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Alterations of OprD in Carbapenem-Intermediate and -Susceptible Strains of *Pseudomonas aeruginosa* Isolated from Patients with Bacteremia in a Spanish Multicenter Study

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We investigated the presence of OprD mutations in 60 strains of metallo-ß-lactamase-negative *Pseudomonas aeruginosa* intermediately susceptible (IS [*n* = 12]; MIC = 8 μg/ml) or susceptible (S [*n* = 48]; MICs ≤ 1 to 4 μg/ml) to imipenem and/or meropenem that were isolated from patients with bacteremia in order to evaluate their impact on carbapenem susceptibility profiles. The presence of mutations in *oprD* was detected by sequencing analysis. OprD expression was assessed by both outer membrane protein (OMP) analysis and real-time PCR (RT-PCR). Fourteen (23%) isolates had an OprD identical to that of PAO1, and OprD modifications were detected in 46 isolates (77%). Isolates were classified as OprD "full-length types" (T1 [*n* = 40, including both wild-type OprD and variants showing several polymorphisms]) and OprD "deficient types" (T2 [*n* = 3 for OprD frameshift mutations] and T3 [*n* = 17 for premature stop codons in *oprD*]). RT-PCR showed that 5 OprD type T1 isolates presented reduced transcription of *oprD* (0.1- to 0.4-fold compared to PAO1), while *oprD* levels increased more than 2-fold over that seen with PAO1 in 4 OprD type T1 isolates. A total of 50% of the isolates belonging to OprD "deficient types" were susceptible to both carbapenems, and 40% were susceptible to meropenem and intermediately susceptible to imipenem. Only one isolate (5%) within this group was intermediately susceptible to both carbapenems, and one (5%) was susceptible to imipenem and intermediately susceptible to meropenem. We concluded that OprD inactivating mutations in clinical isolates of *P. aeruginosa* are not restricted only to carbapenem-resistant isolates but are also found in isolates with imipenem or meropenem MICs of only 0.06 to 4 μg/ml.

Pseudomonas aeruginosa has become an important and frequent opportunistic nosocomial pathogen. This organism is characterized by an innate resistance to multiple classes of antimicrobials (12), causing difficult-to-treat infections, which are therefore associated with significant morbidity and mortality (28).

The broad-spectrum resistance of *P. aeruginosa* is mainly due to a combination of different factors: (i) low outer membrane permeability (27), (ii) the presence of the inducible AmpC chromosomal ß-lactamase (19, 20), (iii) synergistic action of several multidrug efflux systems (32, 33), and (iv) the prevalence of transferable resistance determinants, in particular, carbapenem-hydrolyzing enzymes (mainly metallo-ß-lactamases [MBLs]) (11).

Among the several mutation-mediated resistance mechanisms existing in *P. aeruginosa* are those conferring decreased susceptibility or resistance to carbapenems. These antimicrobial agents are commonly used to treat infections produced by multiresistant strains of *P. aeruginosa*, as they are stable against most clinically relevant ß-lactamases (including broad-spectrum, extended-spectrum, and AmpC-type enzymes). Carbapenems exert their action primarily by inhibiting the peptidoglycan-assembling transpeptidases (penicillin-binding proteins [PBP]) located on the outer face of the cytoplasmic membrane. In general, carbapenems can efficiently cross the outer membrane of the bacterium, as they are small hydrophilic antibiotics. They enter the cell by passing through the aqueous channels provided by porin proteins. The main porin for uptake of carbapenems in *P. aeruginosa* is the outer membrane protein (OMP) OprD, a specialized porin which has a specific role in the uptake of positively charged amino acids such as lysine and glutamate (14). Other routes for carbapenem uptake have been proposed (29), but their actual relevance has not been consistently proved.

Inactivating mutations in OprD have been documented to confer resistance to imipenem and to a lesser extent to meropenem and doripenem (8, 10, 11, 30, 34, 35, 39, 43). It is also remarkable that mutations leading to the upregulation of the MexAB-OprM active efflux system may increase the resistance to meropenem and doripenem but with no effect on the susceptibility of *P. aeruginosa* to imipenem, which is not a substrate for this system (16). Additionally, other resistance-nodulation-cell division (RND) efflux systems such as MexCD-OprJ and MexXY-OprM were previously suggested to be involved in reduced susceptibility to meropenem (1, 23, 24, 44); nevertheless, their

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TABLE 1 Primers used in this study

Gene	Primer name	Sequence 5′–3′	Source or reference	Use
oprD	OprD_FlankF	CGGCTGAGGGGAAAGTCGCC	This study	Sequencing of *oprD*
	OprD_FlankR	TACGCGGTCATTCTCGGGCG		
	OprD.F	GGAACCTCAACTATCGCCAAG	36	
	OprD.R	GTTGCCTGTCGGTCGATTAC		
	OprD2.F	ACTTCACCGAGGGCAAGG		
	OprD2.R	CAGAGTTGGCGAGGAAAATC		
	RTiOprD_F	GGTTTCCGCAGGTAGCACTCAGT	This study	Real-time PCR
	RTiOprD_R	AAGCCGGATTCATAGGTGGTGAG		
proC	ProC_for	CCTGCTCCACCAGTGCTT	25	
	ProC_rev	CTGTCCAGCGAGGTCGAG		
mexT	MexT_F1	CATCAGCACCACGTCGCCAT	This study	Sequencing of *mexT*
	MexT_R1	ACTTCGGCGACGCTGACCTT		
	MexT_F2	CGCGAGCCCAGGAAATCTTC		
	MexT_R2	TAGCTGACGCCCACCGAGAT		

implication in carbapenem resistance remains still uncertain. A recent study suggested that OprD inactivation combined with AmpC overexpression was the most relevant mechanism to confer resistance to doripenem in clinical isolates and laboratory mutants of *P. aeruginosa* (35).

Less frequently, downregulation of *oprD* can happen in the so-called *nfxC* mutants, in which MexEF-OprN is overexpressed. This coregulation is mediated by MexT, a positive regulator of the efflux system MexEF-OprN, which also downregulates OprD at the transcriptional and posttranscriptional levels. This phenotype is sometimes selected by quinolones but rarely by carbapenems (17). There are a few studies suggesting a role of the penicillin-binding proteins (PBPs) in acquired resistance to carbapenems (2, 13, 45). Carbapenem resistance can also arise from production of MBLs, but this mechanism is a less commonly found mechanism than the mutation-driven resistance mechanisms (4).

Regardless of the fact that efflux system upregulation, constitutive AmpC hyperproduction, and OprD alterations have been well described as carbapenem resistance mechanisms in laboratory mutants, there is still little information on how these mechanisms interplay in clinical isolates of *P. aeruginosa*, particularly in those presenting a low level of resistance. A recent study has addressed the issue of how the carbapenem resistance patterns observed in clinical isolates of *P. aeruginosa* are not fully explained by some of the aforementioned mechanisms (8).

In this study, we have analyzed the alterations of OprD in a number of clinical isolates of *P. aeruginosa* with different carbapenem susceptibility profiles, ranging from susceptible to intermediately susceptible. Sequence and computerized structure analyses of OprD were carried out in order to correlate inactivating mutations and other mechanisms with the resistance patterns observed. The presence of additional mechanisms involved in carbapenem resistance in clinical isolates of *P. aeruginosa* was also investigated.

MATERIALS AND METHODS

Selection of bacterial strains. A total of 60 nonduplicate clinical isolates of metallo-ß-lactamase-negative *P. aeruginosa* with different carbapenem susceptibility profiles, ranging from susceptible to intermediately susceptible, were included in this study. These isolates were selected from a collection of 190 strains previously studied that were recovered from bloodstream infections in a Spanish multicenter study carried out in 2008, for which OprD analysis had been carried out in 25 imipenem-resistant strains (MIC $>$ 8 mg/liter) (4).

