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Extended-Spectrum Cephalosporinases in *Pseudomonas aeruginosa*▽

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The characterization of AmpC-type β-lactamases was performed in a collection of 32 clinical *Pseudomonas aeruginosa* isolates with intermediate susceptibility or resistance to imipenem and ceftazidime. Twenty-one out of those 32 isolates overexpressed AmpC β-lactamase, and the MICs of ceftazidime and imipenem were reduced after cloxacillin addition. Cloning and sequencing identified 10 AmpC β-lactamase variants. Reduced susceptibility to imipenem, ceftazidime, and cefepime was observed only with recombinant *P. aeruginosa* strains expressing an AmpC β-lactamase that had an alanine residue at position 105. The catalytic efficiencies (k_{cat}/K_m) of the AmpC variants possessing this residue were increased against oxyiminocephalosporins and imipenem. In addition, we show here that those AmpC variants constitute a favorable background for the in vitro selection of imipenem-resistant strains. This report identified a novel resistance mechanism that may contribute to imipenem resistance in *P. aeruginosa*.

Most AmpC-type β-lactamases naturally produced by gram-negative bacteria hydrolyze amino- and ureido-penicillins, cephamycins (cefoxitin or cefotetan), and, at low levels, oxyiminocephalosporins such as ceftazidime, cefotaxime, or ceftriaxone, and monobactams such as aztreonam (4). Zwitteronic cephalosporins such as cefepime and cefpirome and carbapenems such as imipenem and meropenem usually are excluded from the spectrum of activity of AmpC β-lactamases (10).

However, cephalosporinases with broadened substrate activity have been reported in several enterobacterial isolates, including *Enterobacter cloacae*, *Enterobacter aerogenes*, *Serratia marcescens,* and *Escherichia coli* (16, 23). These extended-spectrum AmpC (ESAC) β-lactamases confer reduced susceptibility to all cephalosporins, including cefepime and cefpirome (16, 23). Those enzymes differ from wild-type cephalosporinases by amino acid substitutions or insertions in four regions in the vicinity of the active site: the Ω loop, the H-10 helix, the H-2 helix, and the C-terminal end of the protein (1, 2, 7, 8, 11, 16, 18–21, 24, 25, 28, 29).

In *P. aeruginosa*, the overexpression of the naturally occurring AmpC is associated with a decreased susceptibility or resistance to expanded-spectrum cephalosporins such as ceftazidime. Resistance to those cephalosporins also may be related to clavulanic acid-inhibited extended-spectrum β-lactamases (ESBLs) (31). Resistance to cefepime and susceptibility to ceftazidime have been related to the overexpression of an efflux pump (MexXY-OprM) or to the production of OXA-30-like β-lactamases (12). Resistance to imipenem is associated mostly with structural changes or the loss of the OprD outer membrane protein and rarely to metallo-β-lactamases or specific Ambler class A β-lactamases such as GES derivatives and KPC-2 (30, 31).

Recently, several ESACs have been described from *E. coli* contributing to reduced susceptibility to imipenem (17). The aim of this study was to characterize the AmpC β-lactamases expressed by *P. aeruginosa* clinical isolates that were of intermediate susceptibility or resistant to imipenem and to search for the putative involvement of those AmpC proteins in that resistance pattern.

MATERIALS AND METHODS

Bacterial strains and plasmids. Nonduplicated *P. aeruginosa* clinical isolates were identified using the API 32GN system (bioMérieux, Marcy-l'Étoile, France). Thirty-two *P. aeruginosa* clinical isolates recovered at the Bicêtre hospital (K.-Bicêtre, France) in 2007 were included in this study (12 pulmonary pathogens, 11 urinary pathogens, 7 abscesses, and 2 from blood cultures). They were selected on the basis of intermediate susceptibility or resistance to ceftazidime and intermediate susceptibility or resistance to imipenem. AmpC production was further confirmed using cloxacillin (200 μg/ml)-containing plates, since cloxacillin inhibits AmpC β-lactamase activity and may restore susceptibility to ceftazidime (23).

E. coli TOP10 (Invitrogen, Life Technologies, Cergy-Pontoise, France), *P. aeruginosa* KG2505 (a knockout for the *ampC* gene), and *P. aeruginosa* 12B (an overproducer of the efflux pump MexXY-OprM) (gifts from P. Plésiat) were used as hosts for cloning and expression experiments. The kanamycin-resistant pCR-BluntII-TOPO vector (Invitrogen) and the low-copy tetracycline-resistant pBBR1MCS.3, a shuttle vector for *E. coli* and *P. aeruginosa* (15), were used for cloning experiments. *P. aeruginosa* PAO1 was used as the reference strain in susceptibility testing and β-lactamase assays. Bacterial cells were grown in trypticase soy (TS) broth or on TS agar plates (Sanofi Diagnostics Pasteur, Marnes-La-Coquette, France) with antibiotics when required.

Antimicrobial agents and MIC determinations. The antimicrobial agents and their sources have been described elsewhere (26). MICs were determined by the Etest technique (AB Biodisk, Solna, Sweden) and the agar dilution method as previously described (26). The results of susceptibility testing were recorded according to the guidelines of the CLSI (5).

