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Transferable Imipenem Resistance in *Pseudomonas aeruginosa*

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We isolated an imipenem-resistant strain, GN17203, of *Pseudomonas aeruginosa*. The strain produced a β-lactamase that hydrolyzed imipenem. The β-lactamase was encoded by a 31-MDa plasmid, pMS350, which belongs to incompatibility group P-9. The plasmid conferred resistance to β-lactams, gentamicin, and sulfonamide and was transferable by conjugation to *P. aeruginosa* but not to *Escherichia coli*. The molecular weight of the purified enzyme was estimated to be 28,000, and the isoelectric point was 9.0. The enzyme showed a broad substrate profile, hydrolyzing imipenem, oxyiminocephalosporins, 7-methoxycephalosporins, and penicillins. The enzyme activity was inhibited by EDTA, iodine, *p*-chloromercuribenzoate, $CuSO_4$, and $HgCl_2$ but not by clavulanic acid or sulbactam.

Imipenem is a carbapenem antibiotic with strong activity against many species, including *Pseudomonas aeruginosa* (17). Recently, imipenem-resistant clinical isolates of *P. aeruginosa* have been found (15, 19), and the resistance was due to decreased drug permeation with diminished production of outer membrane proteins (4, 23). Enzymatic inactivation of imipenem has not been reported in *P. aeruginosa*. In this study, we isolated a strain which produced an imipenem-hydrolyzing β-lactamase. The enzyme was a novel type of β-lactamase mediated by a transferable plasmid.

MATERIALS AND METHODS

Bacterial strains and plasmids. *P. aeruginosa* GN17203 was a clinical isolate collected in 1988 in Japan. The laboratory strains used in this study are listed in Table 1. β-Lactamase-deficient strain PAO4089 and PAO1808 were kindly provided by H. Matsumoto of Shinshu University (16). Plasmid pMS350 was isolated from GN17203. Thirteen plasmids were tested for incompatibility (3, 20). They were RP4 (incompatibility group P-1), R931 (P-2), RIP64 (P-3), Rlb679 (P-4), Rms163 (P-5), Rms149 (P-6), Rms148 (P-7), FP-2 (P-8), Rsu2 (P-9), RP1-1 (P-11), R716 (P-12), pMG26 (P-13), and FP5 (incompatibility group unknown).

Antimicrobial agents. Cephaloridine, cephalothin, cefazolin, cefoperazone, cefuroxime, ceftizoxime, cefotaxime, cefoxitin, and penicillins were commercially available materials. Other antimicrobial agents and sources were as follows: imipenem, Banyu Pharmaceutical Co., Ltd.; meropenem, Sumitomo Pharmaceuticals Co., Ltd.; ceftazidime, Nippon Glaxo Co., Ltd.; cefotiam, cefmenoxime, and cefsulodin, Takeda Chemical Industries, Ltd.; moxalactam, Shionogi & Co., Ltd.; aztreonam, Squibb Inc.; clavulanic acid, SmithKline Beecham Seiyaku, Ltd.; sulbactam, Pfizer Pharmaceutical Co., Ltd.

Susceptibility tests. Antibacterial susceptibility was measured by an agar dilution method using sensitivity disk agar (Nissui, Tokyo, Japan) (24).

Conjugation and transformation. Conjugation and transformation were carried out by the method of Kato et al. (12). Plasmid pMS350 was transferred to *P. aeruginosa* PAO1Nf, PAO2142Rp, and PAO4089Rp by conjugation. Transconjugants were selected on sensitivity disk agar containing norfloxacin (25 μg/ml) or rifampin (100 μg/ml) plus imipenem (12.5 μg/ml) or ceftazidime (25 μg/ml). The frequency of transfer was expressed relative to the number of donor cells at the start of mating.

Incompatibility test. Determination of incompatibility was performed by the method of Sagai et al. (20). A donor [PAO2142Rp(pMS350)] culture was mixed with a recipient (PAO1808) culture harboring each of the plasmids for the incompatibility test described above. Transconjugants were selected on medium A-glucose agar (20) plates containing ceftazidime (50 μg/ml) and leucine (200 μg/ml). The colonies that grew on the plates were purified by using the same medium, and their drug resistances were examined.