P. aeruginosa strains PA-14 and PAO1 and two PAO1-derived mutants, PA3047 (hyperproducing AmpC) from the *P. aeruginosa* transposon mutant collection of the University of Washington (Seattle, WA) and PAO45 (OprD deficient) (42), were also included in the study. MICs of antibiotics other than carbapenems and expression levels of the genes encoding the chromosomal β-lactamase AmpC (*ampC*) protein and the four major *P. aeruginosa* efflux pump systems, MexAB-OprM (*mexB*), MexXY-OprM (*mexY*), MexCD-OprJ (*mexD*), and MexEF-OprN (*mexF*), were determined in the 60 clinical isolates in comparison to the wild-type PAO1 strain by real-time PCR (RT-PCR) in a previous work (4).

Molecular typing and antimicrobial susceptibility testing of clinical isolates. Clonal relatedness was evaluated through pulsed-field gel electrophoresis (PFGE) analysis following previously described protocols (38). The PFGE profiles were analyzed according to the criteria of Tenover et al. (41).

The MICs of imipenem and meropenem were determined by broth microdilution as described before (4). For some strains, the MICs of imipenem and/or meropenem were also determined by Etest (bioMérieux, Marcy l'Etoile, France) as described by the manufacturer. In all cases, MIC breakpoints were those defined by CLSI (5).

Carbapenem hydrolysis assay. Carbapenemase activity in crude sonicated bacterial extracts was quantified in a spectrophotometer, according to previously described protocols (18), with slight modifications. Briefly, reactions were performed in 1 ml of 50 mM assay buffer (AB) consisting of HEPES (pH 7.0) supplemented with 5 μM $ZnSO_4$ (Sigma, Madrid, Spain) at 25°C. Hydrolysis of both imipenem and meropenem was monitored at 300 and 297 nm, respectively, using an initial substrate concentration of 150 μM. *P. aeruginosa* strains COL-1 (VIM-2-producing strain) (31) and PAO1 were used as positive and negative controls, respectively.

DNA sequencing and computer analyses of sequence data. The presence of inactivating mutations in *oprD* was investigated in all the isolates through PCR amplification of the entire gene and its upstream flanking region, containing the promoter, and downstream of the structural gene with specific primers, OprD_FlankF and OprD_FlankR. These primers and two additional pairs of oligonucleotides that were previously described (36) were employed for sequencing of *oprD*. The *mexT* gene was also sequenced in those strains showing high expression levels of *mexF*. The primers used for this purpose are listed in Table 1. PCR amplifications were conducted in a total volume of 50 μl containing 0.25 mM deoxynucleoside triphosphates (dNTPs), 1 mM $MgCl_2$, 0.2 μM each primer, 2 U of AmpliTaq Gold DNA polymerase (Applied Biosystems), and 500 ng of template DNA. PCR conditions were as follows: 95°C for 5 min and 30

TABLE 2 Prevalence of OprD types among the clinical isolates of *Pseudomonas aeruginosa* showing different carbapenem resistance phenotypes contrasted to overexpression of AmpC ß-lactamase and efflux pump systems

Resistance phenotype (μg/ml)[a]	Total no. of strains (%)	No. of isolates within indicated OprD type[b] (%)			No. of isolates showing overexpression of indicated product (%)					
		T1	T2	T3	AmpC	MexB	MexY	MexF	MexD	Others[c]
All	60 (100)	40 (68)	3 (5)	17 (28)	11 (18)	4 (7)	5 (8)	4 (7)	0	6 (10)
Group 1										
I^{IS}-M^{IS}	1 (2)	0	0	1 (100)	1 (100)	0	0	1 (100)	0	1[d] (100)
Group 2										
I^{IS}-M^{S}	9 (15)	1 (33)	0	8 (89)	0	0	1 (11)	1 (11)	0	0
Group 3										
I^{S}-M^{IS}	2 (3)	1 (50)	1 (50)	0	1 (50)	1 (50)	1 (50)	1 (50)	0	1[d], 1[e] (100)
Group 4										
I^{S}-M^{S}	48 (80)	38 (79)	2 (4)	8 (17)	9 (19)	3 (6)	3 (6)	1 (2)	0	3 (6)
Subgroups	48 (80)	38 (79)	2 (4)	8 (17)	9 (19)	3 (6)	3 (6)	1 (2)	0	3 (6)
$I^{S\,(\leq 1)}$-$M^{S\,(\leq 1)}$	16 (27)	14 (87)	1 (7)	1 (6)	4 (25)	0	0	0	0	0
$I^{S\,(2-4)}$-$M^{S\,(2-4)}$	9 (15)	7 (78)	1 (11)	1(11)	2 (22)	1 (11)	0	0	0	1[f]
$I^{S\,(\leq 1)}$-$M^{S\,(2-4)}$	3 (5)	3 (100)	0	0	0	2 (67)	1 (33)	1 (33)	0	1[e], 1[g] (33)
$I^{S\,(2-4)}$-$M^{S\,(\leq 1)}$	20 (33)	14 (70)	0	6 (30)	3 (15)	0	2 (10)	0	0	0

[a] I, imipenem; M, meropenem; R, resistant; IS, intermediately susceptible; S, susceptible.
[b] Classification is indicated according to the type of mutation present in *oprD* and OprD as follows: T1, full-length type (including wild-type OprD and OprD variants showing several polymorphisms in the amino acid sequence); T2 and T3, "deficient types" (i.e., mutations result in earlier termination of translation); T2, premature stop codon, T3, frameshift mutation due to insertions or deletions.
[c] "Other"data represent combinations of different resistance mechanisms as indicated.
[d] AmpC and MexF resistance mechanisms.
[e] MexB and MexY resistance mechanisms.
[f] AmpC and MexB resistance mechanisms.
[g] MexB and MexF resistance mechanisms.

cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 2 min. For each gene, DNA sequences on both strands were determined by using an external resource (Macrogen Inc., Seoul, South Korea). Computer analysis of the DNA sequence data was performed with Vector NTI Advance version 9.0.0 software (InforMax; Invitrogen). Protein alignments were carried out using ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/). In every case, both the nucleotide and the amino acid sequences were compared between the clinical isolates, the PAO1 reference strain, strains PA14 (GenBank accession no. ABJ10119), PA7 (accession no. YP_001349899), LESB58 (accession no. YP_002441940), and MFY72 (accession no. CAL23856), and clinical isolates 59 and 120, two imipenem-resistant strains analyzed in a previous study (4). OprD secondary structures were compared to the one reported by Biswas et al. (3), deposited in the Protein Data Bank (http://www.pdb.org) under accession code 2ODJ, and visualized by using the ESPript 2.0 software (http://espript.ibcp.fr/ESPript/ESPript/). Images of OprD tridimensional (3D) structures were represented using PyMol (http://www.pymol.org/).

OMP analysis. Cultures of *P. aeruginosa* were grown overnight at 37°C in 5 ml of Mueller-Hinton Broth medium (Difco/Becton Dickinson, Sparks, MD) and then diluted 100-fold into fresh medium. Bacterial cells were incubated for approximately 5 h with shaking at 37°C to yield late-logarithmic-phase cells. Outer membrane protein (OMP) profiles were examined using a previously reported method (26). OMPs were run on standard 12% sodium dodecyl sulfate (SDS)-polyacrylamide gels and stained with Coomassie blue. OprD profiles from clinical isolates were compared with those of the PAO1 reference strain and PAO45.