β-Lactamase assays. AmpC β-lactamase-specific activity (in micromoles of cephalothin hydrolyzed per minute and per milligram of protein) was determined by UV spectrophotometry with culture extracts, as previously described (9). Cell extracts were obtained from noninduced overnight cultures. Strains were considered overproducers of AmpC β-lactamase when their specific β-lactamase activity was at least 10-fold higher than that of wild-type reference strain PAO1, which has been used as the reference strain in other studies (9, 14). To assess the specific production of AmpC β-lactamases, the β-lactamase activity also was determined after the incubation of culture extracts with 50 μM cloxacillin (AmpC β-lactamase inhibitor) for 15 min, as described previously (9). Extracts with >90% reduction of β-lactamase activity after cloxacillin addition were

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considered to produce AmpC-type β-lactamase as a major contributor of β-lactamase activity.

Amplification of bla_{ampC} genes and sequence analysis. Whole-cell DNA was extracted as described previously (3). For each PCR experiment, 500 ng of total DNA was used in a standard PCR. PCR amplifications of the *ampC*-type genes were performed with external primers PreAmpC-PA1 (5′-ATGCAGCCAACG ACAAAGG-3′) and PostAmpC-PA2 (5′-CGCCCTCGCGAGCGCGCTTC-3′), yielding a 1,243-bp amplification product encompassing the entire *ampC* gene of *P. aeruginosa* but excluding its promoter sequences. Internal primers ampC-PA-A (5′-CTTCCACACTGCTGTTCGCC-3′) and ampC-PA-B (5′-TTGGCC AGGATCACCAGTCC-3′) also were used for sequencing reactions. Sequence analyses were performed using software available at www.ncbi.nlm.nih.gov and http://www.ebi.ac.uk/clustalw/.

Cloning of β-lactamase genes. PCR amplification with primers PreAmpC-PA1 and PreAmpC-PA2 gave products that did not contain the original promoters of *ampC* genes. Those PCR products were cloned into pCR-BluntII-Topo (Invitrogen), and corresponding recombinant plasmids subsequently were transformed into *E. coli* strain TOP10, as described previously (19). Recombinant plasmids were selected on TS agar plates containing kanamycin (30 μg/ml) and amoxicillin (50 μg/ml). The XbaI-SpeI-digested fragments containing the *ampC* genes then were subcloned into the pBBR1MCS.3 vector in order to express those genes from an identical promoter, P_{T3}, in *P. aeruginosa* KG2505 and *P. aeruginosa* 12B. The recombinant clones were selected on TS agar plates containing tetracycline (20 μg/ml), amoxicillin (50 μg/ml), or piperacillin (100 μg/ml).

β-Lactamase purification. Recombinant *E. coli* TOP10 strains producing different AmpC β-lactamases were grown overnight at 37°C in 4 liters of TS broth containing amoxicillin (100 μg/ml) and kanamycin (30 μg/ml), resuspended in 40 ml of 100 mM phosphate buffer (pH 7), disrupted by sonication, and centrifuged at 20,000 × *g* at 4°C, as described previously (18). β-Lactamase extracts were dialyzed overnight at 4°C against 50 mM sodium phosphate buffer (pH 7) and loaded onto a preequilibrated SP-Sepharose column (GE Healthcare, Orsay, France). The flowthrough fractions containing the β-lactamase activity were recovered and dialyzed against 50 mM bicine buffer (pH 8.2) before being loaded onto a preequilibrated SP-Sepharose column (GE Healthcare, Orsay, France). The proteins were eluted by a linear NaCl gradient (0 to 1 M) in the same buffer. The eluted fractions with the highest β-lactamase activity (as determined by nitrocefin tests) were pooled and dialyzed against 100 mM phosphate buffer (pH 7). To assess the purity of the extracts and to determine the molecular weight of the AmpC β-lactamases, purified enzymes were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis (3 and data not shown).

Kinetic measurements. Purified β-lactamases PDC-1 (*Pseudomonas*-derived cephalosporinase 1), PDC-2, PDC-3, and PDC-5 were used for kinetic measurements (K_m and k_{cat}), which were carried out at 30°C in 100 mM sodium phosphate (pH 7.0). The rates of hydrolysis were determined with a Pharmacia ULTROSPEC 2000 UV spectrophotometer and were analyzed using the SWIFT II software. K_m and k_{cat} values were determined by analyzing the ß-lactam hydrolysis under initial rate conditions by using the Eadie-Hofstee linearization of the Michaelis-Menten equation, as described previously (6). When the K_m value was <5 μM, the K_i value was determined instead of the K_m by using benzylpenicillin as the substrate, and the k_{cat} value was determined from initial rates at saturating substrate concentrations ($[S] \gg K_m$). Values were the means of three independent measures.

Frequency of selection of mutants. To evaluate whether strains might become resistant to imipenem more easily when possessing an ESAC-type PDC enzyme instead of *P. aeruginosa* wild-type PDC-1, the frequency of the selection of mutants was determined by plating ~10^9 viable microorganisms on Mueller-Hinton agar medium with or without imipenem (1 to 2 μg/ml) using recombinant *P. aeruginosa* KG2505 Δ*ampC* mutant strains. The colony count was determined after 48 h at 37°C from three independent assays. Mutation frequencies were expressed as the number of resistant mutants recovered as a fraction of total viable bacteria from Mueller-Hinton agar medium without antibiotic (22).

