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Carbapenem Resistance Mechanisms in *Pseudomonas aeruginosa* Clinical Isolates

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In order to define the contributions of the mechanisms for carbapenem resistance in clinical strains of *Pseudomonas aeruginosa*, we investigated the presence of OprD, the expressions of the MexAB-OprM and MexEF-OprN systems, and the production of the β-lactamases for 44 clinical strains. All of the carbapenem-resistant isolates showed the loss of or decreased levels of OprD. Three strains overexpressed the MexAB-OprM efflux system by carrying mutations in *mexR*. These three strains had the amino acid substitution in MexR protein, Arg (CGG) → Gln (CAG), at the position of amino acid 70. None of the isolates, however, expressed the MexEF-OprN efflux system. For the characterization of β-lactamases, at least 13 isolates were the depressed mutants, and 12 strains produced secondary β-lactamases. Based on the above resistance mechanisms, the MICs of carbapenem for the isolates were analyzed. The MICs of carbapenem were mostly determined by the expression of OprD. The MICs of meropenem were two- to four-fold increased for the isolates which overexpressed MexAB-OprM in the background of OprD loss. However, the elevated MICs of meropenem for some individual isolates could not be explained. These findings suggested that other resistance mechanisms would play a role in meropenem resistance in clinical isolates of *P. aeruginosa*.

Pseudomonas aeruginosa is a clinically important pathogen with intrinsic resistance to various antimicrobial agents. This intrinsic multidrug resistance results from the synergy between broadly specific drug efflux pumps and a low degree of outer membrane permeability. For the carbapenem antimicrobials, the resistance is mostly mediated by OprD loss, which primarily confers a resistance to imipenem but also confers a low grade resistance to meropenem (12, 16). But the multidrug efflux systems which mediate the resistance to quinolone, chloramphenicol, and many other antimicrobial agents, also contribute to the carbapenem resistance. The strains which overexpress the MexAB-OprM system or express the MexEF-OprN system exhibit the carbapenem resistance by pumping the drug out or repressing the transcription of *oprD*, respectively (13, 20, 25). On the other hand, the NfxB mutants which expressed MexCE-OprJ became more susceptible to imipenem, bipenem, and some β-lactams (22). In addition to the OprD loss or drug efflux pumps, chromosomal AmpC β-lactamase plays an important role in carbapenem resistance in *P. aeruginosa* (16, 21). Although the contributions of the OprD loss, the efflux systems, and β-lactamase in the carbapenem resistance have been well characterized in the laboratory strains, little data is available for how such factors play together in the clinical isolates of *P. aeruginosa* (2, 4, 31).

Based on the carbapenem susceptibility patterns, the clinical isolates of carbapenem-resistant *P. aeruginosa* could be divided into three groups as the imipenem-resistant and meropenem-sensitive group, the imipenem-sensitive and meropenem-resistant group, and the imipenem-resistant and meropenem-resistant group (2, 4, 17). This suggests that carbapenem resistance occurs by several mechanisms in concert for the clinical isolates. Therefore, in order to evaluate the contributions of the carbapenem resistance mechanisms in clinical strains of imipenem-resistant *P. aeruginosa*, we investigated the presence of OprD, expressions of the MexAB-OprM and the MexEF-OprN systems, and the production of the β-lactamases.

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MATERIALS AND METHODS

Bacterial strains. A total of 117 strains of imipenem-resistant *P. aeruginosa* were collected from four hospitals in Korea during 1997: 80 strains from Hallym University Hospital (HUH), 17 strains from Dankook University Hospital, 14 strains from Asan Medical Center, and 6 strains from Samsung Medical Center. The 80 strains from HUH showed only two patterns of antibiogram which differed in their susceptibilities to amikacin and ciprofloxacin. To examine a clonal spread, we performed ribotyping for 30 of 80 isolates from HUH. Because the ribotyping of 30 isolates showed two patterns which matched well with the antibiogram, we randomly selected seven isolates from 80 strains and included them in this study. Thus, 44 *P. aeruginosa* isolates were studied: 7 strains from HUH and 37 strains from other hospitals.

