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Pseudomonas aeruginosa Ceftolozane-Tazobactam Resistance Development Requires Multiple Mutations Leading to Overexpression and Structural Modification of AmpC

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We compared the dynamics and mechanisms of resistance development to ceftazidime, meropenem, ciprofloxacin, and ceftolozane-tazobactam in wild-type (PAO1) and mutator (PAOMS, Δ*mutS*) *P. aeruginosa*. The strains were incubated for 24 h with 0.5 to 64× MICs of each antibiotic in triplicate experiments. The tubes from the highest antibiotic concentration showing growth were reinoculated in fresh medium containing concentrations up to 64× MIC for 7 consecutive days. The susceptibility profiles and resistance mechanisms were assessed in two isolated colonies from each step, antibiotic, and strain. Ceftolozane-tazobactam-resistant mutants were further characterized by whole-genome analysis through RNA sequencing (RNA-seq). The development of high-level resistance was fastest for ceftazidime, followed by meropenem and ciprofloxacin. None of the mutants selected with these antibiotics showed cross-resistance to ceftolozane-tazobactam. On the other hand, ceftolozane-tazobactam resistance development was much slower, and high-level resistance was observed for the mutator strain only. PAO1 derivatives that were moderately resistant (MICs, 4 to 8 μg/ml) to ceftolozane-tazobactam showed only 2 to 4 mutations, which determined global pleiotropic effects associated with a severe fitness cost. High-level-resistant (MICs, 32 to 128 μg/ml) PAOMS derivatives showed 45 to 53 mutations. Major changes in the global gene expression profiles were detected in all mutants, but only PAOMS mutants showed *ampC* overexpression, which was caused by *dacB* or *ampR* mutations. Moreover, all PAOMS mutants contained 1 to 4 mutations in the conserved residues of AmpC (F147L, Q157R, G183D, E247K, or V356I). Complementation studies revealed that these mutations greatly increased ceftolozane-tazobactam and ceftazidime MICs but reduced those of piperacillin-tazobactam and imipenem, compared to those in wild-type *ampC*. Therefore, the development of high-level resistance to ceftolozane-tazobactam appears to occur efficiently only in a *P. aeruginosa* mutator background, in which multiple mutations lead to overexpression and structural modifications of AmpC.

The growing prevalence of nosocomial infections produced by multiresistant *Pseudomonas aeruginosa* strains severely compromises the selection of appropriate treatments and is therefore associated with significant morbidity and mortality (1–3). While the incidences of concerning transferable resistance determinants, such as those encoding class B carbapenemases (or metallo-β-lactamases), are increasing, especially in certain areas (4, 5), the current global threat of antimicrobial resistance in *P. aeruginosa* mainly still results from the extraordinary capacity of this microorganism to develop resistance to almost any available antibiotic by the selection of mutations in chromosomal genes (6, 7). Among the particularly noteworthy mutation-mediated resistance mechanisms are those leading to the repression or inactivation of the carbapenem porin OprD, the hyperproduction of the chromosomal cephalosporinase AmpC, or the upregulation of one of the several efflux pumps encoded in the *P. aeruginosa* genome (8, 9). Furthermore, the accumulation of these various chromosomal mutations can lead to the emergence of multiresistant strains that eventually may be responsible for notable outbreaks in the hospital setting (7, 10). Therefore, strategies to overcome *P. aeruginosa* mutation-driven resistance mechanisms are urgently needed.

Ceftolozane (formerly CXA-101) is a new cephalosporin under clinical development in combination with tazobactam (ceftolozane-tazobactam, formerly CXA-201) that shows promising characteristics for the treatment of *P. aeruginosa* infections. Although tazobactam does not have a major impact on the activity of ceftolozane against *P. aeruginosa*, it significantly enhances the coverage of *Enterobacteriaceae* isolates producing extended-spectrum β-lactamases (11). Indeed, several recent studies revealed a potent *in vitro* activity of ceftolozane against *P. aeruginosa*, including in many cystic fibrosis and multiresistant strains not producing horizontally acquired β-lactamases (12–16). Additionally, *in vitro* studies have shown that ceftolozane appears to be stable against the most common resistance mechanisms driven by mutation in this species, particularly the overexpression of the chromosomal cephalosporinase AmpC or efflux pumps, conserving activity against pan-β-lactam-resistant clinical strains (17, 18). Previous studies have also revealed that the spontaneous mutation rate for the development of 4× MIC of ceftolozane-resistant mutants was below the detection limit ($<10^{-10}$) even for DNA mismatch-repair-deficient mutator strains (19). Based on these previous find-

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ings, the objective of this work was to compare the dynamics and mechanisms of *in vitro* development of resistance to ceftolozane-tazobactam with the currently available antipseudomonal agents, using wild-type and mutator strains, under long-term exposure to growing drug concentrations.

MATERIALS AND METHODS

Strains. The wild-type reference strain *P. aeruginosa* PAO1 and its mismatch-repair-deficient (Δ*mutS*) mutator derivative (PAOMS) were used (19).

Dynamics of resistance development. To determine the dynamics of resistance development to ceftazidime, meropenem, ciprofloxacin, and ceftolozane-tazobactam, 10-ml Mueller-Hinton tubes containing 0.5×, 1×, 2×, 4×, 8×, 16×, 32×, and 64× MIC values of each antibiotic were inoculated with approximately 10^6 CFU/ml of exponentially growing PAO1 or PAOMS strains and incubated for 24 h at 37°C and 180 rpm. All experiments were performed in triplicate. The tubes from the highest antibiotic concentration showing growth were reinoculated (at a 1:1,000 dilution) in fresh medium containing concentrations up to 64× MIC for 7 consecutive days. Two colonies per strain, antibiotic, resistance step, and replicate experiment were purified in antibiotic-free LB agar plates for further characterization.

Susceptibility testing. The MICs of ceftolozane, ceftolozane-tazobactam, ceftazidime, cefepime, piperacillin, piperacillin-tazobactam, aztreonam, imipenem, meropenem, and ciprofloxacin were determined by broth microdilution according to CLSI guidelines (20).

