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Pan-β-Lactam Resistance Development in *Pseudomonas aeruginosa* Clinical Strains: Molecular Mechanisms, Penicillin-Binding Protein Profiles, and Binding Affinities

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We investigated the mechanisms leading to *Pseudomonas aeruginosa* pan-β-lactam resistance (PBLR) development during the treatment of nosocomial infections, with a particular focus on the modification of penicillin-binding protein (PBP) profiles and imipenem, ceftazidime, and ceftolozane (former CXA-101) PBP binding affinities. For this purpose, six clonally related pairs of sequential susceptible-PBLR isolates were studied. The presence of *oprD*, *ampD*, and *dacB* mutations was explored by PCR followed by sequencing and the expression of *ampC* and efflux pump genes by real-time reverse transcription-PCR. The fluorescent penicillin Bocillin FL was used to determine PBP profiles in membrane preparations from all pairs, and 50% inhibitory concentrations (IC_{50}s) of ceftolozane, ceftazidime, and imipenem were analyzed in 3 of them. Although a certain increase was noted (0 to 5 2-fold dilutions), the MICs of ceftolozane were ≤4 μg/ml in all PBLR isolates. All 6 PBLR isolates lacked OprD and overexpressed *ampC* and one or several efflux pumps, particularly *mexB* and/or *mexY*. Additionally, 5 of them showed modified PBP profiles, including a modified pattern ($n = 1$) or diminished expression ($n = 1$) of PBP1a and a lack of PBP4 expression ($n = 4$), which correlated with AmpC overexpression driven by *dacB* mutation. Analysis of the essential PBP IC_{50}s revealed significant variation of PBP1a/b binding affinities, both within each susceptible-PBLR pair and across the different pairs. Moreover, despite the absence of significant differences in gene expression or sequence, a clear tendency toward increased PBP2 (imipenem) and PBP3 (ceftazidime, ceftolozane, imipenem) IC_{50}s was noted in PBLR isolates. Thus, our results suggest that in addition to AmpC, efflux pumps, and OprD, the modification of PBP patterns appears to play a role in the *in vivo* emergence of PBLR strains, which still conserve certain susceptibility to the new antipseudomonal cephalosporin ceftolozane.

β-Lactam antibiotics, including antipseudomonal penicillins, cephalosporins, monobactams, and carbapenems, remain key components of our antimicrobial armamentarium for the treatment of life-threatening nosocomial infections by *Pseudomonas aeruginosa* (23). Nevertheless, resistance to these first-line antibiotics is increasing and frequently associated with multidrug resistance (MDR) phenotypes (4, 19). While the acquisition of potent exogenous β-lactamases such as class B carbapenemases (or metallo-β-lactamases [MBLs]) or extended-spectrum β-lactamases (ESBLs) through horizontal gene transfer is a growing threat, β-lactam resistance is still much more frequently caused by the selection of a complex repertoire of chromosomal mutations (19, 20, 31, 32). Particularly noteworthy among them are those leading to the repression or inactivation of the porin OprD, conferring resistance to carbapenems (8, 14, 30, 33), or those leading to the hyperproduction of the chromosomal cephalosporinase AmpC (4, 15, 24), causing resistance to penicillins, cephalosporins, and monobactams. Also, mutations leading to the upregulation of one of the several efflux pumps encoded in the *P. aeruginosa* genome, particularly MexAB-OprM and MexXY-OprM, may significantly contribute to β-lactam resistance phenotypes, in addition to reducing the activity of fluoroquinolones and aminoglycosides (4, 5, 22, 31). While the combination of these mechanisms leads to the emergence of resistance to all currently available β-lactams, some derivatives under clinical development, such as the new cephalosporin ceftolozane (formerly CXA-101), appear to be much less affected by them and thus represent a promising future approach for the treatment of *P. aeruginosa* infections (3, 16, 21, 25, 35).

Another potentially relevant resistance mechanism is the modification of the target of β-lactam antibiotics, the essential penicillin-binding proteins (PBPs), which are PBP1a, PBP1b, PBP2, and PBP3 (37). While the acquisition of modified PBPs showing low affinity for β-lactams is well known to be a major resistance mechanism in Gram-positive cocci, *Haemophilus* spp., and *Neisseria* spp., the role of PBPs in resistance has remained elusive, controversial, or ignored for most species of Gram-negative nosocomial pathogens (37).