P. aeruginosa PAO1, *nalB* mutant OCR1, OprD(−) mutant (2297 β-con D2$^-$), and *nfxC* mutant PAO4222*nfxC* were used as standard strains (16, 28, 13). For secondary β-lactamases, *P. aeruginosa* carrying the genes encoding β-lactamases OXA-10 (R151), OXA-2 (pUD11), and *Escherichia coli* carrying PSE-1 (pMON811) served as the standards (5, 10, 11). For evaluation of the IMP-1 enzyme, *Serratia marcescens* TN9106 was used as the standard (26).

Antibiotics. Antimicrobials tested were imipenem (Choongwae Pharma Co., Seoul, Korea); meropenem, piperacillin, and cloxacillin (Yuhan Co., Seoul, Korea); ceftazidime (Glaxo Korea Co., Seoul, Korea); aztreonam (Dong-A Biotech Co., Seoul, Korea); clavulanic acid (II-Sung Pharmaceuticals, Seoul, Korea); amikacin (Young Jin Pharmaceutical Co., Seoul, Korea); ciprofloxacin (Bayer Korea, Ltd., Seoul, Korea); and tetracycline and carbenicillin (Chong Kun Dang Co., Seoul, Korea).

Susceptibility tests. MICs of antibiotics were determined by the agar dilution method on Mueller-Hinton agar (Difco, Detroit, Mich.) with a Steers multiple inoculator according to National Committee for Clinical Laboratory Standards (24). *E. coli* ATCC 25922 and *P. aeruginosa* ATCC 27853 were used as quality control strains.

Isolation of total membranes, SDS-PAGE, and immunoblot assay. The total membranes of the cells were isolated as described previously (8), electrophoresed using 10% (wt/vol) sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels, and electrophoretically transferred to a nitrocellulose membrane (0.45-μm pore size; Bio-Rad, Hercules, Calif.). For the detection of OprD, OprM, or OprN, anti-OprD antibody YD010 (25), anti-OprM antibody TM002 (8), or anti-OprN antibody TN005 (8, 13) was used as primary antibodies, and alkaline phosphatase-conjugated goat antibodies to mouse immunoglobulin G (Cappel) were used as the secondary antibodies. For color development, AP Conjugate Substrate Kit (Bio-Rad) was used (8).

PCR and sequencing of the *mexR* gene. The *mexR*-coding regions of the *P. aeruginosa* isolates were amplified with primers M1 (5′-ACC AAT GAA CTA CCC CGT GA-3′) and M2 (5′-AAT GTT CTT AAA TAT CCT CAA-3′), which corresponded to nucleotides 273 to 292 and nucleotides 707 to 727 of the *mexR* gene, respectively. The PCR amplification mixture (total volume, 100 μl) contained 10 mM Tris-HCl (pH 7.4), 2.5 mM $MgCl_2$, 50 mM KCl, 0.1% Triton X-100, 200 μM concentrations of each deoxynucleoside triphosphate, 0.1 pmol of primers M1 and M2, 2.5 U of *Taq* polymerase (AmpliTaq; Perkin-Elmer Cetus, Norwalk, Conn.), and 10 μl of template DNA. The mixture was denatured at 95°C for 30 s and annealed at 50°C for 1.5 min, and then the chain was extended at 72°C for 1 min in a thermal cycler (Perkin-Elmer Cetus). This cycle was repeated 35 times. For sequencing, M1 and M3 (5′-TCG CGG CGG ACC AGG TTT-3′), corresponding to nucleotides 510 to 527 of the downstream *mexR* gene, were used. The amplified PCR products were purified by using a Qiaex gel extraction kit (Qiagen, Chatsworth, Calif.) and were used as templates for sequencing by a dideoxy termination cycle sequencing kit (Perkin-Elmer Cetus).