Characterization of the in vitro mutants obtained from *P. aeruginosa*. Fifteen randomly selected mutants obtained from the imipenem (2 μg/ml)-containing plates during the mutant selection assays were further studied to evaluate the mechanisms involved in reduced susceptibility or resistance to imipenem. The relative level of *oprD* mRNA was quantified by real-time PCR by following a previously described protocol (9) using primers OprD-For (5′-GCTCGACCTC GAGGCAGGCCA-3′) and OprD-Rev (5′-CCAGCGATTGGTCGGATGCC A-3′), yielding a 242-bp amplification product. Mutants were considered to have reduced *oprD* expression with a relative *oprD* expression of ≤70% of those of the *P. aeruginosa* KG2505 parental strain (27).

Genomic DNA was extracted as described previously (3). The PCR amplification of *oprD* was performed by using primers OprD-F (5′-CGCCGACA AGAAGAACTAGC-3′) and OprD-R (5′GTCGATTACAGGATCGACAG-3′), yielding a 1,412-bp amplification product encompassing the entire *oprD* gene of *P. aeruginosa*. PCR products were fully sequenced using these primers and the internal primer OprD-F2 (5′-GCCGACCACCGTCAAATCG-3′), as previously described (9), and the resulting sequences were compared to that of the reference strain *P. aeruginosa* PAO1.

Nucleotide sequence accession numbers. The nucleotide sequences reported in this paper have been submitted to the EMBL/GenBank nucleotide sequence database under the accession numbers FJ666065 for PDC-1, FJ666064 for PDC-2, FJ666066 for PDC-3, FJ666067 for PDC-4, FJ666068 for PDC-5, FJ666069 for PDC-6, FJ666070 for PDC-7, FJ666071 for PDC-8, FJ666072 for PDC-9, and FJ666073 for PDC-10.

RESULTS

AmpC production. Thirty-two (6.7%) out of 474 nonduplicate *P. aeruginosa* isolates that had been recovered at the Bicêtre hospital in 2007 met the selection criteria, with intermediate susceptibility or resistance to ceftazidime and intermediate susceptibility or resistance to imipenem. Most of the isolates selected were resistant to all expanded-spectrum cephalosporins. The addition of clavulanic acid did not antagonize resistance to ceftazidime, likely ruling out the production of ESBLs. Overall, 87.5% of the selected isolates were resistant to imipenem. Using cloxacillin-containing plates, the susceptibility to ceftazidime was restored for 25 out of 32 strains, suggesting the overproduction of AmpC; this was confirmed by β-lactamase activity. In 21 out of those 25 isolates, the addition of cloxacillin significantly reduced ceftazidime and imipenem MICs (Table 1).

Identification of AmpC variants. The determination of the AmpC amino acid sequences of all 32 isolates allowed us to identify 10 variants from the AmpC sequence of *P. aeruginosa* PAO1, which is defined as the wild type (Table 2). Those variants were named according to a nomenclature specifically designed for *P. aeruginosa*, namely PDC (similarly to the ADC nomenclature for *Acinetobacter baumannii* AmpC proteins [13]). Ten variants were obtained (Table 2), and the most frequent variant was PDC-2 ($n = 13$), which contained G27D, A97V, T105A, and V205L substitutions compared to AmpC of *P. aeruginosa* PAO1 (PDC-1). The PDC-3 ($n = 4$), PDC-4 ($n = 3$), and PDC-5 ($n = 2$) variants had the T105A, T105A, and K108E; T105A; and R79Q substitutions, respectively (Table 2). All of these substitutions were located inside or near helix H-2 (16). All variants, except PDC-6, contained the T105A substitution (Table 2).

Functional characterization of different AmpC β-lactamases. The functional properties of PDC-2, PDC-3, PDC-4, and PDC-5 cephalosporinases and of AmpC of *P. aeruginosa* PAO1 (PDC-1) were compared after cloning the genes into the low-copy plasmid vector pBBR1MCS.3, followed by expression in either *P. aeruginosa* KG2505 Δ*ampC* or *P. aeruginosa* 12B (MexXY-OprM overproducer). *P. aeruginosa* recombinant clones expressing PDC-2, PDC-3, PDC-4, or PDC-5 β-lactamase showed reduced susceptibility for all β-lactams tested, including ceftazidime, cefepime, cefpirome, aztreonam, imipenem, and meropenem, compared to that of the clone expressing PDC-1 (Table 3 and data not shown), thus demonstrating an ESAC property. The expression of those PDC-type β-lactamases led to a fourfold increase in the MIC of imipenem and a 4- to 16-fold increase in the MIC of cefepime for *P. aeruginosa* KG2505 Δ*ampC* and for *P. aeruginosa* 12B (MexXY-OprM overproducer) when used as recipient strains.

