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Molecular Epidemiology and Mechanisms of Carbapenem Resistance in *Pseudomonas aeruginosa*▽

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The contributions of different mechanisms of resistance to carbapenems among a collection of imipenem- and meropenem-nonsusceptible *Pseudomonas aeruginosa* isolates were investigated. This screening included the recently reported extended-spectrum cephalosporinases (ESACs) weakly hydrolyzing carbapenems. Eighty-seven percent of the studied isolates were resistant to imipenem. Genes encoding metallo-β-lactamases or carbapenem-hydrolyzing oxacillinases were not identified. The main mechanism associated with imipenem resistance was the loss of outer membrane protein OprD. Identification of overexpressed ESACs and loss of OprD were observed for 65% of the isolates, all being fully resistant to imipenem. Resistance to meropenem was observed in 78% of the isolates, with all but one also being resistant to imipenem. Overexpression of the MexAB-OprM, MexXY-OprM, or MexCD-OprJ efflux systems was observed in 60% of the isolates, suggesting the contribution of efflux mechanisms in resistance to meropenem. The loss of porin OprD and the overproduction of ESACs were observed in 100% and 92% of the meropenem-resistant isolates, respectively. *P. aeruginosa* can very often accumulate different resistance mechanisms, including ESAC production, leading to carbapenem resistance.

Carbapenems remain the main antimicrobials for treating infections due to multidrug-resistant *Pseudomonas aeruginosa*, but the development of carbapenem resistance may significantly compromise their efficacy. In the absence of carbapenem-hydrolyzing enzymes (mainly metallo-β-lactamases [MBLs]), carbapenem resistance is usually multifactorial. Increased production of AmpC chromosome-encoded cephalosporinase, reduced outer membrane porin OprD expression, and associated factors are known to contribute to carbapenem resistance (12, 13, 21). Overexpression of the MexAB-OprM efflux system is known to affect meropenem efficacy but not that of imipenem (14, 23). In addition, the MexCD-OprJ and MexXY-OprM efflux systems may also be involved in reduced susceptibility to meropenem (1, 14, 20).

We have recently reported extended-spectrum cephalosporinases (ESACs) of *P. aeruginosa* with broadened hydrolytic activity toward imipenem (22). All those naturally occurring chromosome-encoded AmpC-type β-lactamases possess an alanine residue at position 105 that has a slight carbapenemase activity that is sufficient to compromise the efficacy of carbapenems when the enzyme is overexpressed (22). The isolates studied here were selected on the basis of nonsusceptibility to carbapenems, and we aimed at evaluating the contributions of the different mechanisms to their resistance to carbapenems.

MATERIALS AND METHODS

Bacterial strains. Thirty-two nonduplicated *P. aeruginosa* clinical isolates recovered at the Bicêtre Hospital (K.-Bicêtre, France) in 2007 were included in this study (Tables 1 and 2). Those isolates were selected on the basis of intermediate susceptibility (MIC = 4 μg/ml) or resistance (MIC ≥ 8 μg/ml) to imipenem and meropenem (3). *P. aeruginosa* clinical isolates were identified using the API 32GN system (bioMerieux, Marcy-l'Étoile, France).

P. aeruginosa PAO1 was used as a reference strain in susceptibility testing and β-lactamase assays. *P. aeruginosa* H729 lacking the *oprD* gene was used as a OprD-negative reference strain. The MBL VIM-2-producing *P. aeruginosa* isolate COL-1 (19) was used as a positive control for carbapenemase production. Bacterial cells were grown in Trypticase soy broth or on Trypticase soy agar plates (Sanofi Diagnostics Pasteur, Marnes-la-Coquette, France) supplemented with antibiotics when required.