Analytical IEF and enzyme inhibition assay. Isoelectric focusing (IEF) was performed using sonicated extracts by the method of Matthew et al. (23), by using an LKB Multiphor apparatus on prepared PAGplates (pH 3.5 to 9.5; Pharmacia Biotech Asia Pacific, Hong Kong, Republic of China) or the Mini-IEF cell system (Bio-Rad). Enzyme activities were detected by overlaying the gel with 0.5 mM nitrocefin and comparing active bands with reference enzymes.

An inhibition assay was performed by overlaying the gels with 0.5 mM nitrocefin with or without 0.3 mM cloxacillin or 0.3 mM clavulanic acid in 0.1 M phosphate buffer (pH 7.0) (6).

Assays of β-lactamase activity. Cultures were grown overnight at 37°C in tryptic soy broth, transferred with a 10% inoculum, and incubated for 4 h with shaking. The cells were subsequently harvested by centrifugation, resuspended in 1 ml of 0.1 M phosphate buffer (pH 7.0), and sonicated. The supernatants were assayed against 0.1 mM nitrocefin in 0.1 mM phosphate-buffer (pH 7.0). The activity was standardized against the protein concentration and assayed by the method of Lowry et al. (18).

Characterization of secondary β-lactamases. To amplify PSE-related genes from clinical isolates, the following oligonucleotide primers were synthesized and used for PCR: P1 (5′-TTA TTG GCA TTT TCG CTT TTA-3′), upstream 21-mer from nucleotide 155; and P2 (5′-CGC ATC ATT TCG CTC TG-3′), downstream 17-mer from nucleotide 942 of the PSE gene, respectively (10). For OXA-10-specific PCR, primers ABD1 (5′-TAT CGC GTG TCT TTC GAG TA-3′) and ABD4 (5′-TTA GCC ACC AAT GAT GCC C-3′) were synthesized, and the PCR amplification was carried out as described previously (6). For the sequencing of the OXA-related gene, ABD2 (5′-CGG AAA GCC AAG AGC C-3′) was used (6). For IMP-1-specific PCR, primers IMP1 (5′-CGC AGC AGG GCA GTC-3′) and IMP2 (5′-CAA AAG CGC AAC TTA CAA AC-3′) were synthesized and used (26).

The amplimers of the PSE-specific PCR were transferred to a nylon membrane by the vacuum transfer method after agarose electrophoresis (19) and hybridized with a digoxigenin (DIG DNA Labelling and Detection Kit; Boehringer Mannheim, Mannheim, Germany)-labeled probe (the probe was a PCR product of the *bla*PSE-1 gene of *E. coli* carrying PSE-1 [pMON811]) (10).

Ribotyping. The chromosomal DNA of the organisms was extracted as described earlier and digested with *Pvu*II (Promega, Madison, Wis.) (9, 19). After electrophoresis, the digests of chromosomal DNA were transferred to a nylon membrane by the vacuum transfer method and then hybridized with the digoxigenin-labeled cDNA copy of *E. coli* rRNA (Boehringer Mannheim), manufactured by reverse transcription using AMV-RT (Promega).

RESULTS AND DISCUSSION

Expressions of OprD. The presence of OprD was examined by Western analysis using an anti-OprD antibody. The result unambiguously showed that 5 isolates expressed OprD, 4 isolates showed decreased OprD, and 35 isolates showed OprD loss (Table 1). Five isolates (group 5 in Table 1) which expressed OprD were all sensitive to carbapenem. The isolates (isolates 1, 6, 71, and 85) with decreased OprD were similarly resistant against carbapenem compared to those with OprD loss. Therefore, all carbapenem-resistant isolates expressed the loss of or decreased levels of OprD.