TABLE 1. AmpC-type variants (PDC), residue at position 105, specific β-lactamase activity, and β-lactam MICs for *P. aeruginosa* isolates included in this study

Isolate	PDC variant	Residue at position 105	AmpC overproduction	β-Lactamase sp act[a]	MIC[b] (μg/ml)					
					CAZ	CAZ+CLOXA	FEP	FEP+CLOXA	IPM	IPM+CLOXA
PAO1	PDC-1	T	−	1	1	1	2	2	1	1
Paeβ-01	PDC-1	T	−	2 ± 1	8	8	16	16	4	4
Paeβ-02	PDC-1	T	−	5 ± 1	8	8	16	16	4	4
Paeβ-03	PDC-2	A	+	62 ± 6	16	2	64	16	16	2
Paeβ-04	PDC-2	A	+	100 ± 9	32	4	32	8	16	4
Paeβ-05	PDC-2	A	−	3 ± 1	32	32	16	16	16	16
Paeβ-06	PDC-2	A	+	42 ± 3	16	2	32	8	16	2
Paeβ-07	PDC-2	A	+	35 ± 5	16	2	32	8	16	2
Paeβ-08	PDC-2	A	+	34 ± 10	16	2	32	8	16	2
Paeβ-09	PDC-2	A	+	45 ± 8	16	2	32	8	16	2
Paeβ-10	PDC-2	A	+	67 ± 11	16	2	32	8	16	2
Paeβ-11	PDC-2	A	+	94 ± 13	16	2	32	8	16	2
Paeβ-12	PDC-2	A	+	25 ± 4	16	1	32	8	16	1
Paeβ-13	PDC-2	A	+	120 ± 14	4	0.12	16	1	16	0.5
Paeβ-14	PDC-2	A	+	54 ± 9	16	2	32	8	16	2
Paeβ-15	PDC-2	A	+	310 ± 23	32	8	128	32	16	4
Paeβ-16	PDC-3	A	+	15 ± 2	32	4	32	8	32	4
Paeβ-17	PDC-3	A	+	62 ± 7	64	8	32	8	32	8
Paeβ-18	PDC-3	A	+	600 ± 25	128	32	128	32	64	16
Paeβ-19	PDC-3	A	−	9 ± 2	8	4	32	16	4	4
Paeβ-20	PDC-4	A	+	34 ± 7	8	1	16	4	16	4
Paeβ-21	PDC-4	A	+	200 ± 15	32	4	32	4	16	2
Paeβ-22	PDC-4	A	+	220 ± 9	16	1	16	4	8	0.5
Paeβ-23	PDC-5	A	+	315 ± 21	64	8	32	8	32	8
Paeβ-24	PDC-5	A	+	290 ± 17	64	16	64	16	32	8
Paeβ-25	PDC-6	T	+	64 ± 8	32	4	32	32	8	8
Paeβ-26	PDC-6	T	+	57 ± 6	32	4	16	16	4	4
Paeβ-27	PDC-7	A	+	17 ± 4	16	4	64	32	32	16
Paeβ-28	PDC-7	A	+	84 ± 8	16	2	16	8	16	4
Paeβ-29	PDC-8	A	−	2 ± 1	8	8	32	32	16	16
Paeβ-30	PDC-8	A	−	2 ± 1	8	8	8	8	8	8
Paeβ-31	PDC-9	A	−	3 ± 1	32	16	32	32	16	16
Paeβ-32	PDC-10	A	+	104 ± 10	32	8	32	8	64	16

[a] Relative values of β-lactamase activities, with 1 being considered the obtained basal activity for the reference strain PAO1 (6 nmol of cephalothin hydrolyzed per min per mg of protein).
[b] CAZ, ceftazidime; CAZ+CLOXA, ceftazidime plus cloxacillin; FEP, cefepime; FEP+CLOXA, cefepime plus cloxacillin; IPM, imipenem; IPM+CLOXA, imipenem plus cloxacillin. Cloxacillin was added at 250 μg/ml.

P. aeruginosa 12B recombinant clones producing β-lactamases PDC-2, PDC-3, PDC-4, and PDC-5 also were resistant to ceftazidime. A twofold meropenem MIC increase was observed for these variants in both recipient strains.

A comparison of MICs (Table 3) and sequence determina-

TABLE 2. Comparison of amino acid sequences of the AmpC variants to that of the wild-type AmpC from *P. aeruginosa* PAO1 (PDC-1)

AmpC	Amino acid residue at position:										
	21	27	55	79	97[a]	105[a]	108	155	176	205	356
PDC-1	T	G	A	R	A	T	K	Q	L	V	V
PDC-2		D			V	A				L	
PDC-3						A					
PDC-4						A	E				
PDC-5				Q		A					
PDC-6				Q							
PDC-7		D				A				L	I
PDC-8						A			R		
PDC-9		V	T			A		R		L	
PDC-10	A					A					

[a] Positions 97 and 105 were located inside of the helix H-2 motif.