Characterization of resistance mechanisms. The expression of the genes encoding the chromosomal β-lactamase AmpC (*ampC*) and four *P. aeruginosa* efflux pumps, MexAB-OprM (*mexB*), MexCD-OprJ (*mexD*), MexXY-OprM (*mexY*), and MexEF-OprN (*mexF*), were determined from late-log-phase Luria-Bertani (LB) broth cultures at 37°C and 180 rpm by real-time reverse transcription-PCR (RT-PCR), as previously described (8). The quinolone resistance determining regions (QRDR) of *gyrA*, *gyrB*, *parC*, and *parE* were sequenced in ciprofloxacin-resistant mutants (7). Outer membrane protein (OMP) profiles were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and stained with Coomassie blue (8). The obtained OprD profiles were compared with those of PAO1 and its OprD-deficient mutant. Penicillin-binding proteins (PBPs) were labeled with Bocillin FL fluorescent penicillin, separated through SDS-PAGE, and visualized using a Bio-Rad FX Pro molecular imager (21).

RNA sequencing. RNA sequencing (RNA-seq) was performed in duplicate on the parental strains PAO1 and PAOMS and on two low-level (MICs, 4 to 8 μg/ml) PAO1 and 3 high-level (MICs, 32 to 128 μg/ml) PAOMS ceftolozane-tazobactam-resistant mutants. Total RNA was isolated from three replicate cultures (optical density at 600 nm [OD_{600}], 1; using LB broth and at 37°C and 180 rpm) and after rRNA depletion by the use of a commercial capture and depletion system (MICROB*Express* kit; Ambion), strand-specific bar-coded cDNA libraries were generated, and all samples were sequenced using a lane of an Illumina HiSeq 2500. The raw sequence output consisted of 263.7 million reads, with a length of 100 nucleotides. Computational analysis was slightly modified from that used by Dötsch et al. (22). Briefly, the reads were mapped using Stampy (23), differential gene expression was calculated using the DESeq package (24), and mutations were identified using SAMtools (25). The *Pseudomonas* genome database was used for gene function analysis (26, 27).

Characterization of *ampC* mutations. The obtained *ampC* mutant derivatives were cloned in parallel with the wild-type *ampC* gene from PAO1. For this purpose, PCR products obtained with upstream (AmpC-F-EcoRI, 5′-TCGAATTCACGACAAAGGACGCCAATCC-3′) and downstream (AmpC-R-HinDIII, TCAAGCTTTCAGCGCTTCAGC GGCACC) primers were digested with EcoRI or HinDIII, ligated to pUCP24 (28), and transformed into *Escherichia coli* XL1-Blue made competent by $CaCl_2$. Transformants were selected in 5 μg/ml gentamicin MacConkey agar plates. The cloned genes obtained from three independent experiments were fully sequenced to ascertain the absence of mutations introduced during PCR amplification. The resulting plasmids were transformed into an *ampC* knockout mutant of PAO1 (PAΔC) (29) and characterized through the determination of the MICs for ceftolozane, ceftolozane-tazobactam, ceftazidime, cefepime, piperacillin, piperacillin-tazobactam, aztreonam, and imipenem using broth microdilution, according to CLSI guidelines.

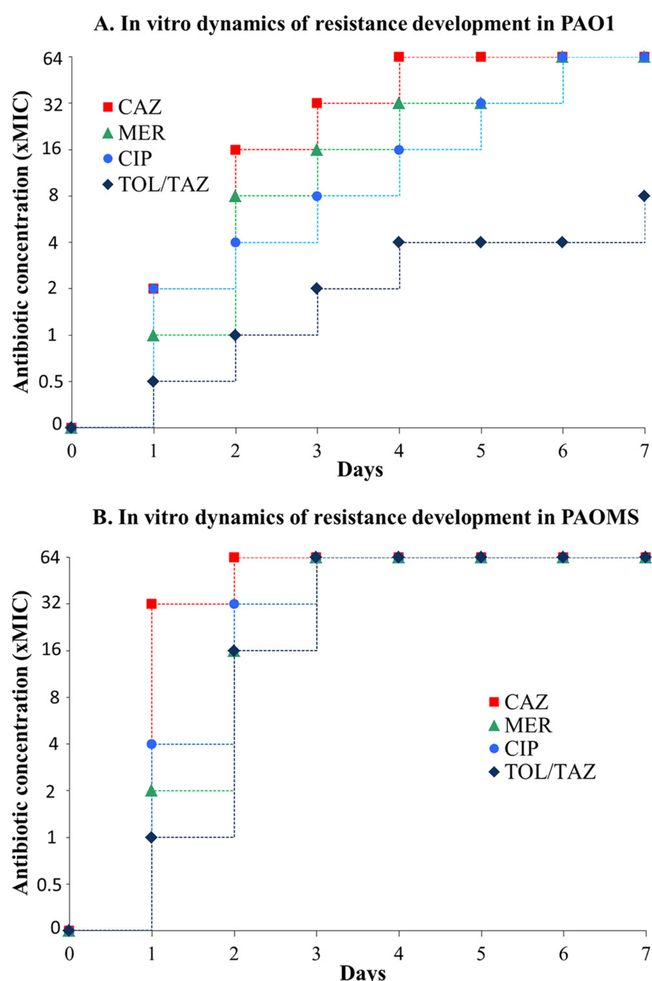

FIG 1 Dynamics of resistance development to ceftolozane-tazobactam and comparators. The modal values for three experiments are shown.

***In vitro* competition experiments.** *In vitro* competition experiments between each of the resistant mutants and a gentamicin-tagged (*att* intergenic neutral chromosomal locus) wild-type PAO1 were performed (29, 30). Exponentially growing cells were mixed in a 1:1 ratio and diluted in 0.9% saline solution. Approximately 10^3 cells from each of the mixtures were inoculated into eight 10-ml LB broth flasks and grown at 37°C and 180 rpm for 16 to 18 h, corresponding to approximately 20 generations. Serial 10-fold dilutions were plated in duplicate onto LB agar alone and with 15 μg/ml of gentamicin. The competition index (CI) was defined as the mutant-to-wild-type ratio.