Previous studies have demonstrated that *Escherichia coli* PBP2 mutants showing reduced affinity for imipenem can be selected *in vitro* upon antibiotic exposure (36), but the only current evidence of the natural occurrence of such mutants in *Enterobacteriaceae* is a single *Proteus mirabilis* clinical isolate (28). Particular attention has raised the potential role of PBPs in *Acinetobacter baumannii*

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imipenem resistance. Fernández-Cuenca et al. (10) demonstrated reduced expression of PBP2 in some imipenem-resistant clinical isolates, although a recent work did not find mutations in PBP-encoding genes that could be linked to resistance phenotypes (6). Likewise, there is very little information on the potential role of PBPs in *P. aeruginosa* β-lactam resistance; a previous work found no correlation between the expression of genes encoding PBP2 or PBP3 and carbapenem resistance (2), but results from two other studies suggest the possibility of a decreased expression in some resistant isolates (9, 13). Although not strictly related to target modification, recent studies have shown that nonessential PBPs may also play an important role in *P. aeruginosa* β-lactam resistance, since mutation of *dacB*, encoding the putative PBP4, triggers AmpC overexpression and resistance to penicillins, cephalosporins, and monobactams (24). Nevertheless, there are no current data correlating *dacB* mutations with a lack of PBP4 expression; it is also not known whether the absence of PBP4 could eventually modify the expression profiles of other PBPs or their relative binding affinities for β-lactam antibiotics.

In order to advance our knowledge in this field, we characterized clonally related pairs of *P. aeruginosa* clinical isolates that had developed pan-β-lactam resistance *in vivo* during treatment of infections in intensive care unit (ICU) patients. Along with characterizing classical β-lactam resistance mechanisms (expression of AmpC, OprD, and efflux pumps), we followed an integral approach to evaluate the involvement of PBPs in resistance phenotypes through the comparative analysis of the clonally related pairs of PBP profiles and the sequence and expression of PBP-encoding genes. Moreover, we comparatively evaluated in the clonally related pairs the PBP binding affinities of imipenem, ceftazidime, and ceftolozane, since this relevant information is currently available for wild-type strains but not for pan-β-lactam-resistant (PBLR) clinical isolates (25).

MATERIALS AND METHODS

Strains and susceptibility testing. Six clonally related pairs of sequential *P. aeruginosa* isolates that developed resistance to all β-lactams during the treatment of nosocomial infections in ICU patients were used. The clonal relatedness had been previously assessed through pulsed-field gel electrophoresis (PFGE) (25). The MICs of ceftolozane, ceftazidime, cefepime, piperacillin-tazobactam, aztreonam, imipenem, meropenem, ciprofloxacin, and tobramycin were determined by standard CLSI broth microdilution (7) in the previous study (24). PAO1 was used as the control strain.

PCR amplification and sequencing of *ampD*, *dacB*, and *oprD*. PCR amplification of *ampD*, *dacB*, and *oprD* was performed on whole DNA extracts (DNeasy tissue kit; Qiagen, Hilden, Germany) from both the susceptible and the PBLR isolates from each of the 6 pairs of *P. aeruginosa* strains using previously described conditions (14, 15, 24) and primers (Table 1). At least two independent PCR products for each isolate and gene were sequenced on both strands. The BigDye Terminator kit (PE-Applied Biosystems) was used for performing the sequencing reactions, and sequences were analyzed with the ABI Prism 3100 DNA sequencer (PE-Applied Biosystems).

Determination of the expression of AmpC and efflux pumps. The expression of the genes encoding the four major *P. aeruginosa* efflux pumps, MexAB-OprM (*mexB*), MexCD-OprJ (*mexD*), MexEF-OprN (*mexF*), and MexXY-OprM (*mexY*), and AmpC (*ampC*) was determined by real-time reverse transcription-PCR (RT-PCR) for the 6 pairs of susceptible and PBLR isolates and PAO1 (as a control) following previously described protocols (15, 29). For the quantification of *ampC* induction the strains were incubated in the presence of 50 μg/ml of cefoxitin (15). Briefly, total RNA from logarithmic-phase-grown LB cultures was obtained with an RNeasy minikit (Qiagen, Hilden, Germany). Fifty nanograms of purified RNA was then used for one-step reverse transcription and real-time PCR using a QuantiTect SYBR green reverse transcription-PCR kit (Qiagen) in a SmartCycler II apparatus (Cepheid, Sunnyvale, CA). Previously described conditions and primers were used (15, 29). The *rpsL* housekeeping gene was used to normalize the expression levels, and results were always referenced against PAO1 basal expression. All RT-PCRs were performed in duplicate, and the mean values of mRNA expression resulting from three independent experiments were considered in all cases. Overexpression was considered when the corresponding mRNA level was at least 3-fold (*mexB*) or 10-fold (*ampC*, *mexD*, *mexF*, *mexY*) higher than that for PAO1 (4).

Complementation of AmpC hyperproduction phenotypes. Plasmids pUCPAD (harboring the wild-type *ampD* gene) and pUCPADE (harboring the complete wild-type *ampDE* operon) were electroporated into the different PBLR strains or PAO1 (as a control) following previously described protocols (24). The complementation of AmpC hyperproduction phenotypes was then evaluated in selected transformants through the determination of β-lactam MICs and the quantification of *ampC* expression as described above.