TABLE 3. MICs of β-lactams for *P. aeruginosa* KG2505 and *P. aeruginosa* 12B and for *P. aeruginosa* reference strains harboring recombinant plasmid pBB-PDC-1, pBB-PDC-2, pBB-PDC-3, pBB-PDC-4, or pBB-PDC-5

Strain	MIC (μg/ml)			
	Ceftazidime	Cefepime	Imipenem	Meropenem
P. aeruginosa KG2505[a]	0.5	0.5	0.12	0.06
pBB-PDC-1[b]	4	1	0.25	0.12
pBB-PDC-2	16	16	1	0.25
pBB-PDC-3	32	4	1	0.25
pBB-PDC-4	16	16	1	0.25
pBB-PDC-5	32	4	1	0.25
P. aeruginosa 12B[a]	1	1	1	1
pBB-PDC-1	8	4	1	1
pBB-PDC-2	32	32	4	2
pBB-PDC-3	32	16	4	2
pBB-PDC-4	64	32	4	2
pBB-PDC-5	32	16	4	2

[a] *P. aeruginosa* KG2505 is deficient for the *ampC* gene; *P. aeruginosa* 12B overproduces the MexXY-OprM efflux pump.
[b] PDC-1, AmpC wild-type from *P. aeruginosa* PAO1.

TABLE 4. Kinetic parameters of the different AmpC-type β-lactamases of *P. aeruginosa*[a]

β-Lactam	PDC-1			PDC-2			PDC-3			PDC-5		
	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (mM^{-1} s^{-1})	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (mM^{-1} s^{-1})	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (mM^{-1} s^{-1})	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (mM^{-1} s^{-1})
Penicillin G	35	15	430	30	35	1,200	30	35	1,200	35	30	860
Cephalothin	85	44	510	50	110	2,200	45	120	2,750	50	115	2,300
Amoxicillin	20	0.5	25	30	2	70	25	2.5	100	30	2.5	80
Piperacillin	100	2	**20**	40	5.5	**135**	40	5	**125**	40	5	**125**
Cefoxitin	0.5	0.015	30	0.5	0.08	160	0.5	0.1	200	0.5	0.08	160
Cefotaxime	6	0.02	**3**	5	0.15	**30**	8	0.15	**20**	5	0.1	**20**
Ceftazidime	20	0.004	0.2	20	0.01	0.5	35	0.02	0.6	30	0.015	0.50
Aztreonam	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Cefepime	800	0.08	**0.1**	850	2	**2**	1,300	2	**1.5**	1,700	2.5	**1.5**
Imipenem	20	0.01	**0.5**	35	0.2	**5**	20	0.1	**5**	15	0.1	**6**

[a] Data are the means of three independent experiments. Standard deviations were within 15% of the means. ND, no detectable hydrolysis (<0.01 s^{-1}) for a maximum amount of 5 μg of purified enzyme and up to 200 nmol of substrate. The main differences between PDC-1 and ESACs PDC-2, PDC-3, and PDC-5 are indicated in boldface.

tion (Table 2) showed that reduced susceptibility to imipenem was related to the T105A substitution. Additionally, amino acid changes G27D, A97V, and V205L in PDC-2 and K108E in PDC-4 led to increased cefepime MICs (32 μg/ml for *P. aeruginosa* 12B) compared to those obtained for PDC-3 and PDC-5 (16 μg/ml for *P. aeruginosa* 12B).

β-Lactamase substrate profile. β-Lactamases PDC-1, PDC-2, PDC-3, and PDC-5 were purified to near homogeneity (>99%) as deduced from the sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis (data not shown). The molecular mass of those proteins was experimentally determined to be 44 kDa, and the predicted pI value was 8.34 to 8.65 (www.expasy.org). The specific activities, determined with 100 μM of benzylpenicillin as the substrate, were 31, 81, 78, and 72 μmol/min/mg of protein for PDC-1, PDC-2, PDC-3, and PDC-5, respectively. The comparison of specific activities before and after purification showed purification factors of 70-, 90-, 60-, and 65-fold for PDC-1, PDC-2, PDC-3, and PDC-5, respectively. The specific activities, determined with 100 μM of imipenem as the substrate, were 0.021, 0.072, 0.061, and 0.059 μmol/min/mg of protein for PDC-1, PDC-2, PDC-3, and PDC-5, respectively.

The K_m and k_{cat} values of PDC-2, PDC-3, and PDC-5 were increased compared to those of PDC-1 for expanded-spectrum cephalosporins, such as cefepime, and for imipenem, thus indicating that these variants presented higher rates of hydrolysis for these compounds, but they displayed slightly lower affinity (Table 4). The resulting catalytic efficiencies (k_{cat}/K_m) of these PDC enzymes were significantly increased against oxyimino-cephalosporins (cefepime) and imipenem, whereas they were only slightly increased for narrow-spectrum cephalosporins compared to those of PDC-1.