Quantification of *oprD* expression by RT-PCR. The levels of expression of the *oprD* gene were determined by real-time PCR (RT-PCR) in a selection of isolates. Primers used for RT-PCR are listed in Table 1. Briefly, strains were grown in 10 ml of LB broth at 37°C and 180 rpm to the late log phase (optical density at 600 nm [OD_{600}] = 0.8 to 1) and collected by centrifugation. Total RNA was extracted with an SV Total RNA isolation system (Promega, Madison, WI), according to the manufacturer's recommendations. DNA was removed with RNase-Free DNase treatment (Ambion, Austin, TX), and RNA preparations were tested for the lack of DNA contamination by RT-PCR with primers specific for the *proC* gene (25). None of the RNA samples used in the study amplified before cycle 35. RNA concentrations were determined in a spectrophotometer (NanoDrop 1000; Thermo Scientific, Wilmington, DE), and the quality of the samples was checked in agarose gels under denatured conditions. Quantification of the *oprD* gene was performed by quantitative PCR (qPCR) using two-step reverse transcription- and RT-PCR and an iScript cDNA synthesis kit (Bio-Rad) and SYBR Premix Ex *Taq* (TaKaRa). The *proC* gene was used as an internal reference to normalize the relative amount of RNA (25). The experiment was repeated in triplicate, using independent RNA extractions in every assay. The expression of the *oprD* gene in each isolate was compared to its expression in the PAO1 reference strain. The genes were considered to be up- or downregulated when the amounts of RNA were at least 2 times higher- or lower than those in the reference strain.

RESULTS AND DISCUSSION

Loss of OprD from the outer membranes is one of the most important mechanisms of resistance to carbapenems in *P. aeruginosa*. Multiple studies have evaluated the importance of OprD alterations in laboratory and clinical isolates of *P. aeruginosa* resistant to carbapenems. However, there is little information on the relevance of this mechanism in intermediately susceptible isolates or with MICs near the breakpoints for susceptibility. Dib et al. studied the implications of loss of OprD and its association with decreases in susceptibility to carbapenems in a collection of 89 clinical isolates of *P. aeruginosa* (6). Those authors demonstrated a correlation between the levels of expression of OprD and the degrees of susceptibility to imipenem. Nevertheless, they detected decreased expression of OprD in some strains showing imipenem MICs of 2 to 4 μg/ml. Subsequent studies have also assessed the relationship between OprD expression and carbapenem susceptibility profiles in clinical strains of *P. aeruginosa.* El Amin et al.

TABLE 3 Different OprD types found among the 60 clinical isolates of *P. aeruginosa*

OprD type(s)[a]	No. of isolates	Resistance phenotype(s) (no. of isolates)[b]	OprD type classification	Alteration(s) or mutation(s)[j]	Effect or OprD structure(s) affected
T1			Full-length type		
T1-I	14	I^S-M^S (13), I^{IS}-M^S (1)	Wild type	None	None
T1-Ia	1	I^S-M^S	Substitution of 1 amino acid residue	$L_{440}P$	None
T1-II	4	I^S-M^S (4)	Several polymorphisms	$T_{103}S$, $K_{115}T$, $F_{170}L$	L4[i], L6
T1-IIa	1	I^S-M^S		$D_{43}N$, $T_{103}S$, $K_{115}T$, $F_{170}L$	L4, L6
T1-IIb	1	I^S-M^S		$V_{82}L$, $T_{103}S$, $K_{115}T$, $F_{170}L$	L4, L6
T1-III	1	I^S-M^S		$T_{103}S$, $K_{115}T$, $F_{170}L$, $Y_{438}L$, $P_{439}S$, $L_{440}A$, $S_{441}V$, $I_{442}D$, $L_{443}P$, $_{443}+53$[c]	L4, L6 C-terminal extreme
T1-IIIa	1	I^S-M^{IS}		$T_{103}S$, $K_{115}T$, $F_{170}L$, $Y_{438}L$, $P_{439}S$, $L_{440}A$, $S_{441}V$, $I_{442}D$, $L_{443}P$, $_{443}+52$[d]	L4, L6 C-terminal extreme
T1-IV	3	I^S-M^S		$T_{103}S$, $K_{115}T$, $F_{170}L$, $E_{185}Q$, $P_{186}G$, $V_{189}T$, $R_{310}E$, $A_{315}G$, $G_{425}A$	L4, L6, L13
T1-IVa	1	I^S-M^S		$T_{103}S$, $K_{115}T$, $F_{170}L$, $E_{185}Q$, $P_{186}G$, $V_{189}T$, $R_{310}E$, $A_{315}G$, $G_{425}A$, $S_{441}K$	L4, L6, L13
T1-IVb	1	I^S-M^S		$T_{103}S$, $K_{115}T$, $F_{170}L$, $E_{185}Q$, $P_{186}G$, $V_{189}T$, $R_{310}E$, $A_{315}G$, $G_{425}A$, $Y_{438}S$	L4, L6, L13
T1-V	1	I^S-M^S	Several polymorphisms	$T_{103}S$, $K_{115}T$, $F_{170}L$, $E_{185}Q$, $P_{186}G$, $V_{189}T$, $R_{310}E$, $A_{315}G$, $G_{425}A$, $L_{440}T$, $S_{441}V$, $I_{442}D$, $L_{443}P$, $_{443}+57$[e]	L4, L6, L13 C-terminal extreme
T1-VI	9	I^S-M^S		$D_{43}N$, $S_{57}E$, $S_{59}R$, $E_{202}Q$, $I_{210}A$, $E_{230}K$, $S_{240}T$, $N_{262}T$, $A_{267}S$, $A_{281}G$, $K_{296}Q$, $Q_{301}E$, $R_{310}G$, $V_{359}L$,$_{372}$(VDSSSS-YAGL-)$_{383}$[f]	L2, L7, L8, L9, L15
T1-VIa	1	I^S-M^S		$D_{43}N$, $S_{57}E$, $S_{59}R$, $E_{202}Q$, $I_{210}A$, $E_{230}K$, $S_{240}T$, $N_{262}T$, $A_{267}S$, $A_{281}G$, $K_{296}Q$, $Q_{301}E$, $R_{310}G$, $V_{359}L$, $_{372}$(VDSSSS-YAGL-)$_{383}$, $Y_{438}V$, $P_{439}S$	L1, L7, L8, L9, L15
T1-VII	1	I^S-M^S		$S_{57}E$, $S_{59}R$, $V_{127}L$, $E_{185}Q$, $V_{189}T$, $E_{202}Q$, $I_{210}A$, $E_{230}K$, $S_{240}T$, $N_{262}T$, $T_{276}A$, $A_{281}G$, $K_{296}Q$, $Q_{301}E$, $R_{310}E$, $A_{315}G$, $L_{374}M$, $_{372}$(VDSSSS-YAGL)$_{383}$, $S_{403}A$, $Q_{424}E$, $D_{437}E$	L1, L4, L5, L7, 15
T2, T3			Porin-deficient types		
T2			Premature stop codon		Loss of porin
	2	I^S-M^S		$_{nt\ 180}$G<u>G</u>A→<u>T</u>GA	
	1	I^S-M^{IS}		$_{nt\ 424}$C<u>A</u>A→<u>T</u>AA	
T3			Frame shift mutations due to insertions or deletions		Loss of porin
	1	I^{IS}-M^S		$_{nt\ 22}G_{nt\ 23}$[g]	
	1	I^{IS}-M^S		$_{nt\ 64}C_{nt\ 65}$	
	1	I^S-M^S		$_{nt\ 66}T_{nt\ 67}$	
	1	I^{IS}-M^S		$_{nt\ 157}C_{nt\ 158}$	
	1	I^{IS}-M^S		$_{nt\ 368}A_{nt\ 369}$	
	1	I^{IS}-M^{IS}		$_{nt\ 934}T_{nt\ 935}$	
	1	I^S-M^S		$_{nt\ 936}$CGTA$_{nt\ 937}$	
	6	I^S-M^S (4), I^{IS}-M^S (2)		$_{nt\ 939}C_{nt\ 940}$	
	1	I^{IS}-M^S		$_{nt\ 392}\Delta C$	
	2	I^S-M^S, I^{IS}-M^S		$_{nt\ 1295}\Delta T$[h]	
	1	I^S-M^S		$_{nt\ 1297}\Delta C$[h]	

[a] OprD type T1 has been divided in 7 subtypes based on the amino acid pattern alterations.
[b] Numbers in parentheses represent numbers of strain showing a determined resistance phenotype.
[c] This OprD type has 53 additional amino acids residues at its C-terminal region.
[d] This OprD type has 52 additional amino acids residues at its C-terminal region.
[e] This OprD type has 57 additional amino acids residues at its C-terminal region.
[f] Divergent sequence of 10 amino acid residues previously reported by Epp et al. (9).
[g] This OprD lacks 20 amino acid residues at the start of its N-terminal region.
[h] The isolates have a deletion of 10 amino acid residues from position $_{434}$(LIVDYPLSIL)$_{443}$.
[i] "L" designates any of the OprD loops possibly affected by a determined amino acid substitution.
[j] Underlining indicates the nucleotide that changes.