RESULTS AND DISCUSSION

Analysis of the dynamics of resistance development to ceftolozane-tazobactam and comparators in wild-type and mutator strains. The basal MICs for both PAO1 and PAOMS strains of ceftazidime, meropenem, ciprofloxacin, and ceftolozane-tazobactam were 1, 0.5, 0.125, and 0.5 μg/ml, respectively.

TABLE 1 Susceptibility profiles and resistance mechanisms of mutants selected during stepwise exposure to ceftolozane-tazobactam, ceftazidime, meropenem, and ciprofloxacin in each of three experiments

	MIC (μg/ml) by antibiotic (CLSI breakpoint)[b]								
Strain information[a]	TOL-TAZ	CAZ (S ≤ 8)	FEP (S ≤ 8)	PIP-TAZ (S ≤ 16)	AZT (S ≤ 8)	IMP (S ≤ 4)	MER (S ≤ 4)	CIP (S ≤ 1)	Resistance mechanism(s)[c]
PAO1	0.5	1	1	4	4	1	0.5	0.12	None
1, P1.1, TOL-TAZ^r (0.5×)	1	4	2	8	**16**[d]	1	1	0.12	None
1, P1.2, TOL-TAZ^r (0.5×)	1	4	2	8	8	1	1	0.12	None
1, P1.3, TOL-TAZ^r (0.5×)	1	4	2	8	8	1	1	0.12	None
2, P1.1, TOL-TAZ^r (1×)	1	4	2	8	**16**	1	1	0.12	None
2, P1.2, TOL-TAZ^r (1×)	1	4	2	8	**16**	1	1	0.12	None
2, P1.3, TOL-TAZ^r (1×)	1	4	2	8	**16**	1	1	0.12	None
3, P1.1, TOL-TAZ^r (2×)	2	4	4	16	**16**	0.5	2	0.25	None
3, P1.2, TOL-TAZ^r (2×)	2	8	4	8	**16**	1	1	0.25	None
3, P1.3, TOL-TAZ^r (2×)	2	4	4	16	**16**	1	1	0.25	None
4, P1.1, TOL-TAZ^r (4×)	4	4	4	16	**16**	2	2	0.25	None
4, P1.2, TOL-TAZ^r (4×)	4	8	4	16	8	2	2	0.25	None
4, P1.3, TOL-TAZ^r (4×)	4	8	4	16	**16**	2	2	0.25	None
7, P1.1, TOL-TAZ^r (8×)	4	4	8	**32**	**16**	1	2	0.25	None
7, P1.2, TOL-TAZ^r (8×)	8	8	4	16	**16**	2	4	0.5	None
7, P1.3, TOL-TAZ^r (8×)	8	8	4	16	**16**	4	4	0.12	None
1, PmS.1, TOL-TAZ^r (1×)	2	**32**	**16**	**128**	**128**	1	0.5	0.25	*ampC* (619)
1, PmS.2, TOL-TAZ^r (1×)	2	**32**	8	**128**	**128**	4	1	0.5	*ampC* (602)
1, PmS.3, TOL-TAZ^r (1×)	4	**64**	**16**	**256**	**256**	4	1	0.25	*ampC* (2,863)
2, PmS.1, TOL-TAZ^r (16×)	16	**32**	2	8	**32**	0.25	1	0.25	*ampC* (264)
2, PmS.2, TOL-TAZ^r (16×)	16	**32**	4	8	**32**	0.25	1	0.25	*ampC* (444)
2, PmS.3, TOL-TAZ^r (16×)	32	**64**	8	8	**32**	0.25	1	0.5	*ampC* (734)
3, PmS.1, TOL-TAZ^r (64×)	128	**256**	8	8	**128**	0.12	1	0.25	*ampC* (169)
3, PmS.2, TOL-TAZ^r (64×)	64	**256**	8	16	**64**	0.12	0.5	0.5	*ampC* (804)
3, PmS.3, TOL-TAZ^r (64×)	32	**64**	4	16	**32**	0.06	0.5	0.03	*ampC* (4,459)
7, PmS.1, TOL-TAZ^r (64×)	128	**>256**	**16**	16	**128**	0.06	0.5	0.06	*ampC* (128)
7, PmS.2, TOL-TAZ^r (64×)	32	**256**	8	16	**128**	0.12	0.5	0.03	*ampC* (268)
7, PmS.3, TOL-TAZ^r (64×)	64	**128**	8	**32**	**64**	0.12	1	0.06	*ampC* (458)
7, P1.1, CAZ^r (64×)	2	**128**	**64**	**>256**	**128**	2	2	0.12	*ampC* (350)
7, P1.2, CAZ^r (64×)	4	**128**	64	**>256**	**128**	2	2	0.12	*ampC* (32)
7, P1.3, CAZ^r (64×)	4	**128**	64	**>256**	**128**	4	2	0.12	*ampC* (67)
7, PmS.1, CAZ^r (64×)	4	**128**	32	**128**	**128**	1	0.25	0.12	*ampC* (244)
7, PmS.2, CAZ^r (64×)	4	**128**	16	**128**	**128**	1	0.5	0.12	*ampC* (672)
7, PmS.3, CAZ^r (64×)	4	**256**	64	**>256**	**256**	1	1	0.12	*ampC* (254)
7, P1.1, MER^r (64×)	2	**16**	4	**256**	**128**	**16**	**64**	1	OprD$^-$ + *mexB* (4.5) + *mexF* (48)
7, P1.2, MER^r (64×)	1	**16**	4	**128**	**64**	**16**	**128**	1	OprD$^-$ + *mexB* (4.4)
7, P1.3, MER^r (64×)	2	**16**	4	**128**	**128**	**16**	**64**	1	OprD$^-$ + *mexB* (5.8)
7, PmS.1, MER^r (64×)	0.25	2	8	**32**	**64**	**16**	**64**	1	OprD$^-$ + *ampC* (17) + *mexB* (5.3) + *mexY* (11) + *mexF* (44)
7, PmS.2, MER^r (64×)	1	8	**16**	**128**	**256**	**16**	**64**	0.5	OprD$^-$ + *mexB* (16)
7, PMS.3, MER^r (64×)	1	8	**16**	**64**	**64**	**8**	**64**	0.5	OprD$^-$ + *mexB* (13)
7, P1.1, CIP^r (64×)	0.25	0.5	4	2	2	<0.12	0.25	**>32**	*mexD* (515) + GyrA T83I + ParC S87L
7, P1.2, CIP^r (32×)	0.25	1	4	4	1	<0.12	0.25	**8**	*mexD* (739) + GyrA E153K + ParC S87L
7, P1.3, CIP^r (64×)	0.5	2	2	16	**32**	1	2	**>32**	*mexB* (8.4) + GyrA T83I + ParC D117E
7, PmS.1, CIP^r (16×)	0.25	0.5	4	4	2	0.5	0.5	**>32**	*mexD* (534) + GyrA T83I + ParC E91K
7, PmS.2, CIP^r (64×)	0.25	0.5	4	4	2	0.25	0.5	**>32**	*mexD* (441) + GyrA T83I + ParC S87L
7, PmS.3, CIP^r (64×)	0.25	0.5	2	4	2	0.5	0.5	**>32**	*mexD* (430) + GyrA T83I + ParC S87L