Expressions of MexAB-OprM and MexEF-OprN efflux system. In order to observe the expression of OprM, the transferred membranes were hybridized with an anti-OprM antibody. Although most isolates expressed OprM, it was difficult to quantitate the expression of OprM by Western blot analysis, especially without comparison to isogenic carbapenem-susceptible parents. But among 44 isolates, at least two strains (isolates 59 and 60) clearly showed elevated amounts of OprM. Thus, in an attempt to support the results of the Western blot analysis, we sequenced the *mexR* genes, which were known to control the *mexA-mexB-OprM* operon negatively. The amplified products were 455 bp sized, a length which contained the entire coding sequences. The nucleotide sequences and deduced amino acid sequence analysis revealed that three isolates (isolates 53, 59, and 60) had the amino acid substitution of Arg (CGG) to Gln (CAG) at the position of amino acid 70 and a Val (GAG) → Glu (GTG) change at amino acid 126. Amino acid 70 was the same position as the amino acid that was substituted in the MexR protein of the *nalB* strain OCR1 previously studied (28), although the changed amino acid was different (Arg [CGG] → Trp [TGG]). With the *mexR* gene sequences and the Western analyses, we concluded that the above three isolates were the mutants overexpressing the MexAB-OprM efflux systems from the mutations in *mexR*.

A total of 25 isolates carried the amino acid change of Val (GAG) to Glu (GTG) at amino acid 126, which was considered not significant (31).

To evaluate the role of the MexEF-OprN efflux system on carbapenem resistance in the clinical isolates, we examined the OprN expressions by Western analysis using an anti-OprN antibody. None of the isolates expressed OprN protein except the control strain (PAO4*222nfxC*).

Assays of β-lactamase activity. The β-lactamases were characterized. Of 44 isolates, 12 isolates produced secondary β-lactamases, and 32 isolates produced only AmpC chromosomal enzymes. The production of the AmpC enzymes was measured for the 32 isolates as described previously (4). When the hydrolysis activities of β-lactamases were compared to that of PAO1 standard strain (35 U/mg of protein), 32 isolates could be divided into three groups based on the activities of the β-lactamases. Eleven isolates (isolates 42, 72, 78, and 151 in group 5 and all isolates in group 2 of Table 1) showed a similar hydrolysis activity to that of PAO1 strain, eight isolates (isolates 59 and 60 in group 1 and all isolates in group 3 of Table 1) produced two- to five-fold-higher activity, and thirteen isolates (isolate 53 in group 1, isolate 57 in group 5, and all isolates belonging to group 4 of Table 1) showed more than fivefold-higher activity than the PAO1 strain. We defined the

TABLE 1. Antimicrobial susceptibilities, the presence of OprD, *mexR* mutations, and AmpC β-lactamase activity of *P. aeruginosa* isolates