Estimation of plasmid size. Preparation of plasmid DNAs and agarose gel electrophoresis were carried out by the method of Kato et al. (12). Plasmids RP4 (36 MDa), pMS108 (24 MDa), pMS101 (14 MDa) (10), and Rlb679 (5.6 MDa) were used as standards to determine the size of pMS350.

Enzyme assays. β-Lactamase activity was assayed spectrophotometrically (9) with a UV-265 spectrophotometer (Shimadzu, Kyoto, Japan). An absorbance change of >0.001 could be detected with this apparatus. Enzyme activity was determined at 30°C in 50 mM phosphate buffer (pH 7.0) or 50 mM morpholinepropanesulfonic acid (MOPS) buffer (pH 7.0). Protein concentration was determined by the method of Lowry et al. (14) or that of Bradford (2) by using bovine serum albumin (Sigma, St. Louis, Mo.) as the standard. One unit of enzyme activity was defined as the amount of enzyme needed to hydrolyze 1 μmol of cephaloridine per min at 30°C.

Induction of β-lactamase. Overnight cultures were diluted 1:20 into fresh sensitivity test broth (Nissui) to a final volume of 10 ml. After 2 h of incubation on a shaker at 37°C, cefoxitin was added to a concentration of 25 or 100 μg/ml and incubation was continued for 2 h. Cells were harvested, washed once, and suspended in 3 ml of 50 mM phosphate buffer (pH 7.0). The cells were disrupted by sonication (1 min at 0°C; output, 54 W; Tomy Seiko Co., Tokyo, Japan), and the cellular debris was removed by centrifugation (15,000 × *g*, 15 min, 4°C). β-Lactamase was measured after dialysis to 50 mM phosphate buffer (pH 7.0) at 4°C (6). Cephaloridine was used as the substrate to determine β-lactamase activity.

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TABLE 1. *P. aeruginosa* strains used

Strain	Origin and/or genotype	Reference or source
GN17203	Clinical isolate	This study
PAO1Nf	Norfloxacin-resistant mutant of PAO1	This study
PAO1808	*leu*	H. Matsumoto
PAO2142Rp	Rifampin-resistant mutant of PAO2142; *ilv lys met tyu*	9
PAO4089Rp	Rifampin-resistant mutant of PAO4089; *met pro blaJ blaP*	13

β-Lactamase purification. *P. aeruginosa* PAO4089Rp (pMS350) cells were disrupted by sonication, and the cellular debris was removed by centrifugation (15,000 × *g*, 15 min, 4°C). Sonic extracts were incubated with DNase I (Sigma) and RNase A (Sigma) at 0°C, and 45 to 80% ammonium sulfate fraction was dialyzed against 50 mM phosphate buffer (pH 7.0). The dialysate was applied to a carboxymethyl—Sephadex C-50 column, and the enzyme was eluted with a linear NaCl gradient. The active fractions were pooled, dialyzed against 10 mM MOPS buffer (pH 7.0) containing 0.1 M NaCl and 1 μM $ZnCl_2$, and concentrated. The enzyme solution was loaded onto a Sephadex G-75 column (1.0 cm [inside diameter] by 100 cm) and eluted with 10 mM MOPS buffer (pH 7.0) containing 0.1 M NaCl and 1 μM $ZnCl_2$. The pooled eluate with activity was concentrated and rechromatographed with a Sephadex G-50 column (1.0 cm [inside diameter] by 100 cm). The enzyme solution was dialyzed against 50 mM MOPS buffer (pH 7.0) and stored at −80°C.

Determination of molecular weight. Molecular weight was estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (13). Purified enzymes and marker proteins (Pharmacia, Uppsala, Sweden) were treated with 1% sodium dodecyl sulfate–3% β-mercaptoethanol at 100°C for 2 min and then subjected to electrophoresis in a 12.5% gel with a current of 20 mA for 5 h at room temperature.

Analytical isoelectric focusing. Electrophoresis was carried out on an LKB Ampholine PAGplate (pH 3.5 to 9.5; LKB Stockholm, Bromma, Sweden) at 4°C by using a power supply (Atto Co., Tokyo, Japan). The gel was cut in two, and each part was used for a single experiment. Each half-size gel was preequilibrated for 1 h at 100 V, and samples were loaded and run for 1.5 h in the constant-power mode (17.5 W). Purified β-lactamase was detected on the gel by using the nitrocefin (0.5 mg/ml) overlay procedure. The pH gradient was determined by using pI-MARKER (Oriental Yeast Co., Ltd., Tokyo, Japan).