OMP analysis. A protocol adapted from those previously published (11, 27) was followed. Briefly, 200 ml of late-log-phase [optical density at 600 nm ($OD_{600\ nm}$) = 1] LB cultures was collected by centrifugation, washed, and suspended in 5 ml of 10 mM Tris-Mg (pH 7.3) buffer. Cells were then sonicated and centrifuged at 7,000 × *g* for 15 min. Membranes were isolated through ultracentrifugation at 100,000 × *g* for 1 h at 4°C. Pellets were suspended in 10 ml of 1% sarcosyl in 25 mM Tris-HCl (pH 8) buffer and incubated for 30 min at room temperature. Outer membrane proteins (OMPs) were collected afterward through ultracentrifugation at 70,000 × *g* for 40 min, suspended in the same buffer, and ultracentrifuged again. OMPs were then suspended in water, separated through SDS-PAGE [11% acrylamide-0.2% bisacrylamide-0.2% SDS-0.375 M (pH 8.8) Tris] and visualized through Coomassie blue staining.

Purification of PBPs. Membranes containing the PBPs of each *P. aeruginosa* strain were obtained following described protocols (26, 38). Briefly, 500 ml late-log-phase ($OD_{600\ nm}$ = 1) Luria-Bertani (LB) (Sigma-Aldrich, St. Louis, MO) cultures were collected by centrifugation (4,400 × *g*, 10 min) and then washed and suspended in 50 ml of 20 mM KH_2PO_4-140 mM NaCl (pH 7.5) (buffer A). Cells were then sonicated using a Digital Sonifier Unit Model S-450D (Branson Ultrasonics Corporation, Danbury, CT) at 20 W for three 30-s bursts (while immersed in an ice bath) and centrifuged at 12,000 × *g* for 10 min. Membranes containing the PBPs were isolated from the supernatant through two steps of ultracentrifugation at 150,000 × *g* for 1 h at 4°C using an Optima L-XP Series Preparative ultracentrifuge (Beckman Coulter Inc., Palo Alto, CA) and suspension in buffer A. The total protein content was measured through the Bradford method using the Quick Start Bradford Protein Assay kit with bovine serum albumin as a standard (Bio-Rad Laboratories, Hercules, CA) following the manufacturer's instructions. For analysis of the PBP profiles, membrane fractions were adjusted to 1 mg/ml, and the adjusted preparations (10 μl) were labeled with 25 μM Bocillin FL (38) and subsequently separated through 10% gel SDS-polyacrylamide gel electrophoresis (Bio-Rad Laboratories). Labeled PBPs were visualized (excitation at 488 nm and emission at 530 nm) using a Bio-Rad Molecular Imager FX Pro (Bio-Rad Laboratories). In order to evaluate the reproducibility and consistency, the profiles were determined on three independent occasions for each of the strains.

Determination of IC_{50}s. Following previously described protocols (26), 20 μl (final volume) of PBP-containing solution was incubated (30 min, 37°C) in the presence of growing concentrations of ceftolozane, ceftazidime, or imipenem (range of concentrations tested, 0.0156 to 2 μg/ml), and PBPs were labeled afterward with a 25 μM concentration of Bocillin FL. The reaction mixtures were then each denatured with 20 μl of SDS-denaturing solution at 100°C for 3 min. PBPs were then separated through 10% SDS polyacrylamide gel electrophoresis. The protein gels were rinsed in water immediately after electrophoresis. Labeled PBPs were