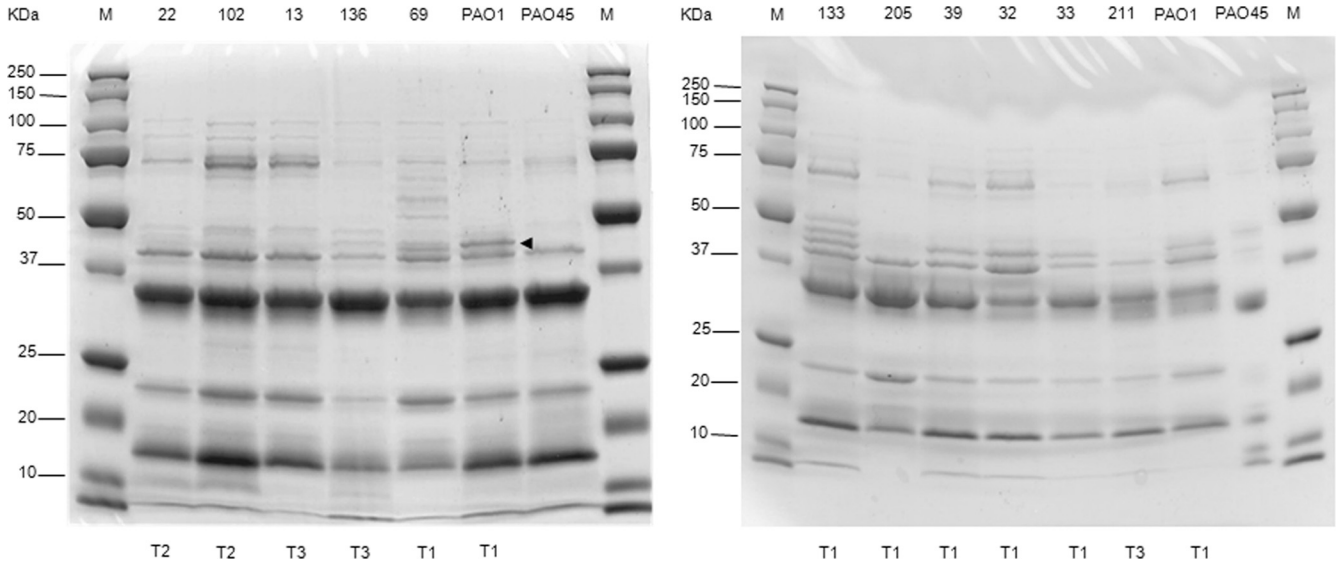


FIG 1 Analysis of *P. aeruginosa* outer membrane proteins (OMPs) in a selection of clinical strains with different OprD types. OMPs were run in a 12% SDS polyacrylamide gel. Lanes M: Precision Plus protein standards (Bio-Rad). OMP profiles of clinical strains were compared to those of PAO1 and PAO45, an OprD-deficient mutant. Strain numbers are shown above the lanes. OprD types are indicated below the lanes. The black arrowhead indicates the PAO1 OprD band.

evaluated the expression of *oprD* among clinical isolates of *P. aeruginosa* and found significant reduction of *oprD* transcription in 5 susceptible and 5 intermediately susceptible strains (8).

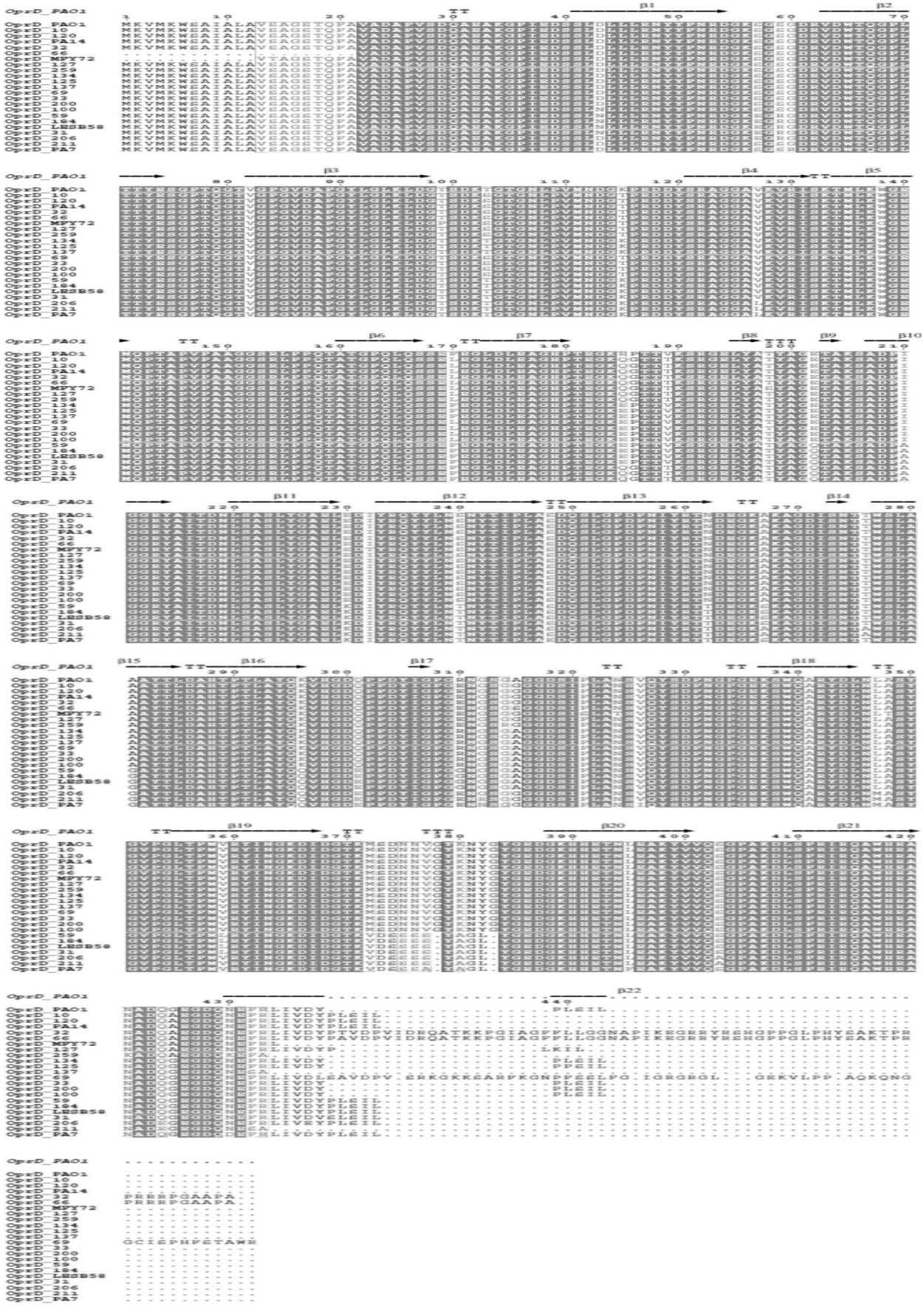
With this study, we aimed to gain an insight into the relationship between OprD alterations and/or expression levels and carbapenem susceptibility profiles in carbapenem-intermediate and -susceptible clinical strains of *P. aeruginosa*. We analyzed the impact of OprD alterations in 60 clinical isolates of metallo-ß-lactamase-negative *P. aeruginosa*, many of them with carbapenem MICs close to the susceptible breakpoints. These isolates belonged to a well-characterized collection of 190 *P. aeruginosa* strains from which all the carbapenem resistance mechanisms known so far were previously evaluated (4). Selection of the isolates was based on their carbapenem susceptibility profiles, including organisms with a broad range of susceptibility: susceptible (MICs $\leq$ 1 to 4 μg/ml) or intermediately susceptible (MIC = 8 μg/ml) to both imipenem and meropenem. The evaluation of the alterations and mutations found in OprD of clinical strains was complemented with the analysis of the OprD secondary structure based on the PAO1 OprD crystal structure recently published (3).