Distribution of ESAC β-lactamases. Out of the 32 clinical isolates, 2 isolates possessed a wild-type PDC-1 sequence. Twenty-two had an ESAC β-lactamase of the PDC-2, PDC-3, PDC-4, or PDC-5 variant (Table 1). Out of those 22 isolates, no difference was observed between the imipenem MIC with or without cloxacillin for 2 strains (Paeβ-05 and Paeβ-19). In those strains, the AmpC enzyme was not expressed at a high level. The imipenem MICs for strains producing PDC-6 did not show any reduction after cloxacillin addition, and the PDC-6 enzyme did not have an alanine residue at position 105. The remaining strains produced PDC-type β-lactamase PDC-7, PDC-8, PDC-9, or PDC-10, which have not been characterized biochemically in this study. All of these variants had an alanine residue at position 105, which is consistent with an ESAC activity. For strains Paeβ-27, Paeβ-28, and Paeβ-32, producing either PDC-7 or PDC-10, the high-level production of AmpC and the reduction of imipenem MICs after cloxacillin addition argue for their ESAC properties. No reduction of imipenem MIC was detected for strains producing either PDC-8 or PDC-9 after cloxacillin addition, but none of those enzymes was overproduced in those clinical isolates (Table 1).

Impact of ESAC-type PDC variants on selection of carbapenem resistance. To evaluate whether strains expressing an ESAC-type PDC enzyme constitute a favorable background for the selection of imipenem-resistant strains after selective pressure, the frequency of the selection of mutants with imipenem at a low concentration (1 to 2 μg/ml) was estimated using the clones obtained in the genetic background of *P. aeruginosa* KG2505 Δ*ampC*. The frequency of the selection of mutants with imipenem resistance or of intermediate susceptibility was 10- to 100-fold higher in an ESAC-type PDC background than that observed in a non-ESAC-type PDC background (Table 5). No mutant was obtained at a selection concentration of 4 μg/ml of imipenem. Further analysis by real-time reverse transcription-PCR and PCR sequencing showed reduced *oprD* expression (less than 70% compared to that of the

TABLE 5. Frequency of the selection of imipenem-resistant or intermediate-susceptibility strains of *P. aeruginosa* KG2505 Δ*ampC* with or without recombinant plasmid pBB-PDC-1, pBB-PDC-2, pBB-PDC-3, pBB-PDC-4, or pBB-PDC-5

Strain	Imipenem selection frequency at[a]:	
	1 μg/ml	2 μg/ml
KG2505	$(<3.4 \pm 1.2) \times 10^{-9}$	$(<3.4 \pm 1.2) \times 10^{-9}$
pBB-PDC-1	$(<3.2 \pm 1.1) \times 10^{-9}$	$(<3.2 \pm 1.1) \times 10^{-9}$
pBB-PDC-2	$(5.0 \pm 1.8) \times 10^{-6}$	$(4.0 \pm 1.7) \times 10^{-7}$
pBB-PDC-3	$(9.3 \pm 2.0) \times 10^{-7}$	$(4.6 \pm 1.1) \times 10^{-8}$
pBB-PDC-4	$(2.5 \pm 1.2) \times 10^{-6}$	$(1.2 \pm 0.5) \times 10^{-7}$
pBB-PDC-5	$(8.1 \pm 1.8) \times 10^{-7}$	$(3.6 \pm 1.1) \times 10^{-8}$

[a] Standard deviations are indicated.

P. aeruginosa KG2505 parental strain) for 11 out of 15 (73%) mutants, and the lack of OprD porin was observed for 3 out of 15 (20%) mutants. The lack of OprD biosynthesis was due to a frameshift mutation produced by a 1-bp insertion in two mutants and a 1-bp deletion in the other one. The imipenem MICs for those mutants ranged between 4 and 16 μg/ml (data not shown).

DISCUSSION

Intermediate susceptibility or resistance to imipenem in *P. aeruginosa* has been widely associated with the modification of the OprD protein, the production of metallo-β-lactamase, and the overexpression of the cephalosporinase associated with decreased outer membrane permeability. Here, we showed that peculiar AmpC enzymes of *P. aeruginosa* with expanded-spectrum activity toward imipenem also may contribute to decreased susceptibility to imipenem. An association was found between AmpC overproduction and increased susceptibility to imipenem and cefepime after cloxacillin addition and a T105A substitution in the AmpC sequence. Gutiérrez et al. reported an association between carbapenem resistance and AmpC overproduction in *P. aeruginosa* (9). The inhibition of the AmpC enzyme by cloxacillin allowed the recovery of susceptibility to imipenem for 12 isolates and the recovery of intermediate susceptibility for 5 others (Table 1).

The most frequently AmpC-type variant was PDC-2, containing the substitutions G27D, A97V, T105A, and V205L. The region containing residues 97 and 105 is located inside of helix H-2 when AmpC from *E. coli* is taken as reference. This helix, H-2, is close to the active Ser64 and interacts with the Ω-loop through hydrogen bonding (28). Substitutions in this region have been previously linked to the extension of the hydrolysis spectrum, facilitating the attack on compounds like ceftazidime (16, 28).

The peculiar activities of ESAC β-lactamases have been attributed to changes in positions located in the vicinity of the active site (16, 20). Here, we report a novel substitution, T105A, leading to a broadened hydrolysis spectrum, including oxyiminocephalosporins and imipenem in *P. aeruginosa*. Decreased susceptibility to imipenem was observed in all isolates that overexpressed this AmpC variant. In addition, the K108E substitution identified in PDC-4 may play an additional role for conferring resistance to cefepime.