TABLE 1 Primers used in this work

Primer	Primer sequence (5′—3′)	PCR product size (bp)	Use	Reference or source
ACrnaF	GGGCTGGCCTCGAAAGAGGAC	246	Quantification of *ampC* mRNA	15
ACrnaR	GCACCGAGTCGGGGAACTGCA			
MexB-U	CAAGGGCGTCGGTGACTTCCAG	273	Quantification of *mexB* mRNA	29
MexB-L	ACCTGGGAACCGTCGGGATTGA			
MexD-U	GGAGTTCGGCCAGGTAGTGCTG	236	Quantification of *mexD* mRNA	29
MexD-L	ACTGCATGTCCTCGGGGAAGAA			
MexF-U	CGCCTGGTCACCGAGGAAGAGT	254	Quantification of *mexF* mRNA	29
MexF-L	TAGTCCATGGCTTGCGGGAAGC			
MexY-Fa	TGGAAGTGCAGAACCGCCTG	270	Quantification of *mexY* mRNA	29
MexY-Ra	AGGTCAGCTTGGCCGGGTC			
ponArnaF	GAAGCCGTGACCTGGGACAGC	231	Quantification of *ponA* mRNA	This work
ponArnaR	GGAGAAGCCGCCGACCAGCG			
mrcBrnaF	CAACCTGGTGCTCGACGTGCTC	217	Quantification of *mrcB* mRNA	This work
mrcBrnaR	CGGATCGAAGCTGGTGAAGATGC			
dacBrnaF	GGCCCGACCTACCAGTGGAAG	217	Quantification of *dacB* mRNA	This work
dacBrnaR	AACGGCTTGGTGTCGTCGCCG			
PBP2rnaF	GTGACTCCATCGACCGGCCGC	227	Quantification of PBP2 mRNA	This work
PBP2rnaR	GTAGATCGCCGACTCCAGGCTC			
PBP3rnaF	CGGCAGCTTGGTGATCATGGAC	223	Quantification of PBP3 mRNA	This work
PBP3rnaR	CGGGTAGACGTCGACGATATCG			
dacCrnaF	CGCCTTCGCCGACATGATGAAC	218	Quantification of *dacC* mRNA	This work
dacCrnaR	AGCAGGTTGCGGTTCGGCTGC			
PA-DEF	GTACGCCTGCTGGACGATG	910	*ampD* amplification and sequencing	17
PA-DER	GAGGGCAGATCCTCGACCAG			
dacBF	CGACCATTCGGCGATATGAC	1,400	*dacB* amplification and sequencing	24
dacBR	CGCGTAATCCGAAGATCCATC			
oprD-F	CGCCGACAAGAAGAACTAG	1,413	*oprD* amplification and sequencing	16
oprD-R	GTCGATTACAGGATCGACAG			
ponAF	CGAAGGCCAGGCAAATGGC	2,636	*ponA* amplification and sequencing	This work
ponAR	CTCCCGTCGTCGCCAACG			
ponAF2	CCTGCAGGACGCGGATCG		*ponA* sequencing	This work
ponAR2	CTCAAGCACCTGGGCCAGC			
ponAR3	CGCTCGAGGATCCAGTTGC			
mrcBF	CATTATGGCGGGAAGGGGTG	2,551	*mrcB* amplification and sequencing	This work
mrcBR	GCGACACACCATGGTGGTTC			
mcrB-F2	GAACCACCATGGTGTGTCGC		*mrcB* sequencing	This work
mrcB-R2	CGAGGCCGAGCTTGGCGG			
PBP2F	GAGCAGCGCTGGTCGCTG	2,135	PBP2 amplification and sequencing	This work
PBP2R	GCAGGCGCTGCAACAGGC			
PBP3F	GGCCGGTTGATTCTCGAGC	1,921	PBP3 amplification and sequencing	This work
PBP3R	GGTCAGCTCGCGGATCAGC			

visualized using a Bio-Rad Molecular Imager FX Pro (excitation at 488 nm and emission at 530 nm), and 50% inhibitory concentrations (IC_{50}s) of ceftolozane, ceftazidime, and imipenem for the different PBPs were determined from triplicate independent experiments using the Quantity One software (Bio-Rad Laboratories, Hercules, CA) and compared using Student's *t* test. *P* values <0.05 were considered statistically significant.

Determination of the sequence and expression of PBP genes. The genes encoding PBPs (PBP1a, PBP1b, PBP2, PBP3 or PBP4) showing modified patterns in any pan-β-lactam-resistant isolate were further analyzed through complete sequencing. PCR amplification was performed on genomic DNA extracts (DNeasy tissue kit; Qiagen, Hilden, Germany) of the susceptible and PBLR isolate of each pair using the primers described in Table 1. Two independent PCR products for each isolate and gene were sequenced on both strands. The BigDye Terminator kit (PE-Applied Biosystems) was used for performing the sequencing reactions that were analyzed with the ABI Prism 3100 DNA sequencer (PE-Applied Biosystems). The sequences were analyzed and compared within each susceptible-resistant pair and with the reference strain PAO1. The protocol described above was used for the quantification of the expression of PBP genes through real-time RT-PCR using the primers listed in Table 1.

RESULTS AND DISCUSSION

Involvement of OprD, AmpC, and efflux pumps in pan-β-lactam resistance development. Results of the characterization of classical resistance mechanisms in the six isogenic pairs of sequential *P. aeruginosa* isolates that had developed resistance to all currently available antipseudomonal β-lactams (including penicillins, cephalosporins, monobactams, and carbapenems) during the treatment of nosocomial infections in ICU patients are shown in Table 2. The time lapse between the isolation of the susceptible and the PBLR isolates ranged from 14 to 52 days (average, 33.2 days). During this period all patients received one or several courses of treatment with antipseudomonal β-lactams, including carbapenems (imipenem), cephalosporins (ceftazidime or

TABLE 2 Characterization of isogenic susceptible-PBLR *P. aeruginosa* clinical isolates