High clonal diversity was observed among the 60 isolates, with 54 different PFGE patterns (see Table S1 in the supplemental material). The 60 clinical isolates of *P. aeruginosa* analyzed in this study were divided in 4 groups (and 4 subgroups within the group of isolates susceptible to both imipenem and meropenem) based on carbapenem sensitivity patterns (Table 1; see also Table S1 in the supplemental material). Fifty clinical isolates (83%) showed susceptibility to imipenem and 57 (95%) to meropenem. Ten isolates (17%) were intermediately susceptible to imipenem and 3 isolates (5%) to meropenem. No carbapenemase activity was found among the 60 isolates studied, as confirmed by the carbapenem hydrolysis assay (data not shown).

Sequencing of the entire *oprD* gene and its putative promoter regions permitted analysis of the alterations in OprD in such strains. Most of the isolates presented point mutations consisting of single nucleotide deletions or nucleotide substitutions between positions −62 and −72 (relative to the ATG start codon). Such mutations were observed in both susceptible and resistant isolates; therefore, we considered their involvement in decreased carbapenem susceptibility not relevant.

Classification of the isolates in 3 major types (T1, T2, and T3) was established based on the pattern of alterations or mutations found in OprD (Tables 2 and 3). The OprD type T1 grouped those isolates showing "full-length type" OprD. This type was subdivided in 7 groups, comprising those isolates showing a wild-type PAO1 OprD and several OprD allelic variants, due to amino acid substitutions. The OprD types T2 and T3 were called "deficient types," as their mutations resulted in loss of porin as confirmed by analysis of their OMP profiles (representative results are shown in Fig. 1). The OprD type T2 designates the porin type in which the substitution of a nucleotide in the *oprD* gene resulted in a premature termination of translation, whereas type T3 comprises those *oprD* genes harboring a frameshift mutation due to nucleotide insertions or deletions.

The analysis of the predicted amino acid sequences of the 40 isolates belonging to OprD type T1 revealed that they were highly conserved compared to the sequence of PAO1 OprD. Using the information from the solved X-ray crystal structure of OprD (3) (rather than the indirect structural details used in other studies), we have observed that the less conserved regions within OprD among the clinical strains were those corresponding to the loops connecting the transmembrane strands (Fig. 2). Fourteen isolates within this group showed an OprD sequence identical to that of OprD of PAO1. Following the usual pattern, most of the OprD full-length type isolates were susceptible strains. Nevertheless, isolate SPME 241, displaying an OprD identical to the PAO1 OprD, had imipenem and meropenem MICs of 8 and 1 μg/ml, respectively. This isolate showed a 10.1-fold increase in MexY expression compared to PAO1. It has been previously suggested that increased transcription levels of MexXY-OprM up to 10-fold may



affect the susceptibility to meropenem but not to imipenem (7, 34, 44). However, meropenem susceptibility was not altered in this strain. These results seem to be contradictory, and additional studies are warranted to evaluate the actual contribution of increased MexY expression to the carbapenem susceptibility of isolate SPME 241.

Several OprD allelic variants compared to PAO1 OprD were found among the 26 remaining OprD sequences belonging to type T1 (Fig. 2, Table 3). The most frequent amino acid substitutions were $T_{103}S$, $K_{115}T$, and $F_{170}L$, found in 13 susceptible isolates and in the PA14 reference strain, which was also susceptible to both carbapenems. OprD variants showing these amino acid substitutions in loops L3 and L5, initially designated loops L2 and L3 by Huang et al. (14), were described before in clinical isolates of *P. aeruginosa* (8, 11, 37). Other frequently found amino acid substitutions are shown in Table 3; some of them were previously described in isolates with different susceptibility profiles to imipenem (8). OprD types T1-VI, -VIa, and -VII also showed a stretch of 12 amino acid residues located in loop L15 (formerly loop L7) replaced by a sequence of 10 amino acid residues (372-V DSSSSYAGL-384). *P. aeruginosa* strains PA7 and LESB58, both susceptible to carbapenems, also presented this feature (Fig. 2). Shortening of loop L15 ($L15_{10}$) was first described by Epp et al., who proposed its implication in meropenem uptake, as deletions in this loop resulted in a 4-fold decrease of the MIC of meropenem, presumably due to an opening of the OprD channel allowing optimal penetration of this agent (9). This conclusion may be supported by our findings, as all the isolates harboring this OprD alteration were susceptible to both carbapenems. The $L15_{10}$ feature has also been previously reported in OprD of clinical isolates from different geographical areas (37, 39). A replacement of a glutamine (Q) by a glutamate (E) residue at position 301 in loop L13 (formerly loop L5) was found in isolates with the shortened L15 loop. It has been suggested that OprD loops L13, L15, and L16 (formerly loop L8) may contribute to the constriction of the OprD channel opening (9).

The differences in the sequences of some OprD loops do not seem to affect the pore constriction, as their function is only to connect the ß-transmembrane domains. Most of the amino acid substitutions observed in OprD were conserved mutations consisting of changes of one hydrophobic residue to another that was also hydrophobic, generally located in loops connecting the ß-sheets or within the ß-sheets with side chains that point to the outside the ß-barrel. It is expected that such substitutions would not affect the integrity of the porin.

The replacement of an aspartic (D) residue by an asparagine (N) at position 43 was found in 11 isolates, 10 of them having the $L15_{10}$. This alteration may result in a change of pore specificity and conformation, as it consists of a replacement of a positively charged amino acid (Asp) by a neutral-polarity amino acid (Asn). Such an amino acid change was described earlier in clinical strains showing reduced susceptibility to imipenem (8) or resistance to both carbapenems (39), raising the hypothesis of its association with resistance to these agents. However, all the isolates that we found in this study harboring this alteration were susceptible strains; therefore, we concluded that such a change may not itself be involved in carbapenem resistance.