[a] Format for strains: number of days of exposure, P1 (PAO1) or PmS (PAOMS), antibiotic resistance (concentration of antibiotic in tubes from which the mutants were selected). For ceftazidime, meropenem, and ciprofloxacin, only mutants obtained in the final step of the experiment (day 7) are included. For ceftolozane-tazobactam, mutants from the intermediate selection steps (according to data from Fig. 1) are also included with the day 7 mutants.

[b] TOL-TAZ, ceftolozane-tazobactam; CAZ, ceftazidime, FEP, cefepime; PIP-TAZ, piperacillin-tazobactam; AZT, aztreonam; IMP, imipenem; MER, meropenem; CIP, ciprofloxacin; S, susceptible.

[c] Resistance mechanisms studied. *ampC*, *mexB*, *mexD*, *mexF*, and *mexY* expression. Previously described breakpoints (8) were applied for defining overexpression: *ampC*, *mexD*, *mexY*, and *mexF*, >10-fold compared to wild-type PAO1; *mexB*, >3-fold compared to wild-type PAO1. Expression levels are indicated in parentheses. The lack of OprD (OprD$^-$), as evidenced by the analysis of outer membrane proteins (OMPs) through SDS-PAGE and mutations in the QRDR regions of GyrA, GyrB, ParC, and ParE are also indicated.

[d] Bold type indicates strains that are not susceptible.

As shown in Fig. 1A, the development of high-level resistance in the PAO1 strain was fastest for ceftazidime, reaching 64× MIC by day 4, followed by meropenem and ciprofloxacin, which reached 64× MIC at day 6. In contrast, resistance development was much slower for ceftolozane-tazobactam, with modal concentrations reaching only 8× MIC after the completion of the 7-day experiments (Fig. 1A). Moreover, a 64× MIC was not reached in any of three cultures even after extended 14-day exposure experiments (not shown).

As shown in Fig. 1B, the development of resistance was dramati-

TABLE 2 Mutations detected by RNA-seq of PAO1 and PAOMS derivative mutants after 7 days of exposure to increasing concentrations of ceftolozane-tazobactam