Patient	Isolate[a]	Treatment before emergence of resistance[b]	MIC (μg/ml)[b]							Resistance mutation(s)[c]			Expression of resistance gene[d]:					
			CAZ	FEP	PTZ	ATM	IMP	MER	TOL	*ampD*	*dacB*	*oprD*	*ampC* basal	*ampC* induced[e]	*mexB*	*mexF*	*mexD*	*mexY*
5	3-B7	TOB, CIP, PTZ, IMP	1	8	8	8	2	0.12	0.25	WT	WT	WT	<5	199	<2	<5	<5	8.3
	3-F4		128	64	128	256	64	64	4	D28G	G427D	T276A; W278X	2,112	4,143	<2	<5	13	14
7	3-A2	IMP, CIP, PTZ, TOB	1	2	2	4	2	0.12	0.12	WT	WT	WT	<5	1,218	<2	<5	<5	<5
	3-D8		128	64	128	128	64	64	4	WT	G366S; A394P	No OprD[f]	1,351	2,846	26	<5	<5	<5
8	1-H9	PTZ, CAZ, TOB	8	8	8	32	64	64	0.5	WT	WT	Δ*oprD*	<5	250	4.0	<5	<5	<5
	2-A1		128	32	128	128	64	64	4	Ins. 1 bp (C) in 481	T428P	Δ*oprD*	1,722	3,058	6.5	<5	<5	<5
14	1-A10	TOB, CIP, IMP,CAZ	1	1	4	4	32	2	0.5	WT	WT	W339X	<5	200	<2	<5	<5	<5
	1-C5		32	16	128	32	64	64	1	Q155X	WT	W339X	317	2,267	14	<5	<5	21
16	2-G5	FEP, TOB, IMP, CIP, CAZ	1	4	4	16	1	0.12	0.5	WT	WT	WT	<5	536	<2	<5	<5	<5
	2-I4		128	128	128	64	64	64	2	Δ*ampDE*	M200I; del D201	Δ*oprD*	1,438	3,388	<2	<5	<5	12
17	3-D9	IMP, PTZ	2	2	8	8	2	0.25	0.5	WT	WT	WT	<5	443	<2	<5	<5	<5
	3-F5		16	64	64	32	64	16	0.5	V10G	WT	NoOprD[f]	67	1,068	<2	<5	<5	<5

[a] Resistance mechanisms for strains 2-A1, 1-C5, and 2-I4 had been previously partially characterized (17, 25).

[b] TOB, tobramycin; CIP, ciprofloxacin; PTZ, piperacillin-tazobactam; IMP, imipenem; CAZ, ceftazidime; FEP, cefepime; ATM, aztreonam; MER, meropenem; TOL, ceftolozane.

[c] WT, wild-type sequence.

[d] Relative mRNA expression compared to wild-type PAO1. According to previous works (4), breakpoints used to define overexpression were ≥10-fold for *ampC*, *mexF*, *mexD*, and *mexY* (5 to 10 borderline) and ≥3-fold for *mexB* (2 to 3 borderline).

[e] Induction experiments were carried out with 50 mg/ml cefoxitin.

[f] Although these strains showed a wild-type *oprD* sequence, OprD was not expressed when outer membrane proteins (OMPs) were analyzed through SDS-PAGE.

TABLE 3 Results of AmpC hyperproduction complementation studies in PBLR strains[a]

Strain[b]	Resistance mutation(s)		Plasmid	MIC (μg/ml)							*ampC* expression[c]
	ampD	*dacB*		CAZ	FEP	PTZ	ATM	IMP	MER	TOL	
PAO1	WT	WT		2	1	4	4	2	0.5	0.5	1
			pUCPAD	2	1	4	4	2	0.5	0.5	ND
			pUCPADE	1	1	4	4	2	0.5	0.5	ND
PAΔ*ampD*	Δ*ampD*	WT		8	4	32	8	2	2	0.5	48
			pUCPAD	1	1	2	2	2	0.5	0.5	1.5
			pUCPADE	1	1	1	2	2	0.5	0.5	1.3
PAΔ*dacB*	WT	Δ*dacB*		32	8	64	16	2	0.5	1	34
			pUCPAD	8	4	4	2	2	0.5	1	27
			pUCPADE	1	1	2	2	1	0.5	0.5	2.5
PAΔ*ampD dacB*	Δ*ampD*	Δ*dacB*		64	8	256	64	2	2	2	1,859
			pUCPAD	16	8	64	16	2	1	1	460
			pUCPADE	4	1	4	8	1	0.5	1	5.2
3F4	D28G	G427D		256	32	256	512	32	32	4	2,112
			pUCPAD	128	32	128	64	32	32	2	64
			pUCPADE	16	8	32	16	16	4	1	3.8
3D8	WT	G366S; A394P		128	32	256	256	32	32	4	1,351
			pUCPAD	16	16	32	32	32	16	2	504
			pUCPADE	4	4	8	32	2	16	1	52
2A1	Ins. 1 bp (C) in 481	T428P		256	64	256	128	32	32	4	1,722
			pUCPAD	32	8	64	32	32	16	2	419
			pUCPADE	4	4	8	16	16	16	1	56
1C5	Q155X	WT		32	8	64	64	32	32	1	317
			pUCPAD	4	8	32	32	32	32	1	11
			pUCPADE	4	8	16	16	16	32	1	4.5
2I4	Δ*ampDE*	M200I; del D201		128	32	256	128	32	16	4	1,438
			pUCPAD	16	8	32	16	16	8	1	466
			pUCPADE	2	4	8	4	8	8	1	19
3F5	V10G	WT		16	16	64	16	32	16	1	67
			pUCPAD	16	16	64	16	32	8	1	14
			pUCPADE	4	4	8	8	32	4	1	2.1

[a] Abbreviations are the same as in Table 2. ND, not determined.
[b] PAO1 *ampD* (PAΔ*ampD*), *dacB* (PAΔ*dacB*), and *ampD-dacB* (PAΔ*ampD dacB*) knockout mutants generated in a previous work (24) were used as controls.
[c] Relative mRNA expression compared to that of wild-type PAO1.

cefepime), and penicillin-β-lactamase inhibitor combinations (piperacillin-tazobactam); most of them were additionally treated with aminoglycosides (tobramycin) and/or fluoroquinolones (ciprofloxacin) (Table 2).