Inhibition study. Enzyme solution was preincubated in 50 mM MOPS buffer (pH 7.0) for 5 min at 30°C with each inhibitor (final concentration, 100 μM), and the remaining activity was assayed spectrophotometrically with cephaloridine (100 μM) as the substrate.

Recovery study. Purified β-lactamase was dissolved in 500 μl of 50 mM MOPS buffer (pH 7.0 at 30°C) containing 10 μg of bovine serum albumin (Sigma) per ml and 2 mM EDTA. After incubation at 30°C for 10 min, the mixture was dialyzed twice against 1 liter of 50 mM MOPS buffer (pH 7.3 at 4°C) for 24 h. The dialyzed enzyme solution was then incubated with each of the divalent cations (final concentration, 1 mM) at 30°C for 10 min before enzyme assay. Enzyme activity was assayed with cephaloridine (100 μM) as the substrate and expressed as the percentage of hydrolysis of the untreated enzyme.

RESULTS

Plasmid-mediated imipenem resistance in *P. aeruginosa*. We isolated imipenem-resistant *P. aeruginosa* GN17203, which was also resistant to other antipseudomonal β-lactams (MIC, ≥25 μg/ml) (Table 2). Strain GN17203 produced imipenem-hydrolyzing β-lactamase. To determine the transferability of the imipenem resistance, conjugation was carried out with PAO1Nf as the recipient and transconjugants were selected on agar plates containing ceftazidime and norfloxacin. Resistant transconjugants were obtained by conjugation, the second transfer was performed with a resultant transconjugant strain as the donor and PAO2142Rp as the recipient, and transconjugants were selected for resistance to ceftazidime and rifampin. Retransfer of imipenem resistance was confirmed. Transformation of strain PAO1808 was performed by using plasmid DNA from the transconjugant of PAO2142Rp, and retransfer of imipenem resistance from PAO1808 transformants to recipient PAO2142Rp was confirmed. These results indicated that imipenem resistance was mediated by a transferable plasmid that we designated pMS350. Susceptibilities of various strains harboring pMS350 to β-lactams are shown in Table 2. The resistance to imipenem, meropenem, ceftazidime, cefsulodin, cefoperazone, moxalactam, and carbenicillin was fully expressed in the transconjugants. MICs of piperacillin for the transconjugants were the same as those for the parent strains, but the MICs of aztreonam were two- to fourfold lower. Synergism was not observed when clavulanic acid (5 μg/ml) was combined with either imipenem or ceftazidime and used against these transconjugants.

TABLE 2. Susceptibilities of GN17203 and transconjugants to β-lactam antibiotics

Strain	MIC (μg/ml)[a]									Sp act (U/mg of protein)[b]
	IPM	MPM	CAZ	CFS	CPZ	LMOX	CBPC	PIPC	AZT	
GN17203	50	100	>400	>400	>400	>400	>400	25	25	0.61
PAO1Nf	1.56	3.13	1.56	1.56	3.13	6.25	25	3.13	3.13	0.04
PAO1Nf(pMS350)	12.5	100	400	>400	200	>400	400	3.13	0.78	0.60
PAO2142Rp	0.78	3.13	1.56	3.13	12.5	12.5	200	3.13	12.5	0.02
PAO2142Rp(pMS350)	12.5	50	400	>400	200	>400	>400	3.13	3.13	0.50
PAO4089Rp	0.39	0.78	1.56	1.56	3.13	6.25	25	3.13	3.13	<0.01
PAO4089Rp(pMS350)	6.25	50	400	>400	200	>400	>400	3.13	1.56	0.43

[a] Abbreviations: IPM, imipenem; MPM, meropenem; CAZ, ceftazidime; CFS, cefsulodin; CPZ, cefoperazone; LMOX, moxalactam; CBPC, carbenicillin; PIPC, piperacillin; AZT, aztreonam.

[b] The β-lactamase activity in the uninduced state was determined with 100 μM cephaloridine as the substrate.