Group[a] and isolate no.	MIC[b] (μg/ml) of:									Presence of OprD	Mutations in *mexR* gene[c]	AmpC enzyme activity[d] (U/mg of protein)	PCR	Ribotype[e]
	IPM	MEM	CAR	PIP	CAZ	ATM	AMK	CIP	TET					
PAO1	1	0.5	64	4	2	1	<2	0.25	0.5					
Group 1														
53	16	16	>256	64	16	64	64	32	1		70,126	187		1
59	16	16	>256	32	8	32	64	32	1		70,126	155		2
60	16	16	256	32	16	64	64	32	1		70,126	84		2
Median	16	16	>256	48	16	48	64	32	1					
Group 2														
6	8	2	128	32	32	2	64	16	<0.5	↓		35		3
43	8	4	64	4	1	1	16	0.25	<0.5			11		4
46	8	4	>256	4	1	2	4	0.06	<0.5			28		5
51	4	4	128	32	4	4	512	16	<0.5		126	41		6
58	16	4	64	4	2	4	8	1	<0.5		126	27		7
77	16	8	64	32	16	8	8	0.13	0.5			24		7
153	8	8	256	16	4	8	256	16	0.5		126	47		8
Median	8	4	128	16	4	4	16	1	<0.5					
Group 3														
45	8	8	>256	256	16	16	512	16	<0.5		126	131		6
73	16	8	128	256	32	16	512	16	<0.5		126	99		2
74	8	8	>256	256	32	32	512	16	0.5		126	103		2
75	8	8	256	128	16	16	128	16	<0.5		126	72		2
81	16	8	256	128	32	16	2	0.25	0.5		126	118		6
156	16	16	256	256	32	16	512	32	1		126	155		9
Median	12	8	256	256	32	16	512	16	<0.5					
Group 4														
1	16	2	>256	256	32	16	16	4	8	↓		631		3
5	16	4	>256	>256	32	16	16	2	8		126	2,105		3
9	16	4	>256	256	32	8	64	2	4		126	2,708		3
10	8	4	>256	256	32	32	32	2	8			5,436		3
11	16	4	>256	256	32	8	16	0.5	8		126	3,560		3
52	8	16	>256	256	32	32	32	0.25	128			549		10
76	16	8	64	256	32	16	2	0.125	<0.5			334		9
79	16	8	>256	>256	64	32	8	16	32		126	3,060		9
80	8	8	256	128	16	8	128	8	<0.5		126	180		6
82	16	16	256	128	32	16	8	16	0.5			947		9
83	16	8	128	64	32	16	32	16	0.5			209		11
Median	16	8	>256	256	32	16	16	2	8					
Group 5														
42	0.5	0.5	256	32	4	4	16	1	0.5	+		53		12
57	1	2	>256	>256	32	8	16	16	16	+	126	1,422		13
72	1	1	>256	256	64	32	64	128	64	+	126	81		14
78	1	0.25	64	4	2	4	4	0.125	<0.5	+		23		
151	1	0.5	128	64	8	4	512	16	<0.5	+		16		2
Median	1	0.5	256	64	8	4	16	16	0.5					
Group 6														
4	16	8	>256	32	>256	128	16	16	0.5			ND[h]		3
41	8	16	128	256	16	16	512	16			126	ND	OXA-10[f]	1
44	8	4	256	32	32	4	32	2	<0.5		126	ND		15
71	16	2	>256	32	256	16	128	0.06	32	↓		ND	OXA-10	16
85	8	8	>256	>256	32	16	64	32		↓	126	ND	PSE[g]	17
152	8	8	256	256	32	16	256	16	0.5		126	ND	OXA-10	2
154	8	16	256	128	32	16	>512	16	512		126	ND	OXA-10	2
155	16	16	256	256	32	16	256	16	512		126	ND	OXA-10	2
157	16	8	>256	>256	16	4	256	16			126	ND	PSE	17
158	8	8	>256	>256	16	8	128	16			126	ND	PSE	9
159	8	4	64	128	16	4	4	0.5	0.5			ND	OXA-10	9
160	16	2	>256	256	16	8	32	4			126	ND	PSE	18

[a] The isolates which belonged to each group had the following characteristics: group 1, OprD loss, MexAB-OprM overexpression (+), AmpC β-lactamase production (+); group 2, OprD loss, group 3, OprD loss, AmpC production 2- to 5-fold higher than PAO1; group 4, OprD loss, AmpC production >5-fold higher than PAO1; group 5, OprD (+); group 6, OprD loss, production of secondary β-lactamases.

[b] IPM, imipenem; MEM, meropenem; CAR, carbenicillin; PIP, piperacillin; CAZ, ceftazidime; ATM, aztreonam; AMK, amikacin; CIP, ciprofloxacin; TET, tetracycline.

[c] 70, The amino acid substitution of Arg (CGG) for Gln (CAG) at amino acid 70; 126, Val (GAG) → Glu (GTG) change at amino acid 126.

[d] 1 U = 1 nmol of nitrocefin hydrolyzed/min at 37°C and at pH 7.0. The β-Lactamase activity of PAO1 was 35 U/mg of protein.

[e] Genomic DNAs of the isolates were restricted by *Pvu*II.

[f] pI value of 6.1 on IEF and positive in OXA-10-specific PCR.

[g] pI value of 5.6 on IEF and positive in PSE-specific PCR.