All six PBLR isolates were OprD deficient, due to either a partial deletion of *oprD*, point mutations leading to a premature stop codon, or the lack of OprD expression in the absence of *oprD* mutations, each mechanism found in two of the strains. All the PBLR isolates additionally overexpressed *ampC* due to mutations in *ampD* (2 strains), *dacB* (1 strain), or both genes (3 strains). In order to denote the contribution of *ampC* overexpression to the PBLR profiles, and to confirm the underlying mechanisms, complementation studies were carried out using plasmids with cloned wild-type *ampD* (pUCPAD; complements *ampD* mutants) and the complete *ampDE* operon [pUCPADE; shown in a previous work to complement both *ampD* and *dacB* mutants (24)]. The MICs of the different β-lactams and *ampC* expression levels for the PBLR strains harboring these plasmids are shown in Table 3. Although a marked increase in β-lactam susceptibility was documented, particularly for pUCPADE complementations, the MICs for several β-lactams were still higher than those of the susceptible parent strain in most of the cases, suggesting the involvement of additional resistance mechanisms. Moreover, 2 of the strains overexpressed *mexB*, 2 *mexY*, and 1 both efflux pumps. These results were thus consistent with those of previous studies showing that *P. aeruginosa* pan-β-lactam resistance frequently results from combinations of mutations leading to OprD inactivation, AmpC hyperproduction, and efflux pump overexpression (4, 19). Regarding the activity of ceftolozane, although a certain increase in MICs was noted, ranging from 0 to 5 2-fold dilutions, they remained ≤4 μg/ml and thus within the susceptible category according to breakpoints suggested by pharmacokinetic/pharmacodymic (PK/PD) analysis (12). The greatest increases in ceftolozane MICs were documented for two strains showing extremely high *ampC* expression levels (>2,000-fold compared to that of PAO1) along with *mexB* and/or *mexY* overexpression (Table 2), while the lowest effect (no modification of the MICs) was documented for a strain showing only moderate (100-fold higher than that of PAO1) *ampC* overexpression. Thus, these results are consistent with previous data suggesting that ceftolozane is much less affected than currently available β-lactams by classical mutation-driven resistance mechanisms in *P. aeruginosa* (16, 25, 34).

PBP expression profiles of susceptible-PBLR pairs. As shown in Fig. 1, five of the six susceptible-PBLR pairs showed modified PBP profiles. For four of the pairs (P5, P7, P8, and P16) the PBLR isolate lacked PBP4 expression. These four isolates were those showing acquired mutations in *dacB* (Table 2), leading to AmpC overexpression. Likewise, a lack of PBP4 expression was also ob-

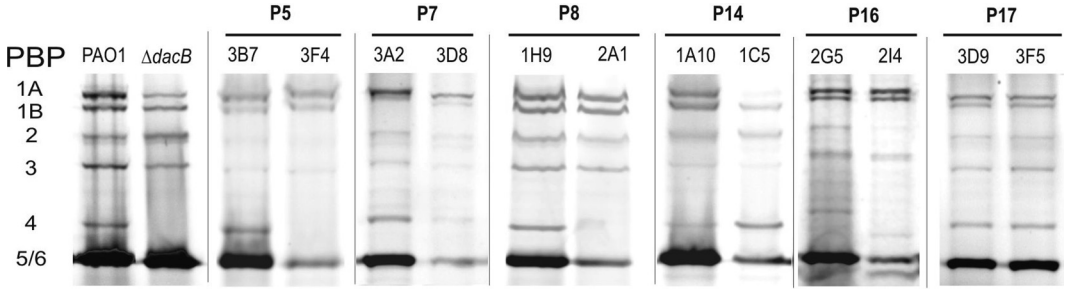

FIG 1 PBP profiles of the six clonally related pairs of sequential isolates. The first two lanes show the profile of PAO1 compared to its isogenic *dacB* mutant. For each pair, the lane on the left represents the profile of the susceptible isolate, while the right lane represents the PBLR isolate. The membrane fractions were adjusted to a 1 mg/ml total protein concentration and labeled with Bocillin FL (25 μM). Ten microliters of the labeled membrane preparations was separated by SDS-PAGE.