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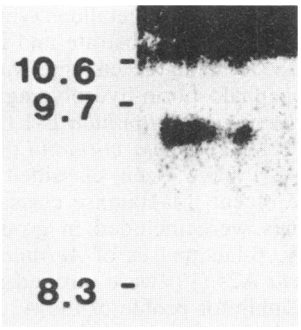


FIG. 1. Analytical isoelectric focusing of pMS350-mediated β-lactamase. The numbers on the left indicate pIs.

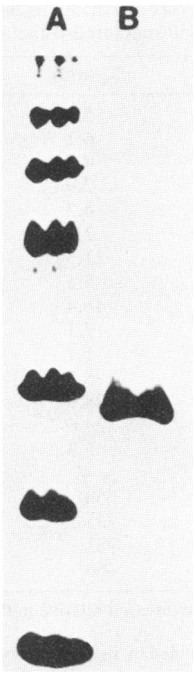


FIG. 2. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of pMS350-mediated β-lactamase. Lanes: A, molecular weight reference markers (from the top, 94,000, 67,000, 43,000, 30,000, 20,100, and 14,400); B, pMS350-mediated β-lactamase.

Characterization of pMS350. The plasmid conferred resistance to gentamicin and sulfonamide, in addition to β-lactams. The incompatibility group of plasmid pMS350 was determined as described in Materials and Methods. Plasmid pMS350 transferred to PAO1808 at a frequency of 8.7×10^{-3} per donor cell by broth mating. A similar frequency was observed when the recipient harbored any resident plasmid except Rsu2. When the recipient harbored plasmid Rsu2, the transfer frequency of pMS350 was reduced to 1.0×10^{-4}. In this case, segregants which lost Rsu2 were obtained among transconjugants after selection by ceftazidime. In contrast, plasmid Rsu2 was transferred to recipient PAO2142Rp at a frequency of 1.5×10^{-6} per donor cell by filter mating but transfer of Rsu2 was not detected when the recipient harbored pMS350. These results indicated that plasmid pMS350 belonged to incompatibility group P-9. Transfer of plasmid pMS350 to *Escherichia coli* or *Acinetobacter calcoaceticus* was not observed. Plasmid pMS350 was stably harbored in PAO2142Rp at 37 and 43°C. The size of the plasmid was estimated as 31 MDa by agarose gel electrophoresis.

Characterization of plasmid-mediated β-lactamase. Crude β-lactamase was obtained from PAO4089Rp(pMS350) by the procedure used for induction experiments. As the host strain produced no chromosomal β-lactamase, the enzyme produced was pMS350 mediated. Production of the enzyme was not induced with cefoxitin (25 or 100 μg/ml) or temperature shift up to 37°C from 20°C.

The β-lactamase was purified as described in Materials and Methods. In preliminary experiments, the enzyme was more stable in MOPS buffer (pH 7.0) containing 1 μM $ZnCl_2$ than in MOPS buffer (pH 7.0) alone. Thus, Sephadex G-75 and G-50 gel filtrations were performed by using the $ZnCl_2$-containing buffer, and the final sample was dialyzed against $ZnCl_2$-free MOPS buffer. The enzyme was purified about 340-fold from the crude cell extract with overall recovery of 8.0%, and the specific activity was 65.3 U/mg of protein. The isoelectric point (pI) of the enzyme was 9.0 by isoelectric focusing chromatography (Fig. 1). The estimated molecular weight of the purified enzyme was 28,000 by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Fig. 2).

The purified enzyme was unstable in ordinary 50 mM phosphate buffer (pH 7.0) at 30°C, and 40% of the activity was lost after 60 min of incubation in the buffer. As the enzyme was more stable in MOPS buffer (pH 7.0) at 30°C than in phosphate buffer, we chose MOPS buffer for assay of the purified enzyme. The pH-activity curve for the enzyme with cephaloridine as the substrate was determined for a range of pH values from 6.0 to 10.0 by using MOPS buffer (pH 6.0 to 7.5), Tris hydrochloride buffer (pH 8.0 to 9.0), or glycine buffer (pH 9.5 to 10.0). The optimal pH was around 9.5, and the enzyme activity was 3.7-fold higher at pH 9.5 than at pH 7.0. The activity of the enzyme in Tris hydrochloride buffer (pH 8.0 to 9.0) after 60 min of incubation at 30°C was 78 to 82% of the initial activity, and more than 90% of the enzyme activity was lost in glycine buffer (pH 9.5 to 10.0) after 60 min of incubation at 30°C. The temperature-activity curve for the enzyme with cephaloridine as the substrate was determined for a range of 20 to 60°C at pH 7.0 in MOPS buffer (20 to 40°C) or Tris hydrochloride buffer (50 to 60°C). The enzyme was most active at 60°C, and the enzyme activity was 9.0-fold higher at 60°C than at 30°C. The activity after 5 min of incubation at 60°C was 77% of the initial activity.