Antimicrobial agents and susceptibility testing. The antimicrobial agents and their sources have been described elsewhere (16). MICs were determined by using the Etest technique (AB Biodisk, Solna, Sweden) and agar dilution method as previously described (3, 16). Results of susceptibility testing were recorded according to the guidelines of the CLSI (3). AmpC overproduction was confirmed using cloxacillin (250 μg/ml)-containing plates, since cloxacillin inhibits AmpC β-lactamase activity (15). The isolates were considering as overexpressing their bla_{ampC} genes when there was at least a twofold dilution difference between the MIC of ceftazidime and the MIC of ceftazidime plus cloxacillin.

Molecular strain typing. Pulsed-field gel electrophoresis was performed according to the instructions of the manufacturer (Bio-Rad, Marnes-la-Coquette, France), as previously described (18). Results were analyzed according to the criteria of Tenover et al. (24).

β-Lactamase analysis. AmpC β-lactamase-specific activity (μmol of cephalothin [cefalotin] hydrolyzed per minute and per milligram of protein) was determined by UV spectrophotometry with crude culture extracts, as described previously (8, 11, 22). Extracts showing >90% reduction of β-lactamase activity after cloxacillin addition were considered to produce AmpC-type β-lactamase as a major contributor of β-lactamase activity.

The carbapenemase activity was determined as previously described (19). Briefly, hydrolyses of imipenem and meropenem (100 μM solution in 100 mM sodium phosphate [pH 7.0]) by crude cell extracts obtained after sonication in phosphate buffer were measured spectrophotometrically at 297 nm and 298 nm, respectively.

Additionally, the presence of genes coding for MBL-type (bla_{IMP}, bla_{VIM}, bla_{SPM}, and bla_{AIM}) and carbapenem-hydrolyzing class D (bla_{OXA-23}, bla_{OXA-40}, bla_{OXA-48}, and bla_{OXA-58}) β-lactamases was searched out by PCR amplification using previously described primers (Table 3). Whole-cell DNA was extracted as described previously (2).

OMP analysis. Outer membrane protein (OMP) profiles were examined using previously reported methods (7). Following sonication, membranes were col-

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TABLE 1. MICs of β-lactamases for the clinical isolates of *P. aeruginosa*