Gene information			Mutation(s) by strain type[a]				
Locus	Name	Product name	7, P1.1, TOL-TAZ^r	7, P1.2, TOL-TAZ^r	7, PmS.1, TOL-TAZ^r	7, PmS.2, TOL-TAZ^r	7, PmS.3, TOL-TAZ^r
Locus	Name	Product name					
PA0123		Probable transcriptional regulator				R131H	R131H
PA0136		Probable ATP-binding component of ABC transporter				Silent	
PA0201		Hypothetical protein			Silent		
Intergenic						A266314G	
PA0347	*glpQ*	Glycerophosphoryl diester phosphodiesterase, periplasmic			Y75H		
PA0352	*yicE*	Probable transporter					P371L
PA0378	*mgtA*	Probable transglycosylase			Y23H		
PA0413	*chpA*	Component of chemotactic signal transduction system				Q947R	
PA0615		Hypothetical protein			Silent		
Intergenic						C788723T	
Intergenic					G811242A		
PA0747		Probable aldehyde dehydrogenase			I5T		
PA0811		Probable major facilitator superfamily (MFS) transporter				Silent	Silent
PA0877		Probable transcriptional regulator				Silent	
PA0887	*acsA*	Acetyl-coenzyme A synthetase				Silent	Silent
PA0895	*aruC*	*N*-Succinylglutamate 5-semialdehyde dehydrogenase			Silent		
PA0902		Hypothetical protein				nt_{858}Ins(G)	nt_{858}Ins(G); A43V
PA0919		Hypothetical protein				D40N	
PA0920		Hypothetical protein				L437P	L437P
PA0923	*dinB*	DNA polymerase IV, DinB			L316F		
PA0928	*gacS*	Sensor/response regulator hybrid			G489S		L531P
Intergenic						A1037625G	A1037625G
PA0964	*pmpR*	*pqsR*-mediated PQS regulator, PmpR			E225G		
PA0971	*tolA*	TolA protein				S12G	S12G
PA0997	*pqsB*	PqsB				Silent	
Intergenic						$nt_{1083918}$Ins(G)	
PA1026		Hypothetical protein				S148P	
PA1069		Hypothetical protein					Silent
PA1124	*dgt*	Deoxyguanosinetriphosphate triphosphohydrolase				H344R	H344R
Intergenic							G1220630A
PA1223		Probable transcriptional regulator				Silent	Silent
Intergenic					G1397241A		
PA1269		Probable 2-hydroxy acid dehydrogenase			Silent		
PA1310	*phnW*	2-Aminoethylphosphonate: pyruvate aminotransferase				A176V	A176V
PA1458	*cheA*	Probable two-component sensor			$nt_{2167}\Delta 1$		
PA1480	*ccmF*	Cytochrome *c*-type biogenesis protein CcmF			F292L		
Intergenic						C1674355T	C1674355T
PA1622		Probable hydrolase				A114V	
PA1662	*clpV2*	ClpV2			A128V		
PA1690	*pscU*	Translocation protein in type III secretion					A11T
PA1730		Conserved hypothetical protein				Silent	Silent
PA1797		Hypothetical protein			Silent		
PA1802	*clpX*	ClpX		G266D			
PA2023	*galU*	UTP-glucose-1-phosphate uridylyltransferase					D64G
PA2040	*pauA4*	Glutamylpolyamine synthetase		Silent			
PA2138		Probable ATP-dependent DNA ligase			G260S		
Intergenic					T2545663C		
PA2232	*pslB*	PslB				V414A	V414A
PA2250	*lpdV*	Lipoamide dehydrogenase-Val				W118X	W118X
Intergenic					T2581013C		
PA2387	*fpvI*	FpvI				Silent	
PA2399	*pvdD*	Pyoverdine synthetase D			R1576Q		
PA2402		Probable nonribosomal peptide synthetase			A2368T		
PA2443	*sdaA*	L-Serine dehydratase					Silent
PA2540		Conserved hypothetical protein			Silent		
PA2597		Hypothetical protein				Silent	Silent
PA2615	*ftsK*	Cell division protein FtsK			Silent		
PA2643	*nuoH*	NADH dehydrogenase I chain H			V101A		
Intergenic						T3108446C	T3108446C
PA2938		Probable transporter			Silent		
PA3047	*dacB*	Probable D-alanyl-D-alanine carboxypeptidase			G427D	E84K	
PA3060	*pelE*	PelE				W102R	W102R
Intergenic						A3481349G	A3481349G
PA3158	*wbpB*	UDP-2-acetamido-2-deoxy-D-glucuronic acid 3-dehydrogenase, WbpB				Silent	Silent
PA3179	*yciL*	Conserved hypothetical protein			Silent		
PA3187	*gltK*	Probable ATP-binding component of ABC transporter				A299T	
PA3206		Probable two-component sensor	I279T				
PA3346		Two-component response regulator			P300S		
PA3516		Probable lyase				T186I	
PA3614		Hypothetical protein				G380D	G380D
PA3624	*pcm*	L-Isoaspartate protein carboxyl methyltransferase type II			T86A		

(Continued on following page)

TABLE 2 (Continued)

Gene information			Mutation(s) by strain type[a]				
Locus	Name	Product name	7, P1.1, TOL-TAZ[r]	7, P1.2, TOL-TAZ[r]	7, PmS.1, TOL-TAZ[r]	7, PmS.2, TOL-TAZ[r]	7, PmS.3, TOL-TAZ[r]
PA3666	*dapD*	Tetrahydrodipicolinate succinylase					Silent
PA3795		Probable oxidoreductase			Silent		
PA3803	*gcpE*	Probable isoprenoid biosynthetic protein GcpE			V224A		
PA3900		Probable transmembrane sensor				R278Q	
PA3919	*ylaK*	Conserved hypothetical protein			L267P		
Intergenic						$nt_{4388937}\Delta1$	$nt_{4388937}\Delta1$
PA3935	*tauD*	Taurine dioxygenase				N241S	
PA3974	*ladS*	Lost adherence sensor				N230S	
Intergenic						T4544558C	
PA4069		Hypothetical protein			Silent		
PA4109	*ampR*	Transcriptional regulator AmpR					D135N
PA4110	*ampC*	β-Lactamase precursor			F147L, Q157R, E247K, V356I	E247K, V356I	G183D
PA4120		Probable transcriptional regulator				Silent	Silent
PA4147	*acoR*	Transcriptional regulator AcoR			nt_{547}Ins(C)		
PA4186		Hypothetical protein				G249S	
PA4208	*opmD*	Probable outer membrane protein precursor				E142G	
PA4290		Probable chemotaxis transducer				Q185X	Q185X
PA4311		Conserved hypothetical protein			E298K		
PA4313		Hypothetical protein			Silent		
Intergenic				G4903413A			
PA4526	*pilB*	Type 4 fimbrial biogenesis protein PilB	$nt_{1478}\Delta3$	$nt_{1478}\Delta3$			
PA4548	*yfiT*	Probable D-amino acid oxidase					Silent
PA4556	*pilE*	Type 4 fimbrial biogenesis protein PilE			N133S		
PA4571		Probable cytochrome *c*			C15Y		
Intergenic					C5141232T		
PA4622		Probable major facilitator superfamily (MFS) transporter			E361K		
PA4659		Probable transcriptional regulator			R239H		
PA4673.1		tRNA-Met				T5242034C	
Intergenic							C5282588T
PA4745	*nusA*	N utilization substance protein A					P87L
PA4771	*lldD*	L-Lactate dehydrogenase				R50W	R50W
PA4783	*yedA*	Conserved hypothetical protein			Silent		
PA4819		Probable glycosyl transferase				I268T	
Intergenic							G5431174A
PA4840	*yciH*	Conserved hypothetical protein				R57C	
PA4846	*aroQ1*	3-Dehydroquinate dehydratase					R65H
PA4848	*accC*	Biotin carboxylase				Silent	Silent
PA4856	*retS*	RetS (regulator of exopolysaccharide and type III secretion)			G197S		
PA4911		Probable permease of ABC branched-chain amino acid transporter				T172A	
Intergenic						G5599844A	G5599844A
PA5006	-	Hypothetical protein			Silent		
PA5197	*rimK*	Ribosomal protein S6 modification protein			$nt_{871}\Delta1$		
PA5398	*dgcA*	DgcA, dimethylglycine catabolism				T276A	T276A
PA5474		Probable metalloprotease					G167D
PA5490	*cc4*	Cytochrome c_4 precursor					D172G
PA5538	*amiA*	*N*-Acetylmuramoyl-L-alanine amidase			P18S		
Intergenic					G6252699A		