served for the control *dacB* knockout mutant of PAO1 (Fig. 1). Thus, this work expands previous findings (23), since it first correlates mutation of *dacB* with the lack of PBP4 expression. Interestingly, while all of the *dacB* mutations lead to the lack of ex pression of a functional PBP4, modified transcription was not observed in any of the cases (not shown). The PBLR isolate from one of these pairs (P7) additionally showed differences in migration of PBP1a and PBP1b, and the PBLR isolate of pair 14 did not express a functional PBP1a and apparently overexpressed PBP4 (Fig. 1). Interestingly, despite these evident modifications of PBP profiles, gene sequencing and transcription analysis revealed no differences between the susceptible and the PBLR isolates (not shown). When compared with PAO1, only silent nucleotide polymorphisms were detected, suggesting the occurrence of posttranscriptional events leading to modified periplasmic PBP expression patterns. In any case, the modification of PBP1a expression profiles could eventually have a relevant impact in β-lactam resistance. Of particular interest, a very recent study, using *Stenotrophomonas malthopilia* as a model organism, showed that the inactivation of PBP1a leads to the overexpression of L1 and L2 β-lactamases (18). Whether a similar effect occurs also in *P. aeruginosa*, and how it might be related to PBP4 activity, is under investigation in our laboratory.

Additionally, as shown in Fig. 1, PBLR strains apparently expressed smaller amounts of PBP5/6 than their susceptible parent strains. Thus, although this PBP is apparently not involved in resistance (1), the expression of *dacC*, encoding PBP5/6, was monitored in all the strains. In all cases, the expression was found similar to that of PAO1 (mean, 0.94; range, 0.47 to 1.23, compared to PAO1). Moreover, no significant differences were observed between PBLR (mean, 0.89; range, 0.47 to 1.11) and susceptible (mean, 0.98; range, 0.72 to 1.23) strains.

Since previous studies have suggested a decreased expression of genes encoding PBP2 or PBP3 in some carbapenem-resistant *P. aeruginosa* isolates (9, 13), we also quantified the expression of these genes in 3 of the susceptible-PBLR pairs, and the results are shown in Table 4. While a slightly lower expression of the PBP3 gene was noted for the 3 strains compared to that of PAO1, we found no significant differences between the susceptible and PBLR isolates in each of the pairs. Similarly, a slightly higher expression of the PBP2 gene was noted in one of the strains, but again without significant differences between the susceptible and the PBLR isolates (Table 4). Moreover, sequencing of the genes encoding PBP2 and PBP3 revealed no differences between the susceptible and the PBLR pairs; besides the presence of silent nucleotide polymorphisms, all sequences were identical to that of wild-type PAO1, except for an L3V substitution in PBP3 found in both the susceptible and the PBLR isolates from one of the patients (Table 4). Thus, our results, first comparing clonally related susceptible-PBLR pairs, suggest that while a certain interstrain variability on the expression of PBP2/PBP3 genes exists, it seems not to be strongly linked to β-lactam resistance development.

PBP binding affinities of ceftazidime, ceftolozane, and imipenem in susceptible-PBLR pairs. Table 5 shows the PBP IC_{50}s of ceftazidime, ceftolozane, and imipenem for the above-described 3 susceptible-PBLR pairs. Interestingly, despite the absence of significant differences in gene expression or sequence, a clear tendency toward increased IC_{50}s was noted when comparing the PBLR with the susceptible isolates for imipenem binding to PBP2 (0.30 ± 0.11 μg/ml vs 0.13 ± 0.02 μg/ml, $P = 0.03$) and for ceftazidime (0.19 ± 0.02 vs 0.12 ± 0.02, $P = 0.004$), ceftolozane (0.18 ± 0.13 vs 0.07 ± 0.02, $P = 0.12$), and imipenem (0.69 ± 0.12 vs 0.34 ± 0.06, $P = 0.008$) binding to PBP3. Additionally, strain-specific variations in PBP1a/b binding affinities of ceftazidime, ceftolozane, or imipenem were noted. In particular, the pair P14 (1A10-1C5) showed a significant increase of the binding affinity of PBP1b for the three antibiotics, likely due to the lack of PBP1a in the PBLR isolate (1C5). All together, these data suggest that the relative binding affinity of a given PBP for a given antipseudomonal agent is influenced by the relative abundance of each of the PBPs, which might be modulated by posttranscriptional events in PBLR isolates.

Concluding remarks. In this work we show that in addition to AmpC hyperproduction, inactivation of OprD, and overexpres-

TABLE 4 Relative expression and sequence of PBP2 and PBP3 genes from 3 pairs of clonally related susceptible-PBLR sequential *P. aeruginosa* isolates[a]

Strain	PBP2 gene expression[b]		PBP2 sequence[c]	PBP3 gene expression		PBP3 sequence
	Susceptible	PBLR		Susceptible	PBLR	
PAO1	1	N/A	WT	1	N/A	WT
P8	2.69 ± 1.89	4.63 ± 1.43	WT	0.57 ± 0.04	0.60 ± 0.33	WT
P14	0.99 ± 0.27	1.01 ± 0.73	WT	0.67 ± 0.31	0.49 ± 0.23	L3V
P16	1.01 ± 0.73	1.95 ± 0.11	WT	0.33 ± 0.07	0.75 ± 0.27	WT