Isolate	MIC (μg/ml)[a]									
	CAZ	CAZ-CLO	FEP	FEP-CLO	IPM	IPM-CLO	MEM	MEM-CLO	ATM	ATM-CLO
PAO-1	1 (S)	1 (S)	2 (S)	2 (S)	1 (S)	1 (S)	1 (S)	1 (S)	2 (S)	2 (S)
Paeβ-01	8 (I)	8 (I)	16 (I)	16 (I)	4 (I)	4 (I)	8 (R)	8 (R)	16 (I)	16 (I)
Paeβ-02	8 (I)	8 (I)	16 (I)	16 (I)	4 (I)	4 (I)	4 (I)	4 (I)	16 (I)	16 (I)
Paeβ-03	16 (I)	2 (S)	64 (R)	16 (I)	16 (R)	2 (S)	8 (R)	4 (I)	16 (I)	8 (I)
Paeβ-04	32 (R)	4 (I)	32 (R)	8 (I)	16 (R)	4 (I)	16 (R)	16 (R)	32 (R)	32 (R)
Paeβ-05	32 (R)	32 (R)	16 (I)	16 (I)	16 (R)	16 (R)	8 (R)	8 (R)	32 (R)	32 (R)
Paeβ-06	16 (I)	2 (S)	32 (R)	8 (I)	16 (R)	2 (S)	8 (R)	2 (S)	16 (I)	4 (I)
Paeβ-07	16 (I)	2 (S)	32 (R)	8 (I)	16 (R)	2 (S)	8 (R)	2 (S)	16 (I)	4 (I)
Paeβ-08	16 (I)	2 (S)	32 (R)	8 (I)	16 (R)	2 (S)	8 (R)	2 (S)	16 (I)	4 (I)
Paeβ-09	16 (I)	2 (S)	32 (R)	8 (I)	16 (R)	2 (S)	8 (R)	4 (I)	16 (I)	4 (I)
Paeβ-10	16 (I)	2 (S)	32 (R)	8 (I)	16 (R)	2 (S)	8 (R)	4 (I)	16 (I)	8 (I)
Paeβ-11	16 (I)	2 (S)	32 (R)	8 (I)	16 (R)	2 (S)	8 (R)	2 (S)	16 (I)	4 (I)
Paeβ-12	16 (I)	1 (S)	32 (R)	8 (I)	16 (R)	1 (S)	8 (R)	2 (S)	16 (I)	2 (S)
Paeβ-13	4 (I)	0.12 (S)	16 (I)	1 (S)	16 (R)	0.5 (S)	8 (R)	2 (S)	16 (I)	2 (S)
Paeβ-14	16 (I)	2 (S)	32 (R)	8 (I)	16 (R)	2 (S)	4 (I)	2 (S)	16 (I)	8 (I)
Paeβ-15	32 (R)	8 (I)	128 (R)	32 (R)	16 (R)	4 (I)	8 (R)	4 (I)	32 (R)	32 (R)
Paeβ-16	32 (R)	4 (I)	32 (R)	8 (I)	32 (R)	4 (I)	4 (I)	2 (S)	8 (I)	4 (I)
Paeβ-17	64 (R)	8 (I)	32 (R)	8 (I)	32 (R)	8 (R)	8 (R)	4 (I)	32 (R)	8 (I)
Paeβ-18	128 (R)	32 (R)	128 (R)	32 (R)	64 (R)	16 (R)	64 (R)	32 (R)	128 (R)	64 (R)
Paeβ-19	8 (I)	4 (I)	32 (R)	16 (I)	4 (I)	4 (I)	4 (I)	4 (I)	32 (R)	32 (R)
Paeβ-20	8 (I)	1 (S)	16 (I)	4 (I)	16 (R)	4 (I)	8 (R)	4 (I)	16 (I)	8 (I)
Paeβ-21	32 (R)	4 (I)	32 (R)	4 (I)	16 (R)	2 (S)	32 (R)	16 (R)	64 (R)	32 (R)
Paeβ-22	16 (I)	1 (S)	16 (I)	4 (I)	8 (R)	0.5 (S)	4 (I)	1 (S)	16 (I)	4 (I)
Paeβ-23	64 (R)	8 (I)	32 (R)	8 (I)	32 (R)	8 (R)	16 (R)	8 (R)	64 (R)	16 (I)
Paeβ-24	64 (R)	16 (I)	64 (R)	16 (I)	32 (R)	8 (R)	32 (R)	16 (R)	64 (R)	32 (R)
Paeβ-25	32 (R)	4 (I)	32 (R)	32 (R)	8 (R)	8 (R)	8 (R)	8 (R)	32 (R)	16 (I)
Paeβ-26	32 (R)	4 (I)	16 (I)	16 (I)	4 (I)	4 (I)	4 (I)	4 (I)	32 (R)	16 (I)
Paeβ-27	16 (I)	4 (I)	64 (R)	32 (R)	32 (R)	16 (R)	64 (R)	64 (R)	64 (R)	64 (R)
Paeβ-28	16 (I)	2 (S)	16 (I)	8 (I)	16 (R)	4 (I)	4 (I)	4 (I)	16 (I)	8 (I)
Paeβ-29	8 (I)	8 (I)	32 (R)	32 (R)	16 (R)	16 (R)	32 (R)	32 (R)	64 (R)	64 (R)
Paeβ-30	8 (I)	8 (I)	8 (R)	8 (I)	8 (R)	8 (R)	8 (R)	8 (R)	8 (I)	8 (I)
Paeβ-31	32 (R)	16 (I)	32 (R)	32 (R)	16 (R)	16 (R)	8 (R)	8 (R)	64 (R)	64 (R)
Paeβ-32	32 (R)	8 (I)	32 (R)	8 (I)	64 (R)	16 (R)	16 (R)	8 (R)	64 (R)	16 (I)