The resulting catalytic efficiencies (k_{cat}/K_m) of those PDC-2, PDC-3, and PDC-5 variants were increased against oxyiminocephalosporins and imipenem, whereas they were (in general) only slightly increased against narrow-spectrum cephalosporins. For some ESACs reported from *S. marcescens* and *E. cloacae* (11, 21, 28) with amino acid changes located in their Ω-loop and H-2 helix, the mutant enzymes had increased k_{cat} values for expanded-spectrum cephalosporins together with increased K_m values. The effect of an alanine residue at position 105 in the sequence of AmpC of *Enterobacteriaceae* also would be interesting to analyze.

The analysis of AmpC determinants in a large collection of *P. aeruginosa* isolates, whatever the resistance phenotype, would be interesting in order to know whether PDC-1 from *P. aeruginosa* reference strain PAO1 (taken here as the wild-type cephalosporinase) or, on the contrary, one of the characterized ESAC is the real wild-type and the most widespread AmpC-type enzyme of *P. aeruginosa*. Taking into account that the overexpression of AmpC is associated with large amounts of enzymes in the periplasmic space, a high quantity of ESAC ß-lactamases, which displayed an increased hydrolytic activity against imipenem, may explain a higher risk for the selection of carbapenem-resistant isolates. These in vitro-obtained results should be confirmed in vivo by analyzing selected carbapenem-resistant *P. aeruginosa* isolates after carbapenem-containing treatment. The screening of those *P. aeruginosa* isolates for AmpC genes may be useful to predict which isolates would be able to develop carbapenem resistance in vivo.

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REFERENCES

1. **Barnaud, G., Y. Benzerara, J. Gravisse, L. Raskine, M. J. Sanson-Le Pors, R. Labia, and G. Arlet.** 2004. Selection during cefepime treatment of a new cephalosporinase variant with extended-spectrum resistance to cefepime in an *Enterobacter aerogenes* clinical isolate. Antimicrob. Agents Chemother. **48:**1040–1042.
2. **Barnaud, G., R. Labia, L. Raskine, M. J. Sanson-Le Pors, A. Philippon, and G. Arlet.** 2001. Extension of resistance to cefepime and cefpirome associated to a six amino acid deletion in the H-10 helix of the cephalosporinase of an *Enterobacter cloacae* clinical isolate. FEMS Microbiol. Lett. **195:**185–190.
3. **Bellais, S., D. Aubert, T. Naas, and P. Nordmann.** 2000. Molecular and biochemical heterogeneity of class B carbapenem-hydrolyzing β-lactamases in *Chryseobacterium meningosepticum*. Antimicrob. Agents Chemother. **44:** 1878–1886.
4. **Bush, K., G. A. Jacoby, and A. A. Medeiros.** 1995. A functional classification scheme for β-lactamases and its correlation with molecular structure. Antimicrob. Agents Chemother. **39:**1211–1233.
5. **Clinical and Laboratory Standards Institute.** 2008. Performance standards for antimicrobial susceptibility testing. 18th informational supplement. Clinical and Laboratory Standards, Wayne, PA.
6. **Cornish-Bowden, A. (ed.).** 1995. Fundamentals of enzyme kinetics, p. 30–37. Portland Press, London, United Kingdom.
7. **Crichlow, G. V., A. P. Kuzin, M. Nukaga, K. Mayama, T. Sawai, and J. R. Knox.** 1999. Structure of the extended-spectrum class C β-lactamase of *Enterobacter cloacae* GC1, a natural mutant with a tandem tripeptide insertion. Biochemistry **38:**10256–10261.
8. **Doi, Y., J. Wachino, M. Ishiguro, H. Kurokawa, K. Yamane, N. Shibata, K. Shibayama, K. Yokoyama, H. Kato, T. Yagi, and Y. Arakawa.** 2004. Inhibitor-sensitive AmpC β-lactamase variant produced by an *Escherichia coli* clinical isolate resistant to oxyiminocephalosporins and cephamycins. Antimicrob. Agents Chemother. **48:**2652–2658.
9. **Gutiérrez, O., C. Juan, E. Cercenado, F. Navarro, E. Bouza, P. Coll, J.-L. Perez, and A. Oliver.** 2007. Molecular epidemiology and mechanisms of carbapenem resistance in *Pseudomonas aeruginosa* isolates from Spanish hospitals. Antimicrob. Agents Chemother. **51:**4329–4335.
10. **Hancock, R. E., and F. Bellido.** 1996. Antibacterial in vitro activity of fourth generation cephalosporins. J. Chemother. **8**(Suppl. 2)**:**31–36.
11. **Hidri, N., G. Barnaud, D. Decré, C. Cerceau, V. Lalande, J. C. Petit, R. Labia, and G. Arlet.** 2005. Resistance to ceftazidime is associated with a S220Y substitution in the omega loop of the AmpC β-lactamase of a *Serratia marcescens* clinical isolate. J. Antimicrob. Chemother. **55:**496–499.
12. **Hocquet, D., P. Nordmann, F. El Garch, L. Cabanne, and P. Plésiat.** 2006. Involvement of the MexXY-OprM efflux system in emergence of cefepime resistance in clinical strains of *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **50:**1347–1351.
13. **Hujer, K. M., N. S. Hamza, A. M. Hujer, F. Perez, M. S. Helfand, C. R. Bethel, J. M. Thomson, V. E. Anderson, M. Barlow, L. B. Rice, F. C. Tenover, and R. A. Bonomo.** 2005. Identification of a new allelic variant of the *Acinetobacter baumannii* cephalosporinase, ADC-7 β-lactamase: defining a unique family of class C enzymes. Antimicrob. Agents Chemother. **49:**2941–2948.
14. **Juan, C., M. D. Macia, O. Gutierrez, C. Vidal, J. L. Perez, and A. Oliver.** 2005. Molecular mechanisms of β-lactam resistance mediated by AmpC