[h] ND, not done.

derepressed mutant as the isolate with a β-lactamase activity more than fivefold higher than that of the PAO1 strain (4). Based on this definition, we concluded at least 13 isolates were derepressed mutants.

We analyzed the effects of AmpC β-lactamase production on the MICs of β-lactams for the strains included in groups 2, 3, and 4 (Table 1). The MIC_{50} values of piperacillin, ceftazidime, and aztreonam for seven isolates which showed a similar hydrolysis activity to that of PAO1 (group 2) were 16, 4, and 4 μg/ml, respectively. For six strains which produced two- to fivefold-higher levels of β-lactamase than PAO1 (group 3), the MIC_{50}s for piperacillin, ceftazidime, and aztreonam were 256, 32, and 16 μg/ml, respectively. The corresponding values for 11 derepressed mutants (group 4 in Table 1) were 256, 32, and 16 μg/ml, respectively. These data clearly showed that the MICs of piperacillin, ceftazidime, and aztreonam for the derepressed mutans were significantly higher than those of the isolates in group 2 (Mann-Whitney U test, $P < 0.001$). However, there were no significant differences in the MICs of β-lactams between the derepressed mutants and the isolates in group 3. We speculated that other mechanisms of resistance against β-lactams, such as permeability change, might work in concert in these strains.

Susceptibility patterns of carbapenem based on the resistance mechanisms in the isolates. The isolates could be divided into six groups based on the results of the expression of OprD, MexAB-OprM efflux system, and β-lactamases (Table 1). A total of 3 isolates showed the loss of OprD, hyperproduced AmpC enzymes, and overexpressed MexAB-OprM efflux system (group 1); 7 isolates showed only the loss of OprD (group 2); 6 isolates lost the OprD and produced AmpC enzymes two- to fivefold higher than PAO1 strain (group 3); 11 isolates lost the OprD and were derepressed (group 4); 5 strains expressed the OprD and produced variable amounts of β-lactamases (group 5); and 12 isolates showed the loss of OprD, and produced secondary β-lactamases (group 6).

The MIC values of carbapenem were compared among six groups. For the isolates which expressed the OprD (group 5), the MIC_{50} values for imipenem and meropenem were 1 and 0.5 μg/ml. However, for 39 isolates with decreased or the loss of OprD (groups 1, 2, 3, 4, and 6) all isolates were resistant to imipenem, and 25 of 39 isolates were resistant to meropenem at 4 μg/ml, respectively. Although 14 of 39 isolates were sensitive to meropenem at 4 μg/ml, their MICs of meropenem were four- to eightfold higher than the MIC_{50}s for the isolates in group 5. These results clearly showed that the presence of OprD primarily determined the MIC values of carbapenem.

The MIC_{50} values of imipenem and meropenem for the isolates in group 1 were 16 and 16 μg/ml, respectively. For the isolates in group 4, the MIC_{50} values of imipenem and meropenem were 16 and 8 μg/ml; the MIC_{50}s of imipenem and meropenem were 12 and 8 μg/ml for the isolates in group 3, and the isolates in group 2 showed the MIC_{50} values of imipenem or meropenem to be 8 or 4 μg/ml, respectively. These results suggested that, in the background of OprD loss, the isolates which overexpressed MexAB-OprM efflux system revealed two- to fourfold-higher MICs for meropenem (group 1 versus groups 3 and 4) (Mann-Whitney U test, $P < 0.05$). The effect of AmpC production on carbapenem resistance could be evaluated by comparing the MICs of carbapenem among the isolates in groups 2 and 4. For the derepressed strains in the background of OprD loss (group 4), the MICs of imipenem were twofold higher (Mann-Whitney U test, $P = 0.057$); the MICs of meropenem were also about twofold higher, but this was not statistically significant.