[a] CAZ, ceftazidime; CAZ-CLO, ceftazidime-cloxacillin; FEP, cefepime; FEP-CLO, cefepime-cloxacillin; IPM, imipenem; IPM-CLO, imipenem-cloxacillin; MEM, meropenem; MEM-CLO, meropenem-cloxacillin; ATM, aztreonam; ATM-CLO, aztreonam-cloxacillin. Cloxacillin was added at 250 μg/ml. S, susceptible; I, intermediate susceptibility; R, resistant.

lected by ultracentrifugation at 50,000 × *g* for 35 min. OMPs were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Amplification of bla_{ampC} genes and sequence analysis. For each PCR experiment, 500 ng of total DNA was used in a standard PCR. The primers used have been previously described (Table 3) (22). Sequence analyses were performed using software available on the Internet at www.ncbi.nlm.nih.gov and http://www.ebi.ac.uk/clustalw/.

PCR amplification and sequencing of *oprD*. PCR amplification of *oprD* was performed by using the primers described in Table 3. Sequences were compared with that of reference strain *P. aeruginosa* PAO1.

Real-time RT-PCR studies. The levels of expression of *oprD*, *mexB*, *mexY*, and *mexD* were quantified using the one-step real-time quantitative reverse transcription PCR (RT-PCR), as recommended by Fey et al. (6). Expression level results were standardized according to the transcription level of the constitutively expressed *rpsL* ribosomal gene (Table 3) (8). Transcript quantification was performed by using the QuantiFast SYBR green RT-PCR kit on a LightCycler 1.0 instrument (Roche Diagnostics, Neuilly-sur-Seine, France). Isolates were considered to be MexAB-OprM, MexXY-OprM, MexCD-OprJ hyperproducers when the level of expression of *mexB*, *mexY*, or *mexD* were at least two-, four-, or twofold higher than that of PAO1, respectively, according to previously defined criteria (10, 21). Reduced *oprD* expression was considered relevant when it was ≤30% compared to that of the *P. aeruginosa* PAO1 reference strain (21).

RESULTS

Epidemiology of carbapenem-resistant isolates. Thirty-two (6.7%) out of the 474 nonduplicate *P. aeruginosa* isolates that had been recovered at the Bicêtre Hospital in 2007 were categorized as nonsusceptible to imipenem and to meropenem and therefore retained for this study. Overall, 87.5% and 78.1% of the selected isolates were resistant to imipenem and meropenem, respectively (Table 1). Genotyping performed using pulsed-field gel electrophoresis revealed high clonal diversity, with 22 distinct clones identified among the 32 isolates. One genotype (clone E) included eight isolates with two different subclones, E1 (two isolates) and E2 (six isolates).

OprD porin. All the isolates had reduced *oprD* expression (Table 2). Twenty-four isolates showed relative *oprD* expression of ≤10% compared to that of *P. aeruginosa* PAO1 (Table 2). Reduced expression of OprD was also assessed by OMP analysis (data not shown).