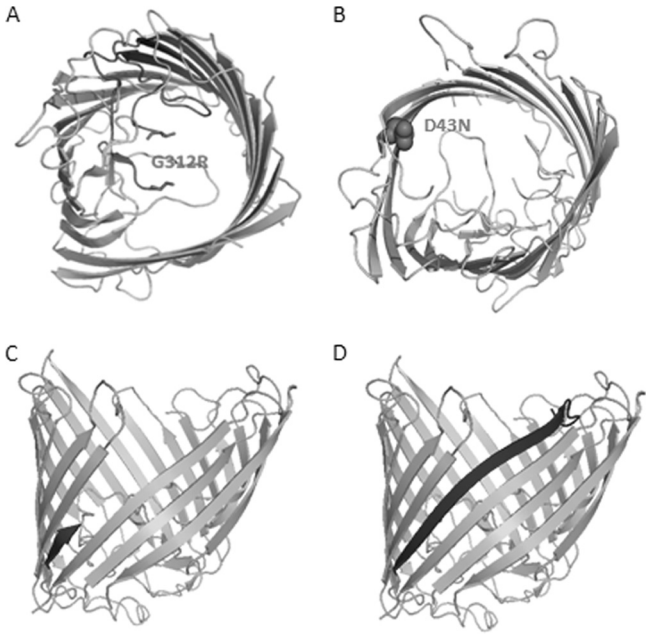


FIG 3 Tridimensional structure of PAO1 OprD based on the model by Biswas et al. (3). (A) Approximate location of mutation $G_{312}R$ (in dark gray) between ß-sheets 16 and 18 from OprD of isolate SPME 211 (type T3). (B) Location of the Asp 43 residue (represented by black balls), replaced by an Asn in isolates SPME 100 (type T1-IIa) and SPME 31 (type T1-VIa). (C and D) Shortening of transmembrane strand 22 (in black) (C) compared to that of PAO1 OprD (D). This was due to a deletion of 10 amino acids from residue 434 in isolates SPME 211, SPME 137, and SPME 259 from OprD type T3.

Another interesting finding was the presence of 3 isolates, SPME 32 (type T1-V), SPME 69 (type T1-III), and SPME 205 (type T1-IIIa), that seem to be new allelic variants of OprD not described before. These isolates have an extended tail of over 50 amino acid residues at the C terminus that provides an additional membrane-spanning stretch (Fig. 2, Table 3). Isolate SPME 32 was susceptible to both carbapenems (MICs of imipenem and meropenem, 1 mg/liter and 0.25 mg/liter) and expressed OprD, as shown by OMP profile analysis (Fig. 1). The *oprD* mRNA transcription levels of strain SPME 32 were similar to that of PAO1 (0.89-fold). The analysis of OMP profiles showed that isolate SPME 69 seemed to express OprD. However, the *oprD* mRNA expression levels in this strain were reduced in comparison with PAO1 (0.42-fold). Although isolate SPME 69 showed overexpression of AmpC (296.6-fold compared to PAO1) and MexB (3.4-fold compared to PAO1), this strain was susceptible to both carbapenems (MICs of imipenem and meropenem, 2 mg/liter and 4 mg/liter). Isolate SPME 205 was susceptible to imipenem (MIC, 2

FIG 2 Multiple sequence alignment by ClustalW of the OprD from PAO1 and clinical isolates belonging to different porin types. OprD sequences from strains PA14, MFY72, LESB58, PA7, 59, and 120 are also included in the alignment. The OprD secondary structure was compared with that of 2ODJ (3) and visualized by using ESPript 2.0 software. Characters on a white background indicate the amino acid substitutions compared to PAO1 OprD. Locations of ß-sheets are indicated with black arrows. The open box shows the stretch of 12 amino acids from residues 372 to 383 located in loop L15 of OprD from isolates of types T1-VI, T1-VIa, and T1-VII, which is replaced by the 10 amino acid residues 372(VDSSSS-YAGL-)384.

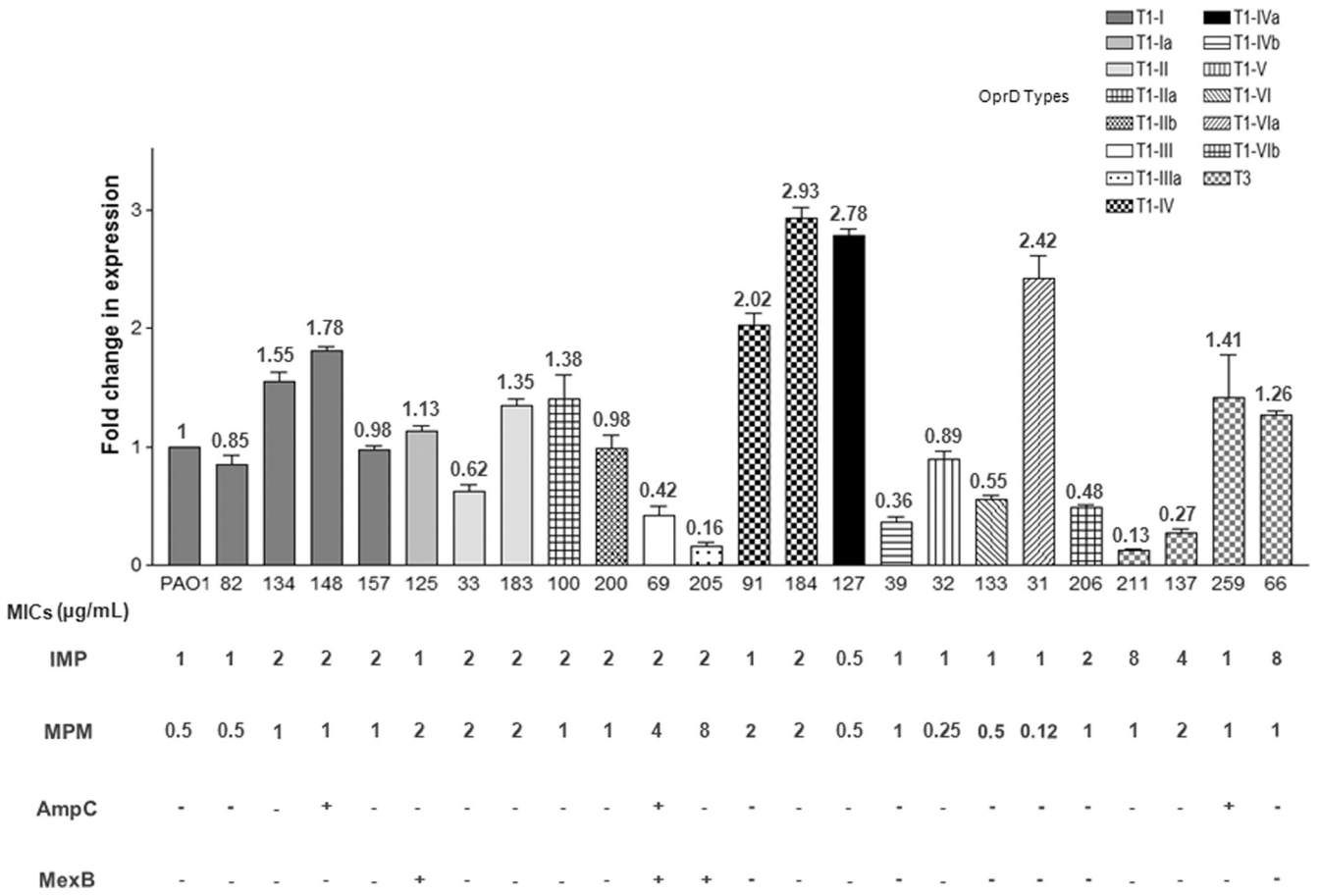


FIG 4 Transcription levels of *oprD*, carbapenem susceptibility profiles, and expression of AmpC and MexB in a selection of clinical isolates of *P. aeruginosa* belonging to OprD types T1 and T3. Increased or decreased expression of *oprD* in clinical isolates was compared to that of PAO1 *oprD*, which was assigned a value of 1.0. MICs of imipenem (IMP) and meropenem (MPM) are shown below the bars. Changes in AmpC and MexB expression are also shown. +, upregulation; −, no significant changes.

mg/liter) and intermediately susceptible to meropenem, probably due to overexpression of MexB (10.3-fold compared to PAO1). Curiously, the OMP analysis proved that this isolate did not produce OprD, which was consistent with the reduced *oprD* expression (0.16-fold) as assessed by RT-PCR. The presence of the additional variable sequence of amino acid residues does not appear to be correlated with the loss of porin or with reduced susceptibility to carbapenems, as it can be found in susceptible isolates that express OprD. The *oprD* mRNA expression was also assessed in 16 other isolates representative of the different OprD T1 subtypes (see Fig. 4; see also Table S1 in the supplemental material). Four isolates (SPME 33 [T1-Ia], SPME 39 [T1-IVb], SPME133 [T1-VI], and SPME 206 [T1-VIb]) were considered to have reduced *oprD* expression (≤60% of that of PAO1). Most of the 16 isolates were susceptible to both carbapenems, with MICs ranging from 0.12 to 2 mg/liter, with the exception of strain SPME 205 which was intermediately susceptible to meropenem (MIC of 8 mg/liter) (see Fig. 4).