[a] Format for strains: number of days of exposure, P1 (PAO1) or PmS (PAOMS), antibiotic resistance.

cally enhanced for all compounds in the mutator strain; 64× MICs were reached by day 2 for ceftazidime and by day 3 for meropenem, ciprofloxacin, and ceftolozane-tazobactam. However, first-step ceftolozane-tazobactam resistance development was very limited even for the mutator strain, with concentrations reaching only 1× MIC after day 1. Thus, these results are consistent with previous findings suggesting that resistance development to ceftolozane cannot be achieved by single-step mutations (19).

Analysis of the susceptibility profiles and resistance mechanisms of the mutants selected during stepwise antibiotic exposure. All mutants selected upon ceftazidime exposure showed high-level resistance to ceftazidime, cefepime, piperacillin-tazobactam, and aztreonam (Table 1), caused by the overexpression of the chromosomal cephalosporinase AmpC (Table 1). Cross-resistances to carbapenems and/or fluoroquinolones were not detected in any of the ceftazidime-selected mutants, and none of them showed efflux pump overexpression. Remarkably, ceftolozane-tazobactam MICs remained at ≤4 μg/ml in all ceftazidime-selected mutants, confirming the much higher stability of ceftolozane-tazobactam against AmpC hydrolysis (17, 18).

Mutants selected upon meropenem exposure developed resistance to imipenem and meropenem through the loss of the expression of the carbapenem porin OprD (Table 1), but they also showed significantly enhanced MICs for ceftazidime, cefepime, piperacillin-tazobactam, aztreonam, and ciprofloxacin due to the overexpression of the efflux pump MexAB-OprM in all of them, occasionally accompanied by the overexpression of other efflux pumps (MexXY-OprM or MexEF-OprN) or AmpC (Table 1). Thus, meropenem exposure selected multidrug-resistant (MDR) profiles. Nevertheless, in contrast to all other antibiotics tested, the MICs of ceftolozane-tazobactam were not increased in meropenem-selected mutants.

Similarly, all ciprofloxacin-resistant mutants showed two QRDR mutations (*gyrA* and *parC*) determining high-level fluoro-

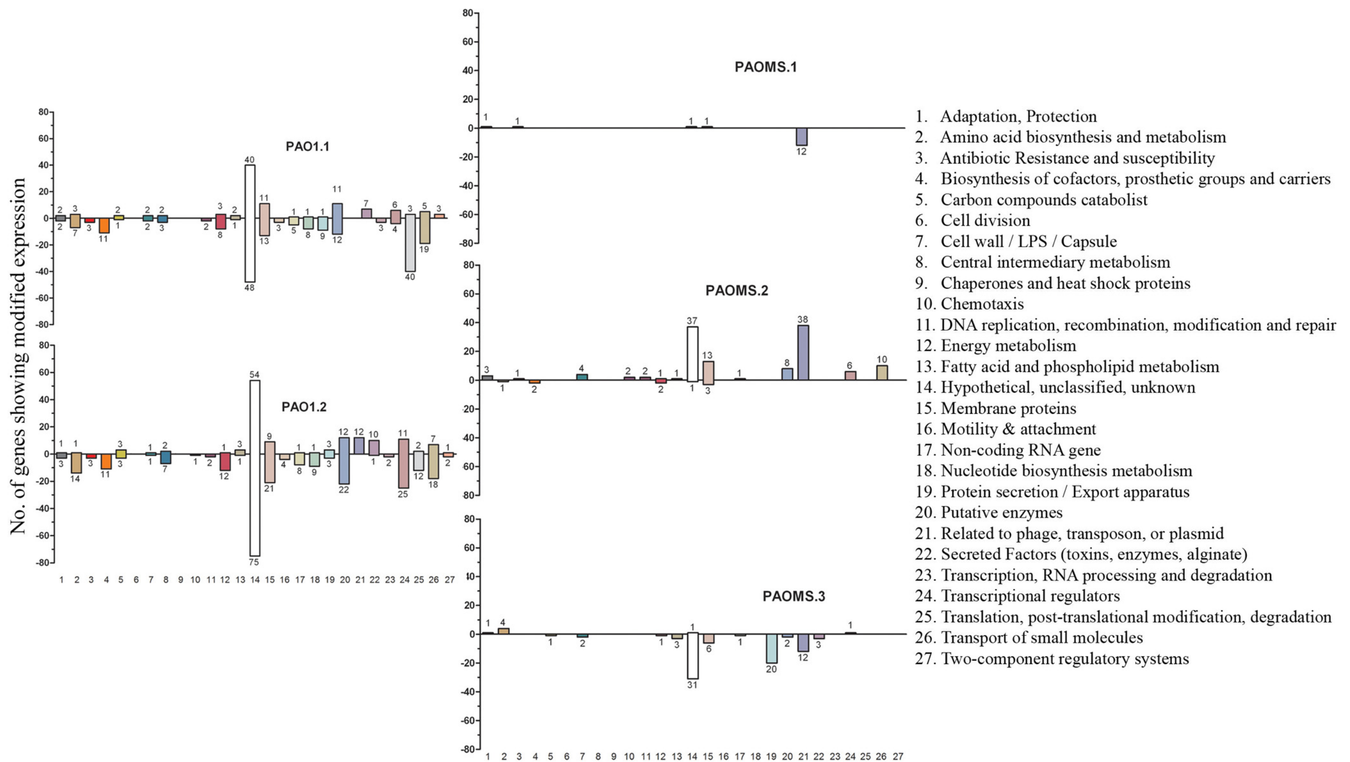

FIG 2 Numbers of genes showing modified expression in the studied ceftolozane-tazobactam resistant mutants compared with wild-type PAO1 in each of the 27 established functional categories. Negative numbers indicate genes with decreased expression, and positive numbers indicate genes with increased expression.

quinolone resistance. Remarkably, while the mutations detected most frequently included the classical GyrA T83I and ParC S87L, mutations not previously described in GyrA (E153K) and ParC (D117E) were also detected, each in one different mutant (Table 1). The specific effects of these new QRDR mutations in quinolone resistance are under investigation in our laboratory. Additionally, all ciprofloxacin-resistant mutants overexpressed efflux pumps (MexAB-OprM or MexCD-OprJ) conferring reduced susceptibility to unrelated antipseudomonal agents (Table 1).