The Michaelis constant (K_m) and the maximum rate of hydrolysis (relative V_{max}) are shown in Table 3. The K_m values and relative V_{max} values were obtained from Lineweaver-Burk plots. The K_m values for cephalosporins were lower than those for penicillins. All of the oxyiminocephalosporins tested were hydrolyzed. 7-Methoxycephalosporins (moxalactam and cefoxitin) and penicillins (penicillin G, ampicillin, and carbenicillin) were also hydrolyzed. Aztreonam was resistant to hydrolysis by the enzyme.

The effects of inhibitors and ions on the enzyme activity are shown in Table 4. The enzyme activity was not inhibited by clavulanic acid and sulbactam but was inhibited by EDTA, iodine, *p*-chloromercuribenzoate, $CuSO_4$, and $HgCl_2$.

Treatment of the enzyme with EDTA caused complete loss of activity. However, activity was restored by addition of divalent cations, such as Zn^{2+}, after dialysis of the EDTA-treated enzyme (Table 5). These results indicated that the β-lactamase was a metalloenzyme.

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TABLE 3. Hydrolysis of various β-lactam antibiotics by pMS350-mediated β-lactamase

Substrate	K_m (μM)	Relative V_{max}[a]
Cephaloridine	5.7	100
Cephalothin	6.1	113
Cefazolin	4.4	77
Cefotiam	2.9	46
Cefuroxime	4.2	35
Cefotaxime	2.3	22
Ceftizoxime	21.3	141
Cefmenoxime	5.1	54
Ceftazidime	46.4	20
Cefoperazone	7.1	98
Cefsulodin	1.1	5
Cefoxitin	6.1	51
Moxalactam	28.9	193
Imipenem	24.6	166
Meropenem	5.3	37
Aztreonam	—[b]	<1
Penicillin G	650	717
Ampicillin	335	215
Carbenicillin	381	391
Piperacillin	468	145

[a] Rates of hydrolysis are expressed relative to that of cephaloridine, which was set at 100.
[b] —, The K_i value, determined by using cephaloridine as the substrate, was >100 μM.

DISCUSSION

Imipenem-resistant clinical isolates of *P. aeruginosa* have been found (15, 19), and these isolates lack an outer membrane protein with a molecular weight of 45,000 to 49,000. This protein was shown to correspond to protein D2 of *P. aeruginosa* PAO1 (4, 23), and the resistance was revealed to be due to decreased imipenem permeation with diminished production of the protein. In this study, we found an imipenem-resistant strain which produced a novel β-lactamase encoded by transferable plasmid pMS350, and the plasmid conferred resistance to various antipseudomonal β-lactams except piperacillin and aztreonam.