The OprD "deficient types" (T2 and T3) included 20 strains showing an earlier termination of translation due to premature stop codons and frameshift mutations caused by nucleotide insertions or deletions in the *oprD* structural gene. OprD type T2 was found in 3 isolates (5%), two of them (SPME 102 and SPME 135) susceptible to both carbapenems (MICs of imipenem ranging from 1 to 4 mg/liter and of meropenem from 0.5 to 2 mg/liter), showing a single-base G→T substitution at nucleotide (nt) 180. The remaining isolate (SPME 22) was susceptible to imipenem (MIC, 4 mg/liter) and intermediately susceptible to meropenem and presented a single-base C→T substitution at nt 424 (Table 3; see also Table S1 in the supplemental material).

OprD type T3 was found in 17 isolates (28%) showing different profiles of susceptibility to both carbapenems, with MICs of imipenem ranging from 1 to 8 mg/liter and of meropenem from 0.06 to 8 mg/liter (Tables 2 and 3). The majority of mutations found within this OprD type consisted of single nucleotide insertions at different positions in the *oprD* gene. Most of the isolates harboring these mutations were intermediately susceptible to imipenem and susceptible to meropenem (Table 3). The insertion of a C at nt 939 was the most frequent mutation, observed in 6 of the 17 isolates; 4 of them were susceptible to both carbapenems, and the 2 remaining were intermediately susceptible to imipenem and susceptible to meropenem.

Isolates SPME 137 and SPME 211 presented a deletion of a T at position 1295, and isolate SPME 259 showed a deletion of a C at position 1297. Such deletions generated a TGA stop codon at position 1301. Earlier termination of OprD translation in these isolates would yield a smaller protein, lacking 10 amino acid residues at the end of the C-terminal extreme from a leucine at position

434. Isolates SPME 137 and SPME 211 had reduced transcription levels of *oprD* (0.27 and 0.13 of PAO1), whereas isolate SPME 259 showed levels of *oprD* mRNA similar to that of PAO1 (1.41-fold). Isolates SPME 259 and SPME 137 were susceptible to both carbapenems (MICs of imipenem and meropenem, 1 and 1 mg/liter and 4 and 2 mg/liter, respectively). Isolate SPME 211 was intermediately susceptible to imipenem and susceptible to meropenem (MIC, 1 mg/liter) and also presented several amino acid substitutions in OprD, including the divergent sequence of 10 amino acids shortening the L15 loop. The replacement of a glycine by the basic amino acid arginine ($G_{312}R$) (Fig. 3A), unique for this isolate, was especially interesting. This residue is located in a disordered loop that is not observed in the OprD structure, but according to the location of the last ordered residues of the loop, this glycine would be located toward the center of the pore, between transmembrane strands 16 and 18 (Fig. 3). Therefore, changes in the sequence of this loop may affect, though slightly, passing of imipenem through the OprD pore, which could explain the differences in the susceptibility profiles for imipenem in this isolate.

Another interesting finding within the OprD T3 group was the presence of an isolate (SPME 66) with an insertion of a G at position 23, leading to a frameshift mutation with a stop codon at position 48. Curiously, the OMP profile analysis of this strain showed expression of an OprD-like protein and the *oprD* mRNA expression was very similar to that of PAO1 (1.26-fold) (Fig. 4; see also Table S1 in the supplemental material). Further analysis of the *oprD* nucleotide sequence led us to think of a possible alternative start codon (GTG) located 64 nucleotides downstream of the usual PAO1 OprD start codon. This OprD allelic variant would have a deletion of 21 amino acid residues at the beginning of the N-terminal extreme (Fig. 2) and would also display some of the mutations mentioned above and a tail of 57 additional residues at the C-terminal extreme (Table 3) due to the insertion in *oprD* of an A between nt 1316 and 1317, leading to a frameshift mutation that affected the stop codon (TGA). Isolate SPME 66 had an imipenem MIC of 8 mg/liter and was susceptible to meropenem (MIC, 1 mg/liter). Among the 20 isolates belonging to OprD-deficient types, only 4 isolates had increased *ampC* expression, and one was considered to have borderline incrase in expression (5.2-fold compared to PAO1). The 15 remaining isolates did not show significant expression of this enzyme (mRNA levels more than 5-fold lower than those of PAO1).

The *mexT* regulator gene that positively regulates the *mexEF-oprN* multidrug efflux system was sequenced in 9 isolates, of which 3 showed increased levels of expression of *mexF*, 4 were considered to have borderline increase in expression (5- to 10-fold compared to PAO1), and the remaining 2 did not have significant *mexT* mRNA expression levels. The sequence of the *mexT* gene obtained from the clinical isolates was compared to that of PAO1. A deletion of 8 nt (GCCGGCCA) at position 240 of *mexT* was found in all the isolates belonging to different OprD types and showing carbapenem resistance profiles from intermediately susceptible to susceptible to both agents. This 8-bp deletion was within a 14-bp direct repeat. A similar deletion in different *P. aeruginosa* strains showing the so-called *nfxC* phenotype was previously described (22). Those authors demonstrated that the deletion of the 8 bp produces an active *mexT*. In a recent work, the role of MexT in MexEF-OprN upregulation was also assessed in 10 *nfxC* mutants harboring the aforementioned 8-bp deletion, resulting in reversion of the nonsense *mexT* gene. Such mutants showed reduced *mexT* expression compared to the wild-type strains (21) In our strains, this mutation in *mexT* was found in strains showing either basal or increased activity of MexF. Several other mutations were found in the *mexT* gene of the clinical isolates (Table 4). Jin et al. compared the *mexT* genes of two laboratory strains, PAO1 and PAK (15). The PAK MexT was found to be a peptide of 304 amino acid residues, in contrast to the 347-amino-acid-long protein of PAO1 MexT. According to those authors, the start codon of the active *mexT* would be the ATG at position 122 (compared to the PAO1 *mexT*). Six of the 9 clinical isolates showed a MexT sequence identical to that of strains PAK, PA14, and 59 (Fig. 5). Isolate SPME 152 differed from the rest of the isolates in a glycine residue replaced by a serine at position 300,

TABLE 4 Analysis of MexT in a selection of clinical isolates of *Pseudomonas aeruginosa*

Strain SPME no.	Resistance phenotype[a]	*mexT* mutations[b]	MexT amino acid substitution(s)[c]	Expression of MexF[d]
22	I^S-M^{IS}	$_{178}C\rightarrow T$, $_{321}G\rightarrow A$, $_{514}T\rightarrow A$, $_{622}C_{623}$	DS-$K_{208}Q$	++
68	I^{IS}-M^S	$_{178}C\rightarrow T$, $_{372}T\rightarrow C$, $_{393}T\rightarrow C$, $_{514}T\rightarrow A$, $_{702}T\rightarrow C$, $_{765}G\rightarrow A$, $_{771}A\rightarrow G$, $_{837}G\rightarrow C$, $_{921}A\rightarrow G$, $_{936}G\rightarrow C$, $_{957}T\rightarrow C$, $_{981}T\rightarrow C$, $_{987}C\rightarrow G$	$F_{172}I$	++
71	I^S-M^S	$_{19}A\rightarrow G$, $_{514}T\rightarrow A$, $_{936}G\rightarrow C$	$F_{172}I$	+
82	I^S-M^S	$_{178}C\rightarrow T$, $_{321}G\rightarrow A$, $_{472}T\rightarrow C$ $_{501}G\rightarrow A$, $_{514}T\rightarrow A$	$F_{172}I$	+
84	I^S-M^S	$_{178}C\rightarrow T$, $_{321}G\rightarrow A$, $_{514}T\rightarrow A$	$F_{172}I$	−
149	I^S-M^S	$_{514}T\rightarrow A$, $_{629}A_{630}$, $_{936}G\rightarrow C$, $_{898}G\rightarrow A$	DS-$T_{211}D$	−
152	I^{IS}-M^{IS}	$_{514}T\rightarrow A$, $_{898}G\rightarrow A$, $_{936}G\rightarrow C$	$F_{172}I$, $G_{300}S$	++
205	I^S-M^{IS}	$_{321}G\rightarrow A$, $_{472}T\rightarrow C$, $_{501}G\rightarrow A$, $_{514}T\rightarrow A$	$F_{172}I$	+
257	I^S-M^S	$_{444}C\rightarrow G$, $_{514}T\rightarrow A$	$F_{172}I$	+