TABLE 2. Description of clinical isolates of *P. aeruginosa* used in this work

Isolate	Type of specimen	Clone	PDC variant[a]	Residue at position 105	AmpC overproducer	β-Lactamase sp act	ESAC phenotype[b]	Presence of OprD porin[c]	mRNA expression for[d]:			
									oprD	*mexB*	*mexY*	*mexD*
PAO-1			PDC-1	T	−	1	−	WT	1	1	1	1
Paeβ-01	Urinary	I	PDC-1	T	−	2 ± 1	−	No	0.2	0.8	**5.3**	**3.8**
Paeβ-02	Urinary	W	PDC-1	T	−	5 ± 1	−	Polymorphism	0.1	0.5	3.1	0.9
Paeβ-03	Urinary	C	PDC-2	A	+	62 ± 6	+	Polymorphism	0.02	0.9	2.3	0.6
Paeβ-04	Abscess	E1	PDC-2	A	+	100 ± 9	+	No	0.3	**5.6**	1.8	1.7
Paeβ-05	Blood	U	PDC-2	A	−	3 ± 1	−	No	0.01	0.5	1.9	1.7
Paeβ-06	Abscess	E2	PDC-2	A	+	42 ± 3	+	IS	0.0001	0.6	**5.9**	**2.2**
Paeβ-07	Pulmonary	E2	PDC-2	A	+	35 ± 5	+	IS	0.0002	0.3	**6.5**	**2.4**
Paeβ-08	Pulmonary	E2	PDC-2	A	+	34 ± 10	+	IS	0.0001	0.4	**5.9**	**3.2**
Paeβ-09	Urinary	E2	PDC-2	A	+	45 ± 8	+	IS	0.0005	0.5	**4.1**	**4.9**
Paeβ-10	Pulmonary	E1	PDC-2	A	+	67 ± 11	+	No	0.001	**5.7**	1.9	**2.2**
Paeβ-11	Urinary	E2	PDC-2	A	+	94 ± 13	+	IS	0.0001	0.5	**5.9**	**3.2**
Paeβ-12	Pulmonary	C	PDC-2	A	+	25 ± 4	+	Polymorphism	0.02	0.8	2.3	0.7
Paeβ-13	Pulmonary	C	PDC-2	A	+	120 ± 14	+	Polymorphism	0.02	0.9	2.4	0.7
Paeβ-14	Urinary	E2	PDC-2	A	+	54 ± 9	+	IS	0.0001	1.1	3.1	1.7
Paeβ-15	Abscess	A	PDC-2	A	+	310 ± 23	+	Polymorphism	0.1	0.8	**4.4**	1.1
Paeβ-16	Urinary	D	PDC-3	A	+	15 ± 2	+	No	0.2	1.1	0.4	1.8
Paeβ-17	Pulmonary	H	PDC-3	A	+	62 ± 7	+	No	0.3	1.2	**10.2**	**9.9**
Paeβ-18	Abscess	N	PDC-3	A	+	600 ± 25	+	No	0.005	**3.9**	**5.6**	1.6
Paeβ-19	Urinary	T	PDC-3	A	−	9 ± 2	−	WT	0.002	**11.9**	2.3	0.8
Paeβ-20	Pulmonary	M	PDC-4	A	+	34 ± 7	+	No	0.05	**5.9**	1.4	**7.7**
Paeβ-21	Pulmonary	O	PDC-4	A	+	200 ± 15	+	Polymorphism	0.1	1.3	**13.5**	1.3
Paeβ-22	Pulmonary	S	PDC-4	A	+	220 ± 9	+	No	0.1	0.9	0.5	**4.9**
Paeβ-23	Pulmonary	J	PDC-5	A	+	315 ± 21	+	No	0.003	0.8	0.3	0.9
Paeβ-24	Abscess	Q	PDC-5	A	+	290 ± 17	+	No	0.1	**5.4**	0.8	0.6
Paeβ-25	Pulmonary	B1	PDC-6	T	+	64 ± 8	−	No	0.1	1.3	0.3	0.6
Paeβ-26	Blood	B2	PDC-6	T	+	57 ± 6	−	Polymorphism	0.05	0.7	0.2	1.1
Paeβ-27	Pulmonary	F	PDC-7	A	+	17 ± 4	+	No	0.1	**3.4**	**5.5**	1.1
Paeβ-28	Abscess	P	PDC-7	A	+	84 ± 8	+	No	0.02	**2.2**	2.1	0.5
Paeβ-29	Urinary	G	PDC-8	A	−	2 ± 1	−	No	0.2	**3.3**	**4.2**	1.1
Paeβ-30	Urinary	V	PDC-8	A	−	2 ± 1	−	No	0.1	1.4	2.5	1.4
Paeβ-31	Abscess	K	PDC-9	A	−	3 ± 1	−	No	0.02	1.4	0.2	1.5
Paeβ-32	Urinary	L	PDC-10	A	+	104 ± 10	+	No	0.1	1.4	1.2	1.7