[a] N/A, not applicable; WT, wild type.
[b] Relative mRNA expression compared to that of wild-type PAO1.
[c] Sequence compared to that of wild-type PAO1. In all cases, the susceptible and the PBLR isolates from each pair yielded an identical sequence.

sion of efflux pumps, modification of PBP patterns appears to play a role in the *in vivo* emergence of PBLR strains, which still conserve certain susceptibility to the new antipseudomonal cephalosporin ceftolozane. In particular, the correlation of *dacB* mutations (leading to AmpC overexpression) with a lack of PBP4 expression is demonstrated for the first time in this work. Additionally, post-transcriptional modifications of PBP1a/b expression patterns are observed in some PBLR isolates. Thus, these results suggest that altered patterns of PBP1a/b may also be involved in β-lactam resistance; therefore, future studies are needed to address this issue. Finally, this work investigated for the first time the binding affinities of antipseudomonal β-lactams to PBP extracts from clonally related susceptible and PBLR isolates, showing a clear tendency toward increased IC_{50}s for PBP2 (imipenem) and/or PBP3 (ceftazidime, ceftolozane, and imipenem). While the underlying genetic or physiological drivers of these findings still need to be elucidated, as well as their specific impact in the resistance profiles, these results highlight the interest of testing β-lactam-resistant strains in the evaluation of the potency of new β-lactam molecules through the determination of their PBP binding affinities.

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TABLE 5 PBP IC_{50}s of ceftazidime, ceftolozane, and imipenem for 3 pairs of susceptible-PBLR sequential *P. aeruginosa* isolates

PBP	Isolate	IC_{50} for indicated isolate											
		Ceftazidime				Ceftolozane				Imipenem			
		P8	P14	P16	PAO1	P8	P14	P16	PAO1	P8	P14	P16	PAO1
1a	Susceptible	0.08 ± 0.01	0.35 ± 0.15	0.35 ± 0.12	0.19 ± 0.10	0.37 ± 0.15	0.21 ± 0.16	0.12 ± 0.06	0.12 ± 0.04	0.66 ± 0.04	0.41 ± 0.26	0.08 ± 0.03	0.21 ± 0.06
	PBLR	0.11 ± 0.02[a]		0.29 ± 0.16	N/A	0.53 ± 0.34[a]		0.21 ± 0.06	N/A	0.75 ± 0.15[a]		0.19 ± 0.12[a]	N/A
1b	Susceptible	1.48 ± 0.73	0.48 ± 0.17	>2	>2	1.19 ± 0.21	1.59 ± 0.34	0.15 ± 0.21	0.89 ± 0.24	0.15 ± 0.05	1.02 ± 0.05	0.14 ± 0.05	0.12 ± 0.05
	PBLR	>2	0.12 ± 0.01[a]	>2	N/A	>2	0.09 ± 0.02[a]	0.19 ± 0.12[a]	N/A	0.24 ± 0.07	0.39 ± 0.15[a]	0.11 ± 0.02	N/A
2	Susceptible	>2	>2	>2	>2	>2	1.89 ± 0.42	>2	1.59 ± 0.42	0.10 ± 0.04	0.14 ± 0.03	0.15 ± 0.07	0.10 ± 0.04
	PBLR	>2	>2	>2	N/A	>2	1.52 ± 0.37	>2	N/A	0.21 ± 0.04[a]	0.42 ± 0.13[a]	0.28 ± 0.17	N/A
3	Susceptible	0.13 ± 0.06	0.13 ± 0.04	0.09 ± 0.02	0.09 ± 0.02	0.09 ± 0.02	0.07 ± 0.01	0.06 ± 0.01	0.04 ± 0.01	0.41 ± 0.04	0.31 ± 0.14	0.41 ± 0.14	0.16 ± 0.04
	PBLR	0.18 ± 0.04	0.19 ± 0.03	0.21 ± 0.20[a]	N/A	0.33 ± 0.22[a]	0.12 ± 0.01[a]	0.09 ± 0.04	N/A	0.55 ± 0.14[a]	0.77 ± 0.19[a]	0.74 ± 0.26[a]	N/A
4	Susceptible	0.89 ± 0.09	>2	1.88 ± 0.13	1.78 ± 0.53	>2	1.72 ± 0.58	0.89 ± 0.18	0.21 ± 0.08	0.38 ± 0.21	0.06 ± 0.02	0.01 ± 0.005	0.03 ± 0.01
	PBLR		>2		N/A		>2[a]		N/A		0.03 ± 0.02		N/A
5/6	Susceptible	>2	>2	>2	>2	>2	>2	>2	>2	0.51 ± 0.34	0.70 ± 0.18	0.26 ± 0.08	0.40 ± 0.10
	PBLR	>2	>2	>2	N/A	>2	>2	>2	N/A	>2[a]	>2[a]	>2[a]	N/A

[a] Statistically significant differences (Student's *t* test, $P < 0.05$) for the comparison of PBP IC_{50}s for the susceptible and the PBLR sequential isolates.

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