To elucidate the biochemical properties of the enzyme, we purified the β-lactamase from PAO4089Rp, a β-lactamase-deficient mutant (16) that harbors the plasmid. The β-lactamase purified by column chromatography showed a broad substrate profile, hydrolyzing imipenem, oxyiminocephalosporins, and 7-methoxycephalosporins; these three groups of antibiotics are resistant to hydrolysis by most β-lactamases. The β-lactamase inhibition and recovery studies indicated that this enzyme was a metalloenzyme. We classified β-lactamase on the basis of substrate and inhibitor profiles (22), and the pMS350-mediated enzyme was classified as a type II oxyiminocephalosporin-hydrolyzing enzyme. β-Lactamases of *Xanthomonas maltophilia* L-1 (21), *Flavobacterium odoratum* (22), *Legionella gormanii* (8), and *Bacteroides fragilis* (7, 25) have been classified in this group. According to the recent β-lactamase classification of Bush (5), these enzymes were included in group 3 (metalloenzymes). Recently, β-lactamases of *Aeromonas hydrophilia* A2h and *A. sobria* A2s (10) were purified and classified in group 3, but the inhibitor profile of the *A. hydrophilia* A2h enzyme for clavulanic acid was unique and different from those of type II oxyiminocephalosporin-hydrolyzing enzymes. It is of great interest to define the relationship of the pMS350-mediated enzyme to other metallo-β-lactamases to speculate on the origin of the enzyme. The molecular weight of the pMS350-mediated enzyme (28,000) was different from those of other metallo-β-lactamases from *X. maltophilia* L-1 (118,000; subunit, 26,000), *B. fragilis* TAL2480 and TAL3636 (44,000), *A. hydrophilia* A2h (31,500), *A. sobria* A2s (35,000), *F. odoratum* (26,000), *B. fragilis* G-237 (26,000), *L. gormanii* (25,000), and *Bacillus cereus* (22,000) (5). The pMS350-mediated enzyme showed higher affinity for cephalosporins than for penicillins, and the substrate profile of the pMS350-mediated enzyme for β-lactams was similar to that of *B. fragilis* G-237 β-lactamase (25).

The purified β-lactamase was unstable in phosphate buffer (pH 7.0), as is the β-lactamase from *B. fragilis* (7), and we used MOPS buffer for assay of the purified enzyme. In the recovery study, we tried some conditions to get high recovery of activity, and the highest recovery was obtained when we used bovine serum albumin-containing MOPS buffer. However, recovery of the activity of the enzyme by cations after EDTA treatment was lower than those of the enzymes of *P. maltophilia* L-1 (21).

The effect of temperature on the activity of the pMS350-mediated enzyme was peculiar. The enzyme was most active at 60°C. The optimal temperatures of penicillinases, cephalosporinases, and type I oxyiminocephalosporin-hydrolyzing enzymes were between 35 and 50°C in most cases (18), but the optimal temperatures of other metallo-β-lactamases have not been elucidated. It would be important to elucidate whether a high optimal temperature is a common feature of metallo-β-lactamases. The optimal pH of the pMS350-mediated enzyme was one of the most alkaline among the β-lactamases previously reported (1, 18). It was postulated

TABLE 4. Effects of inhibitors on the activity of pMS350-mediated β-lactamase

Inhibitor or ion[a]	% Inhibition
$FeSO_4$	57
$FeCl_3$	57
$CuSO_4$	100
$HgCl_2$	100
EDTA	100
PCMB[b]	100
Iodine	100

[a] Addition of KCl, $CaCl_2$, NaCl, $MgSO_4$, $ZnCl_2$, $NiCl_2$, $CoSO_4$, $MnCl_2$, sulbactam, clavulanic acid, or aztreonam had no inhibitory effect on the enzyme.
[b] *p*-Chloromercuribenzoate.

TABLE 5. Effects of divalent cations on the β-lactamase activity mediated by pMS350

Treatment			Relative activity (%)
EDTA	Dialysis	Divalent cation	
−	−	None	100
−	+	None	51
+	−	None	0
+	+	None	0
+	+	Ca^{2+}	7
+	+	Mg^{2+}	0
+	+	Mn^{2+}	5
+	+	Zn^{2+}	54
+	+	Fe^{2+}	5
+	+	Co^{2+}	7
+	+	Cu^{2+}	0
+	+	Hg^{2+}	0

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that the enzymological property of the enzyme, including the environment of the active site, is unique, and we investigated the enzyme by a genetic approach.

Interestingly, the MICs of aztreonam for transconjugants harboring pMS350 were decreased in comparison with those for the parents. To elucidate the phenomenon, we investigated the plasmid-mediated products. Since aztreonam was resistant to hydrolysis by the pMS350-mediated enzyme, an unknown product encoded by the plasmid was speculated to play a role in the increased susceptibility.

Transferable R plasmids play an important role in rapid dissemination of resistance to antibiotics (11, 18). In this study, a transferable plasmid-mediated metallo-β-lactamase was observed in *P. aeruginosa*. Hereafter, this broad-spectrum enzyme will be one of the clinical problems in the therapy of *P. aeruginosa* infections.

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