[a] PDC, *Pseudomonas*-derived cephalosporinase (22). Strains were classified according to their PDC sequence type.
[b] ESAC phenotype toward carbapenems and zwitterionic cephalosporins observed in the presence of cloxacillin added at 250 μg/ml.
[c] Determined by PCR and sequencing. WT, wild-type OprD sequence of *P. aeruginosa* PAO1; no, inactivating mutations detected but no corresponding proteins; polymorphism, amino acid substitutions compared to OprD of *P. aeruginosa* PAO1; IS, disruption by insertion sequence IS*Pa27*.
[d] Relative to the expression of *P. aeruginosa* PAO1, which is assigned a value of 1. Values are geometric means from at least two independent determinations. Standard deviations were within 15% of the geometric means. Isolates were considered to be MexAB-OprM, MexXY-OprM, or MexCD-OprJ hyperproducers when the level of expression of *mexB*, *mexY*, or *mexD* was at least two-, four-, or twofold higher than that of PAO1, respectively, according to previously reported studies (10, 21), and these values are indicated in boldface. Reduced *oprD* expression was considered when it was ≤70% of that of the *P. aeruginosa* PAO1 reference strain (21).

In order to evaluate the possible impact of qualitative modifications of OprD, the sequence of the *oprD* gene was also determined. Twenty-four isolates (17 distinct clones) out of the 32 isolates contained inactivating mutations of the *oprD* gene (Tables 2 and 4). The most frequent mechanisms of *oprD* inactivation resulted from 1-bp insertions/deletions or point mutations, leading to premature stop codons identified in 11 and 5 isolates, respectively. In two isolates, the OprD inactivation was due to a 4- or 5-bp insertion inside the coding sequence (Table 4). Finally, the inactivation of OprD was the result of a disruption of the coding sequence by a novel 1.3-kb insertion sequence (IS) element, named IS*Pa27* (submitted to the IS database at the Internet site http://www-is.biotoul.fr/), in six clonally related isolates (clone E2).

We also identified a shortened putative loop L7 of the OprD porin in the clones B2, O, and W (Table 4). This shortening may open the porin channel to allow optimal penetration of meropenem and increase its activity (5). Conversely, this alteration might not modify the susceptibility to a smaller-sized carbapenem molecule, such as imipenem (5). Interestingly, downregulated expression of the *oprD* gene observed in these clones may modulate the phenotype of imipenem and meropenem resistance (Table 2).

β-Lactamase expression. Synergy tests performed with clavulanic acid-ticarcillin and ceftazidime-containing disks did not evidence any inhibition of ceftazidime resistance for all the *P. aeruginosa* isolates, ruling out the production of extended-spectrum β-lactamases. Using cloxacillin-containing plates, the MICs of ceftazidime were decreased by at least twofold for 25 out of 32 isolates (78% of total), suggesting overproduction of