In contrast, PAO1 ceftolozane-tazobactam mutants reached only moderate resistance (MICs, 4 to 8 μg/ml) after the 7-day exposure experiments. High-level ceftolozane-tazobactam-resistant mutants were selected only in PAOMS experiments and showed cross-resistance to ceftazidime, piperacillin-tazobactam, and aztreonam due to AmpC overexpression. On the other hand, none of the ceftolozane-tazobactam-selected mutants overexpressed efflux pumps. Moreover, high-level ceftolozane-tazobactam-resistant mutants showed increased susceptibility to imipenem and ciprofloxacin (Table 1).

Characterization of ceftolozane-tazobactam resistance mechanisms through whole-genome analysis. PAO1 ceftolozane-tazobactam mutants, reaching only moderate resistance (MICs, 4 to 8 μg/ml) after the 7-day exposure experiments, showed only two to four mutations in the RNA-seq experiments (Table 2). Both mutants showed a deletion in *pilB*, whereas PAO1.1 showed a mutation in PA3206 (a probable two-component sensor) and PAO1.2 in the intracellular protease ClpX. PAO1.2 additionally showed a silent and an intergenic mutation. Despite the small number of mutations, a global transcriptome analysis revealed a remarkable number of genes with modified expression both in PAO1.1 (309 genes) and PAO1.2 (395 genes), perhaps related to the broad regulatory functions of PA3206 and

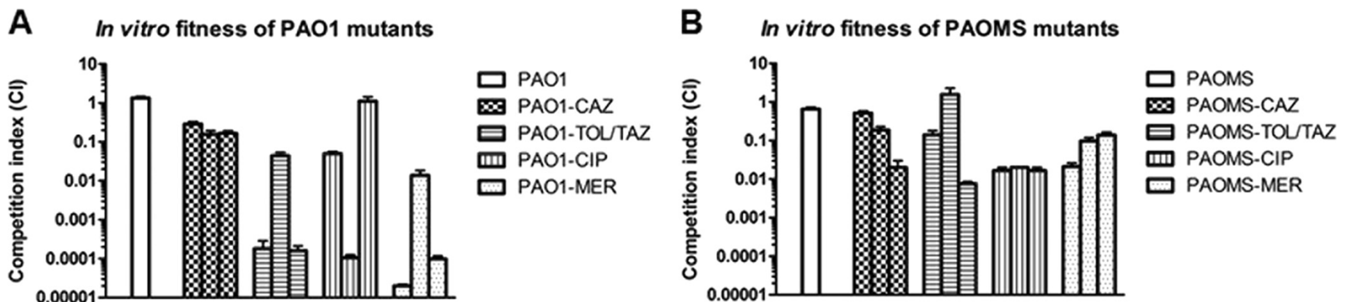

FIG 3 *In vitro* competition assays to assess the fitness cost associated with the development of resistance to ceftolozane-tazobactam (TOL-TAZ), ceftazidime (CAZ), meropenem (MER), and ciprofloxacin (CIP) in the three day 7 PAO1 and PAOMS mutants described in Table 1. Error bars indicate standard deviation.

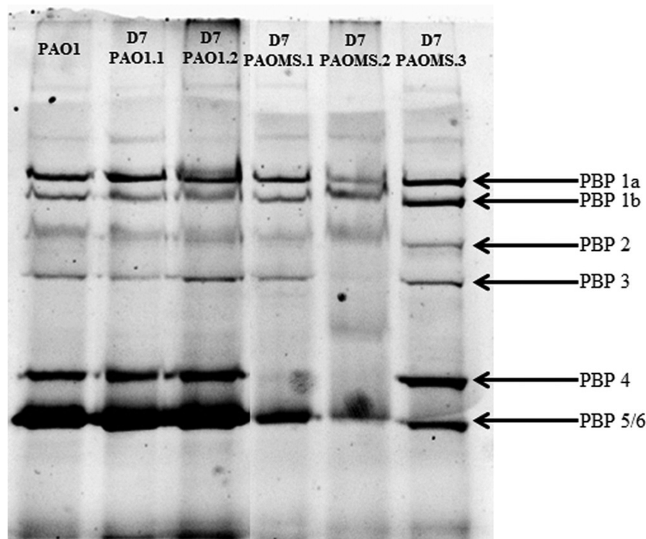

FIG 4 Penicillin-binding protein (PBP) profiles of ceftolozane-tazobactam-resistant mutants obtained from PAO1 and PAOMS strains.

ClpX (Fig. 2; see also Table S1 in the supplemental material). However, neither the mutations detected nor the genes showing modified expression were directly linked to classical antibiotic resistance mechanisms. Moreover, both mutants showed greatly reduced expression of genes belonging to the MDR efflux pumps MexXY-OprM (*mexX* and *mexY*) and MexCD-OprJ (*mexC*) (see Table S1 in the supplemental material). The specific effects on β-lactam resistance of the detected mutations are under investigation in our laboratory; the function of PA3206 is currently unknown, but previous works established a role for intracellular proteases in multiple relevant processes, including antibiotic resistance, motility, biofilm formation, and alginate production (31, 32). Moreover, *in vitro* competition experiments revealed a major fitness cost in both mutants, with a CI of 0.0002 for PAO1.1 and a CI of 0.044 for PAO1.2 (Fig. 3A). Indeed, the CIs of moderately resistant ceftolozane-tazobactam PAO1 mutants were much lower than those of high-level ceftazidime-resistant mutants and comparable only to those of high-level meropenem-resistant mutants (Fig. 4A). Therefore, our data suggest that moderate ceftolozane-tazobactam resistance in PAO1 results from nonspecific mutations with global pleiotropic effects associated with an important fitness cost.