[a] I, imipenem; M, meropenem; S, susceptible; IS, intermediately susceptible.
[b] Compared to the PAO1 *mexT*.
[c] Compared to the PAO1 MexT. The N-terminal sequences of MexT from strains PAK and PA14 and the clinical isolates differ from the PAO1 MexT sequence in the first 78 amino acid residues MNRND[3]LR[11]RVD[5]LNLLIVFETLMHER[24]SVTRAAE KLFL (see Fig. 5). Accordingly, the *mexT* translation would start from the ATG at position 122, generating a 304-amino-acid-long peptide (except for the MexT of SPME 22 and SPME 149, which is 336 amino acids long). DS, divergent sequence starting with the replacement of a lysine (K) by a glutamine (Q) at position 208 in MexT of isolate SPME 22 and a threonine (T) by an aspartate (D) at position 211 in MexT of isolate SPME 149.
[d] ++, overexpression; +, borderline increase in expression; −, no significant expression.

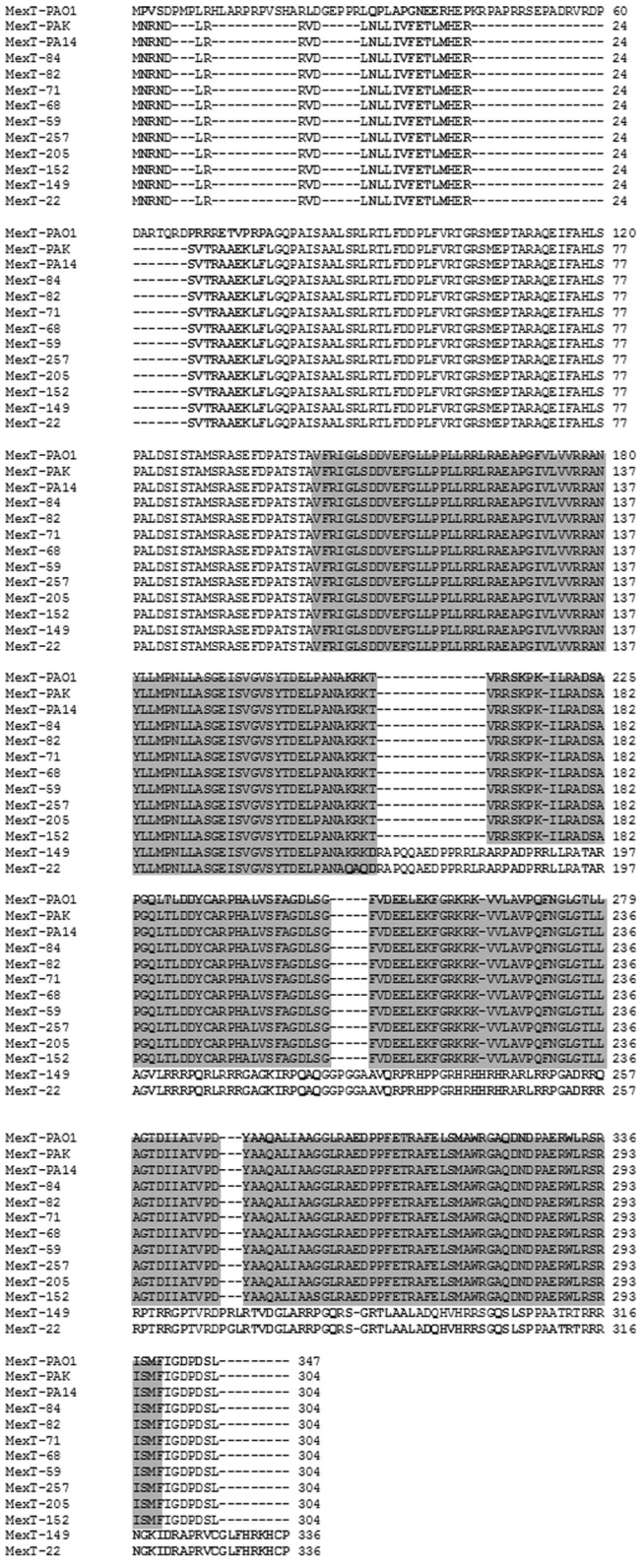


FIG 5 Multiple sequence alignment by ClustalW of MexT from PAO1 and clinical isolates of *P. aeruginosa* analyzed in this study. The MexT sequences from strains PAK, PA14, and 59 are also included. Amino acid residue differences compared to PAO1 MexT are in bold characters. The C-terminal substrate-binding domain of the LysR-type transcriptional regulator is highlighted by gray shading.

taking as a reference the PAO1 MexT. All of these MexT variants kept a conserved domain of the LysR family transcriptional factors from residues 144 to 340 (Fig. 5). The MexT of isolates SPME 22 and SPME 149 had a 336-amino-acid peptide that resulted from a frameshift mutation caused by a nucleotide insertion in the *mexT* gene at positions 622 and 629, respectively (Table 4). The MexT in these isolates differed from the rest of the isolates in the C-terminal extreme from residue 208 in isolate SPME 22 and residue 211 in SPME 149 (Fig. 5). Despite the similarity (98%) of the MexT sequences from these two isolates (data not shown), the mRNA levels of *mexF* in isolate SPME 149 were not significant (see Table S1 in the supplemental material). Similar results were found for the rest of clinical isolates, showing identical MexT sequences (except for the SPME 152 MexT, with 99% similarity). A possible explanation for the basal levels of MexF displayed by some of the clinical strains with an active *mexT* is the presence of a second active regulatory protein, MexS, that was previously shown to repress the activity of MexEF-OprN (40). Llanes et al. also assessed the impact of mutations in *mexS* and *mvaT*, an additional regulatory protein, on the activity of MexEF-OprN, but their contribution to the *nfxC* resistance phenotype is something that has to be further investigated (21).

Conclusions. Mutations in *oprD* caused by nucleotide insertions or deletions in the *oprD* structural gene have been found to be the major mechanisms leading to inactivation of OprD with concomitant loss of the porin from *P. aeruginosa* outer membranes and increases of the MICs of carbapenems. In this study, we have identified a group of clinical isolates of *P. aeruginosa* with distinctive carbapenem resistance phenotypes that confront the previous theories of OprD regulation. The alterations and mutations of OprD found in such strains did not always explain the levels of susceptibility to carbapenems. We found that OprD inactivating mutations in clinical strains of *P. aeruginosa* are not confined only to carbapenem-resistant isolates but can also be present in susceptible strains with MICs of imipenem or meropenem of only 0.06 to 4 mg/liter. We believe that the actual contribution of OprD alterations to the resistance of *P. aeruginosa* has not yet been completely defined, as OprD deficiency does not always result in an increase of the MICs above the susceptible breakpoints.

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