TABLE 3. Primers used in this work

Primer	Sequence (5′–3′)	PCR product size (bp)	Use
PreAmpC-PA1	ATGCAGCCAACGACAAAGG	1,243	Amplification and sequencing of entire *bla*$_{ampC}$ genes
PostAmpC-PA2	CGCCCTCGCGAGCGCGCTTC		
ampC-PA-A	CTTCCACACTGCTGTTCGCC	1,063	Amplification and sequencing of *bla*$_{ampC}$ genes
ampC-PA-B	TTGGCCAGGATCACCAGTCC		
OprD-F	CGCCGACAAGAAGAACTAGC	1,412	Amplification and sequencing of *oprD* genes
OprD-R	GTCGATTACAGGATCGACAG		
OprD-F2	GCCGACCACCGTCAAATCG		Sequencing of *oprD* genes
MxB-U	CAAGGGCGTCGGTGACTTCCAG	272	Quantitative real-time PCR of *mexB*
MxB-L	ACCTGGGAACCGTCGGGATTGA		
MxY-U	GGACCACGCCGAAACCGAACG	522	Quantitative real-time PCR of *mexY*
MxY-L	CGCCGCAACTGACCCGCTACA		
MxD-For	GGACGGCTCGCTGGTCCGGCT	236	Quantitative real-time PCR of *mexD*
MxD-Rev	CGACGAAGCGCGAGGTGTCGT		
OprD-For	GCTCGACCTCGAGGCAGGCCA	242	Quantitative real-time PCR of *oprD*
OprD-Rev	CCAGCGATTGGTCGGATGCCA		
Rspl-1	GCTGCAAAACTGCCCGCAACG	249	Quantitative real-time PCR of *rpsL*
Rspl-2	ACCCGAGGTGTCCAGCGAACC		
ISPa27-A	GTGGCAACAACGGCCACTGGA	239	Amplification of IS*Pa27* insertion sequence
ISPa27-B	CGGCAATCAGCACATCCTCGA		
OXA-IMP1	GCAAATAMAGAATATGTSCC	495	Amplification of *bla*$_{\text{OXA-23}}$ and *bla*$_{\text{OXA-40}}$ genes
OXA-IMP2	CTCMACCCARCCRGTCAACC		
OXA-48A	TTGGTGGCATCGATTATCGG	744	Amplification of *bla*$_{\text{OXA-48}}$ gene
OXA-48B	GAGCACTTCTTTTGTGATGGC		
OXA-58A	CGATCAGAATGTTCAAGCGC	529	Amplification of *bla*$_{\text{OXA-58}}$ gene
OXA-58B	ACGATTCTCCCCTCTGCGC		
IMP-A	GAAGGYGTTTATGTTCATAC	587	Amplification of *bla*$_{\text{IMP}}$-type genes
IMP-B	GTAMGTTTCAAGAGTGATGC		
VIM2004A	GTTTGGTCGCATATCGCAAC	382	Amplification of *bla*$_{\text{VIM}}$-type genes
VIM2004B	AATGCGCAGCACCAGGATAG		
SPM-1A	CTGCTTGGATTCATGGGCGC	783	Amplification of *bla*$_{\text{SPM-1}}$ gene
SPM-1B	CCTTTTCCGCGACCTTGATC		

AmpC that was confirmed by determination of β-lactamase activities (Table 2). Twenty-one out of those 25 isolates overexpressed the AmpC β-lactamase and had reduced MICs of ceftazidime but also of imipenem and cefepime after cloxacillin addition (Table 1), suggesting the presence of an ESAC (Tables 1 and 2). PCR and sequencing identified 10 AmpC β-lactamase variants, with 8 of those variants possessing an alanine residue at position 105 (Table 2). This residue had been previously shown to be the key factor for an ESAC property (Table 2) (22). All isolates were negative for *bla*$_{\text{IMP}}$-, *bla*$_{\text{VIM}}$-, *bla*$_{\text{SPM}}$-, *bla*$_{\text{AIM}}$-, and *bla*$_{\text{OXA}}$-type genes by PCR screening.

Efflux pump expression. Overexpression of the MexAB-OprM, MexXY-OprM, and MexCD-OprJ efflux systems was demonstrated in 28%, 37.5%, and 31% of the carbapenem-nonsusceptible isolates, respectively, suggesting the contribution of efflux in the resistance (Table 2). Seven isolates overexpressed only one efflux pump, and 12 isolates overexpressed two efflux pumps. Sixty percent of the isolates overexpressed at least one efflux pump. No isolate overexpressed all three efflux pumps.

DISCUSSION

Whereas OprD inactivation alone is the source of intermediate susceptibility or resistance to imipenem, the mechanisms leading to meropenem resistance seem to be more complex and are very likely multifactorial, involving overproduction of AmpC or overexpression of the efflux pumps MexAB-OprM, MexXY-OprM, and MexCD-OprJ (4, 21). In our collection, clonal spread played a rather minor role in the epidemiology of infections caused by *P. aeruginosa*, and the main mechanism associated with reduced susceptibility or resistance to imipenem was the absence or weak expression of OprD found in all the isolates.

