactamase because CBPC possesses very high affinity for this enzyme. Furthermore, cefoxitin has an inhibitory activity for chromosome-mediated beta-lactamase and exerts its resistance to hydrolysis by plasmid-mediated beta-lactamase (10). These results suggest that, in beta-lactam antibiotics, the effect of inoculum size is related to both the rate of hydrolysis (V_{max}) by beta-lactamase and the inhibitory activity (K_i) against beta-lactamase.

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