In contrast, high-level (MICs, 32 to 128 μg/ml) ceftolozane-tazobactam-resistant PAOMS mutants showed 45 to 53 mutations (Table 2). However, the number of genes showing modified expression (16 to 136 genes) was lower than for PAO1 mutants but always included *ampC* overexpression (Fig. 2; see also Table S1 in the supplemental material). Consistently, all PAOMS mutants showed mutations in *ampC* regulators (*dacB* [$n = 2$] or *ampR* [$n = 1$]) (Table 2). Interestingly, the detected AmpR mutation (D135N) has been observed among clinical isolates of multiple Gram-negative pathogens, including *P. aeruginosa* (33). The absence of a functional PBP4 was also evidenced in the two *dacB* mutants through the analysis of the PBP profiles (Fig. 4). The PBP profiles also revealed an apparent reduction of PBP3 (one of the main targets of cephalosporins) expression in one of the mutants. Moreover, all PAOMS mutants contained one to four mutations in the conserved residues of AmpC (F147L, Q157R, G183D, E247K, or V356I) (Table 2). Complementation studies on PAΔC revealed that these mutations greatly increased ceftolozane-tazobactam and ceftazidime MICs but reduced those of piperacillin-tazobactam and imipenem compared with wild-type *ampC* (Table 3). Further ongoing structural and biochemical studies with these mutant enzymes will yield relevant information for understanding the plasticity of AmpC enzymes for β-lactam hydrolysis and its impact on resistance.

The specific effects on the susceptibility profiles, if any, of each of the other multiple mutations detected in the PAOMS mutants still need to be explored. It is expected that a number of them should just be nonpositively selected random mutations as a consequence of the very high spontaneous mutation rate of PAOMS. This is likely to be the case for the 12 to 14 silent mutations detected in each of the mutants. However, in addition to those related to AmpC, several others of the nonsynonymous mutations might also play a role in the phenotype. Indeed, at least eight (*gacS*, *pqsB*, *phnW*, *galU*, *nuoH*, *nusA*, *pvdD*, and PA3516) of the mutated genes have been shown to have an impact (increase or decrease) on antimicrobial susceptibility in previous analyses of saturated

TABLE 3 Susceptibility profile of the PAO1 a*mpC* knockout mutant (PAΔC) complemented with wild-type *ampC* and derivatives from ceftolozane-tazobactam-resistant mutants

Strain (*ampC* mutation[s])	MICs (μg/ml)by antibiotic (CLSI breakpoint)[b]							
	TOL	TOL-TAZ	CAZ (S ≤ 8)	PIP (S ≤ 16)	PIP-TAZ (S ≤ 16)	FEP (S ≤ 8)	ATM (S ≤ 16)	IMP (S ≤ 4)
PAO1	0.5	0.5	1	2	2	0.5	2	1
PAΔC	0.5	0.5	1	2	2	0.5	2	0.25
PAΔC + $pUCPAC_{WT}$[a]	1	1	16	128	128	4	32	0.5
PAΔC + $pUCPAC_{PAOMS.1}$ (F147L, Q157R, E247K, V356I)	128	128	256	8	8	4	32	0.25
PAΔC + $pUCPAC_{PAOMS.2}$ (E247K, V356I)	64	64	128	32	32	8	32	0.25
PAΔC + $pUCPAC_{PAOMS.3}$ (G183D)	32	32	32	8	8	2	8	0.25

[a] WT, wild type.
[b] TOL, ceftolozane; TOL-TAZ, ceftolozane-tazobactam; CAZ, ceftazidime, PIP, piperacillin; PIP-TAZ, piperacillin-tazobactam; FEP, cefepime; ATM, aztreonam; IMP, imipenem; S, susceptible.

transposon-mutant libraries (34–37). Among these, mutations in *phnW*, *galU*, and *nuoH* have been shown to increase cephalosporin (ceftazidime) MICs, but remarkably some resulted in increased imipenem (*galU* and *nusA*) or ciprofloxacin (*gacS* and PA3516) susceptibility, consistent with the susceptibility profiles observed in our work (Table 1).

For all antibiotics, the impact on fitness of high-level antibiotic resistance was much lower for PAOMS mutants than for PAO1 mutants, likely reflecting the increased capacity of this strain to acquire cost-compensatory mutations (Fig. 3). Indeed, the fitness costs of high-level ceftolozane-tazobactam-resistant PAOMS mutants were highly variable, ranging from a CI of 0.008 in PAOMS.3 to a CI of 1.57 in PAOMS.2, possibly indicating the absence or presence of cost-compensatory mutations in these mutants (Fig. 3B).

Concluding remarks. The development of ceftolozane-tazobactam resistance was much slower than that of resistance to other antipseudomonal agents. Moreover, ceftolozane-tazobactam remained active against ceftazidime-, ciprofloxacin-, and meropenem-resistant *P. aeruginosa* mutants. After 7 days of exposure, the wild-type strain PAO1 developed only moderate resistance (MICs, 4 to 8 μg/ml), which was associated with a high biological cost. High-level resistance occurred only in the mutator strain, in which multiple mutations led to overexpression and structural modifications of AmpC. These mutations increased cephalosporin resistance but reduced resistances to penicillins and carbapenems. Ceftolozane-tazobactam is therefore envisaged as a valuable option for the treatment of *P. aeruginosa* infections, minimizing the development of self- and cross-resistance and conserving activity against MDR strains selected with other antipseudomonal agents.

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