hyperproduction in *Pseudomonas aeruginosa* clinical strains. Antimicrob. Agents Chemother. **49:**4733–4738.
15. **Kovach, M. E., R. W. Phillips, P. H. Elzer, R. M. Roop, and K. M. Peterson.** 1994. pBBR1MCS: a broad-host-range cloning vector. BioTechniques. **16:** 800–802.
16. **Mammeri, H., and P. Nordmann.** 2007. Extended-spectrum cephalosporinases in *Enterobacteriaceae*. Anti-Infect. Agents Med. Chem. **6:**71–82.
17. **Mammeri, H., P. Nordmann, A. Berkani, and F. Eb.** 2008. Contribution of extended-spectrum AmpC (ESAC) β-lactamases to carbapenem resistance in *Escherichia coli*. FEMS Microbiol. Lett. **282:**238–240.
18. **Mammeri, H., L. Poirel, P. Bemer, H. Drugeon, and P. Nordmann.** 2004. Resistance to cefepime and cefpirome due to a 4-amino-acid deletion in the chromosome-encoded AmpC β-lactamase of a *Serratia marcescens* clinical isolate. Antimicrob. Agents Chemother. **48:**716–720.
19. **Mammeri, H., L. Poirel, N. Fortineau, and P. Nordmann.** 2006. Naturally occurring extended-spectrum cephalosporinases in *Escherichia coli*. Antimicrob. Agents Chemother. **50:**2573–2576.
20. **Mammeri, H., L. Poirel, and P. Nordmann.** 2007. Extension of the hydrolysis spectrum of AmpC β-lactamase of *Escherichia coli* due to amino acid insertion in the H-10 helix. J. Antimicrob. Chemother. **60:**490–494.
21. **Matsumura, N., S. Minami, and S. Mitsuhashi.** 1998. Sequences of homologous β-lactamases from clinical isolates of *Serratia marcescens* with different substrate specificities. Antimicrob. Agents Chemother. **42:**176–179.
22. **Miller, K., A. J. O'Neill, and I. Chopra.** 2002. Response of *Escherichia coli* hypermutators to selection pressure with antimicrobial agents from different classes. J. Antimicrob. Chemother. **49:**925–934.
23. **Nordmann, P., and H. Mammeri.** 2007. Extended-spectrum cephalosporinases: structure, detection and epidemiology. Future Microbiol. **2:**297–307.
24. **Nukaga, M., S. Haruta, K. Tanimoto, K. Kogure, K. Taniguchi, M. Tamaki, and T. Sawai.** 1995. Molecular evolution of a class C β-lactamase extending its substrate specificity. J. Biol. Chem. **270:**5729–5735.
25. **Nukaga, M., K. Taniguchi, Y. Washio, and T. Sawai.** 1998. Effect of an amino acid insertion into the omega loop region of a class C β-lactamase on its substrate specificity. Biochemistry **37:**10461–10468.
26. **Philippon, L. N., T. Naas, A. T. Bouthors, V. Barakett, and P. Nordmann.** 1997. OXA-18, a class D clavulanic acid-inhibited extended-spectrum β-lactamase from *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **41:** 2188–2195.
27. **Quale, J., S. Bratu, J. Gupta, and D. Landman.** 2006. Interplay of efflux system, *ampC*, and *oprD* expression in carbapenem resistance of *Pseudomonas aeruginosa* clinical isolates. Antimicrob. Agents Chemother. **50:**1633–1641.
28. **Raimondi, A., F. Sisto, and H. Nikaido.** 2001. Mutation in *Serratia marcescens* AmpC β-lactamase producing high-level resistance to ceftazidime and cefpirome. Antimicrob. Agents Chemother. **45:**2331–2339.
29. **Vakulenko, S. B., D. Golemi, B. Geryk, M. Suvorov, J. R. Knox, S. Mobashery, and S. A. Lerner.** 2002. Mutational replacement of Leu-293 in the class C *Enterobacter cloacae* P99 β-lactamase confers increased MIC of cefepime. Antimicrob. Agents Chemother. **46:**1966–1970.
30. **Walsh, T. R., M. A. Toleman, L. Poirel, and P. Nordmann.** 2005. Metallo-β-lactamases: the quiet before the storm? Clin. Microbiol. Rev. **18:**306–325.
31. **Weldhagen, G. F., L. Poirel, and P. Nordmann.** 2003. Ambler class A extended-spectrum β-lactamases in *Pseudomonas aeruginosa*: novel developments and clinical impact. Antimicrob. Agents Chemother. **47:**2385–2392.