Our data show that both *oprD* gene downregulation and OprD protein inactivation contributed to resistance to imipenem and reduced susceptibility to meropenem. Noteworthy, the inactivating mutations identified in the *oprD* gene of isolates Paeβ-05 and Paeβ-23 (corresponding to clones U and J, respectively) (Tables 2 and 4) had been previously identified in carbapenem-resistant *P. aeruginosa* isolates from Spain, Portugal, and the United States (8, 17).

Noteworthy, the prevalence of the ESAC-encoding genes in our collection was very high, with 28 out of the 32 isolates. In addition, overproduction of those ESAC enzymes was evidenced in most of the isolates (75%). Basically, when considering the high rate of OprD-deficient isolates, it might be hypothesized that ESAC overproduction could play a secondary but additive role toward reduced susceptibility or resistance to imipenem. Of note, the additive presence of overexpressed ESACs and loss of OprD was correlated with resistance to imipenem in all cases (Tables 1 and 2).

On the other hand, the main mechanism associated with reduced susceptibility or resistance to meropenem was probably overexpression of efflux pumps identified in 60% of the

TABLE 4. Inactivating mutations in *oprD* and modifications affecting carbapenem resistance

Type of inactivating mutation	Clone(s)	Mutation(s)[a]
Frameshift mutation produced by 1-bp insertion or deletion	B1	Insertion of 1 bp (G) at GG repeat (nt 840-841)
	E1	Insertion of 1 bp (C) at CC repeat (nt 872-873)
	F	Insertion of 1 bp (G) at GGGGG repeat (nt 631-635)
	G	Insertion of 1 bp (C) at CC repeat (nt 1,203-1,204)
	K	Insertion of 1 bp (T) at GGC**T**GAC (nt 949-954)
	M	Deletion of 1 bp (T) at CAA**T**CAC (nt 644-650)
	N	Insertion of 1 bp (G) at GG repeat (nt 837-838)
	P	Deletion of 1 bp (G) at GG repeat (nt 373-374)
	S	Insertion of 1 bp (C) at CC repeat (nt 1,203-1,204)
	V	Deletion of 1 bp (A) at AAA repeat (nt 783-785)
Premature stop codon	D	T**G**G→T**A**G at nt 1,016
	I	T**C**C→T**AA** at nt 1,013-1,014
	J	TG**G**→TG**A** at nt 195
	L	T**G**G→T**A**G at nt 830
	U	TG**G**→TG**A** at nt 831
Partial insertion in the coding sequence	H	4-bp insertion beginning at nt 645
	Q	5-bp insertion beginning at nt 1,023
Disruption of the coding sequence by IS insertion	E2	1,363-bp IS (IS*Pa27*) at nt 559; encodes a 415-aa putative IS*256*-type transposase
None	A, C	Several nonunique polymorphisms in OprD: T103S, K115T, F170L, E185Q, P186G, V189T, A293P, R310E, A315G, and G425A
	B2, O, W	Shortening of putative loop L7 (372V-DSSSSYAGL-Y384) (5)

[a] Nucleotide (nt) and amino acid (aa) numbers according to the published *oprD* sequence of PAO1. Boldface indicates mutated nucleotides. Clone T showed the wild-type sequence for OprD compared to that of *P. aeruginosa* PAO1.

isolates, together with the loss of OprD. ESAC overexpression (65% of the isolates) could play an additive role in reduced susceptibility or resistance to meropenem. Thirteen isolates did not overexpress any efflux pump, suggesting that the lack of OprD, overexpression of AmpC, or production of ESAC β-lactamases (or other unknown mechanisms) may explain meropenem resistance (9). The additive presence of overexpressed ESACs and overexpression of at least one efflux pump correlated with resistance to meropenem in 87.5% of the cases (14 out of 16 isolates with this combination of mechanisms of resistance) (Tables 1 and 2).

Our results show that *P. aeruginosa* may very often accumulate different resistance mechanisms (overproduction of AmpC cephalosporinase, ESACs, increased drug efflux, and deficient production or loss of porin OprD). One of the most interesting and original results obtained from the present study is that *P. aeruginosa*-type ESACs probably contribute to carbapenem resistance.

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