In the laboratory strains with the loss of OprD, the MIC of meropenem was known to be usually fourfold lower than that of imipenem (12, 16). However, 28 of 36 isolates which had lost OprD but which normally expressed MexAB-OprM efflux system in this study (excluding 8 isolates belonging to group 1 and 5) showed a twofold range (twofold higher, twofold lower, or the same) of MICs of meropenem compared with those of imipenem. Moreover, for the isolates 52, 41, and 154, the MICs of meropenem were twofold higher than those of imipenem. But these isolates did not overexpress the MexAB-OprM efflux systems. These results suggest that there is probably another unidentified resistance mechanism for meropenem, such as the loss of a different portal of entry for meropenem, that might play a role in these isolates.

Secondary β-lactamases produced by *P. aeruginosa*. Among 44 isolates, 12 isolates produced secondary β-lactamases (Table 1). On IEF, six isolates produced the β-lactamases with a pI value of 6.1, and four isolates produced β-lactamases with a pI of 5.6. The enzyme with a pI of 5.6 was inhibited by 0.3 mM clavulanic acid but not by 0.3 mM cloxacillin on the gel overlaying inhibition test, and the pI 6.1 β-lactamase was not inhibited by clavulanic acid or cloxacillin. On the basis of the MIC profiles, the pI values of the enzymes, and the inhibition profiles, we suspected that the enzyme with a pI value of 5.6 was PSE-1-like and that the pI 6.1 β-lactamase was OXA-10-like. Thus, PCRs with a PSE-specific primers and OXA-10-specific primers were performed with these isolates. All four isolates which produced pI 5.6 enzyme showed the amplified PCR products with PSE-specific PCR. Southern hybridization of the PCR products from four isolates revealed that they were PSE-1-like enzymes. In a PCR with the OXA-10-specific primers, five of six isolates which produced the β-lactamases with a pI value of 6.1 showed a positive amplified product (except isolate 44). Among five isolates which harbored $bla_{OXA\text{-}10\text{-}like}$ genes, the amplified product from isolate 152 was sequenced, which revealed that the nucleotide sequences matched exactly with the known sequences of OXA-10 (11).

For the screening of IMP-1 β-lactamase, we performed the PCR with IMP-1-specific primers. But no amplified product was detected from any isolates, which indicated that there was no IMP-1-producing organism in our isolates.

Ribotyping. We performed the ribotyping for the isolates. The ribotyping experiment showed 18 different patterns in the isolates (Table 1). Because we performed DNA restriction with *Pvu*II only in this experiment, it is possible that the ribotyping could not differentiate some organisms.

The resistance against imipenem is usually mediated by OprD loss, and the imipenem does not select for the multidrug efflux pumps. However, because imipenem was the only carbapenem used in this country until recent times, we selected the isolates based on imipenem resistance for looking at the resistance mechanisms for carbapenems. In the laboratory strains, the organism which lost OprD showed an MIC of meropenem a fourfold lower than that of imipenem (12, 16). However, the MIC values for our clinical strains showed that

25 of 39 strains (excluding five strains which were sensitive to imipenem) were also resistant to meropenem at 4 μg/ml and most of the clinical isolates (33 of 44 isolates) were resistant to ciprofloxacin. Thus, we suspected that other resistance mechanisms such as efflux systems might play a role for the carbapenem resistance in these isolates. However, our strains showed only three isolates overexpressing the MexAB-OprM system and none of the *nfxC*-type mutant.

For the quinolone resistance, we speculate that MexCD-OprJ might play a main role although we did not study the OprJ expression in these isolates. Actually, ofloxacin and ciprofloxacin were the most commonly used quinolones, but enoxacin or sparfloxacin was not much used in Korea. The reason why we could not find any *nfxC* mutant among our imipenem-resistant isolates might be the selection of different efflux pumps by the different quinolones (14).

Recently, there have been several reports which proposed alternative ports of entry for meropenem (12, 27). Moreover, analysis of preliminary sequence data from the *P. aeruginosa* genome suggested the existence of 14 open reading frames sharing significant homology with either OprD or OprE porins (12). Our results also suggested that other mechanisms of meropenem other than MexAB-OprM might exist in the clinical isolates of *P. aeruginosa*.

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