Mutations in β-Lactamase AmpC Increase Resistance of *Pseudomonas aeruginosa* Isolates to Antipseudomonal Cephalosporins

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Mutation-dependent overproduction of intrinsic β-lactamase AmpC is considered the main cause of resistance of clinical strains of *Pseudomonas aeruginosa* to antipseudomonal penicillins and cephalosporins. Analysis of 31 AmpC-overproducing clinical isolates exhibiting a greater resistance to ceftazidime than to piperacillin-tazobactam revealed the presence of 17 mutations in the β-lactamase, combined with various polymorphic amino acid substitutions. When overexpressed in AmpC-deficient *P. aeruginosa* 4098, the genes coding for 20/23 of these AmpC variants were found to confer a higher (2-fold to >64-fold) resistance to ceftazidime and ceftolozane-tazobactam than did the gene from reference strain PAO1. The mutations had variable effects on the MICs of ticarcillin, piperacillin-tazobactam, aztreonam, and cefepime. Depending on their location in the AmpC structure and their impact on β-lactam MICs, they could be assigned to 4 distinct groups. Most of the mutations affecting the omega loop, the R2 domain, and the C-terminal end of the protein were shared with extended-spectrum AmpCs (ESACs) from other Gram-negative species. Interestingly, two new mutations (F121L and P154L) were predicted to enlarge the substrate binding pocket by disrupting the stacking between residues F121 and P154. We also found that the reported ESACs emerged locally in a variety of clones, some of which are epidemic and did not require hypermutability. Taken together, our results show that *P. aeruginosa* is able to adapt to efficacious β-lactams, including the newer cephalosporin ceftolozane, through a variety of mutations affecting its intrinsic β-lactamase, AmpC. Data suggest that the rates of ESAC-producing mutants are ≥1.5% in the clinical setting.

*P*seudomonas aeruginosa is a well-known cause of acute and chronic infections in fragile patients. One of the most remarkable traits of this opportunistic pathogen is its ability to evolve and become resistant to many antibiotics through a variety of mutational and transferable mechanisms (reviewed in reference 1). Some mechanisms tend to prevent the interaction of drugs with their cognate cellular targets, while others result in drug inactivation (1). Like several other Gram-negative species, *P. aeruginosa* harbors a chromosomal drug-inducible gene, bla_{AmpC}, encoding a wide-spectrum class C β-lactamase (2). This enzyme contributes to the natural resistance of the microorganism toward labile and inducing molecules, such as aminopenicillins, first- and second-generation cephalosporins (3). More importantly, when overproduced as a result of mutations altering the peptidoglycan recycling process, AmpC becomes a major cause of resistance to widely used antipseudomonal penicillins (ticarcillin and piperacillin), monobactams (aztreonam), and third-generation (ceftazidime) and fourth-generation (cefepime) cephalosporins (4–7). The so-called "derepressed mutants" are common in the clinical setting and account for a large proportion of strains resistant to ceftazidime and cefepime in various studies (8–11). This worrisome situation has called for the development of new β-lactams such as ceftolozane and novel AmpC inhibitors such as avibactam that show promising activities against this type of mutants (12, 13).

Class C β-lactamase variants showing unusual substrate specificities (named ESACs for extended-spectrum AmpCs) have been characterized in several Gram-negative pathogens (14). However, because of the high sequence polymorphism of AmpC in *P. aeruginosa* (affecting more than 22% of its amino acid residues), the occurrence of ESACs in this species has remained largely unexplored (15). This study demonstrates that such variants have potential therapeutic implications as they are responsible for a higher resistance to ceftazidime and ceftolozane-tazobactam than to piperacillin-tazobactam in clinical strains.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. Thirty-five (31 ESAC and 4 non-ESAC) strains of *P. aeruginosa* isolated between 2009 and 2014 in 21 French hospitals and health care facilities were used in this study as sources of different AmpC variants, named PDCs for *Pseudomonas*-derived cephalosporinases. These strains were referred to the French National Reference Center (NRC) for Antibiotic Resistance (University Hospital, Besançon, France) for analysis of their resistance mechanisms to β-lactams and were found to produce AmpCs differing from that of reference strain PAO1 (16) by at least one amino acid residue. Strain PA14 (17) was used as the source of PDC-34. The resistance phenotypes conferred by the cloned bla_{AmpC} genes were assessed in strain 4098, an AmpC-deficient mutant from PAO1 (18), while the impact of enzymes PDC-1 and PDC-3 on carbapenem susceptibility was investigated more specifically in mutant PAO1ΔoprD (19), which lacks the carbapenem-specific uptake porin OprD. The PBP4-deficient mutant PAO1ΔdacB (6), which constitutively overproduces PDC-1, was used as a control in MIC exper-

iments. In addition, 44 nonredundant, antibiotic-susceptible strains of *P. aeruginosa*, including 39 isolates from the University Hospital of Besançon and 5 isolates from surface waters of eastern France, were selected from the laboratory collection to investigate the sequence polymorphism of AmpC in wild-type *P. aeruginosa*. Molecular biology experiments were performed with *Escherichia coli* DH5α (Life Technologies) as the recipient strain. The bla_{AmpC} genes were cloned into the broad-host-range plasmid vector pUCP24, kindly supplied by Herbert Schweizer (Colorado University) (20). Bacteria were cultivated at 35 ± 1°C on Mueller-Hinton (MH) agar (Bio-Rad) supplemented with gentamicin (5 μg/ml for *E. coli* and 50 μg/ml for *P. aeruginosa*) to maintain pUCP24 and its derivatives where needed.

Drug susceptibility testing. MICs (shown as ≤ susceptible [S] breakpoint/≥ resistant [R] breakpoint) of ticarcillin (16/128 μg/ml), piperacillin-tazobactam (16-4/128-4 μg/ml), ceftazidime (8/32 μg/ml), cefepime (8/32 μg/ml), ceftolozane-tazobactam (breakpoints pending), aztreonam (8/32 μg/ml), imipenem (2/8 μg/ml), and meropenem (2/8 μg/ml) were determined with and without 250 or 1,000 μg/ml cloxacillin using customized Sensititre plates, according to the instructions of the manufacturer (Thermo Fisher Scientific). All plates were incubated at 35 ± 1°C for 18 ± 2 h with inocula of 10^5 CFU/ml. The strains were considered "susceptible" (S), "intermediate" (I), or "resistant" (R) to the tested drugs in reference to the Clinical and Laboratory Standards Laboratory (CLSI) breakpoints (21).

Molecular typing of the strains. The epidemiological relatedness of the clinical *P. aeruginosa* strains was studied by multilocus sequence typing (MLST) based on the allelic variations of 7 housekeeping genes, namely, *acsA*, *aroE*, *guaA*, *mutL*, *nuoD*, *ppsA*, and *trpE* (22). The isolates were assigned a sequence type (ST) number according to the allelic profiles available in the MLST Database (http://pubmlst.org/paeruginosa).

Analysis and cloning of bla_{AmpC} genes. Genomic DNA was extracted and purified from the clinical strains by using QIAamp DNA minikit (Qiagen). For sequencing purposes, the bla_{AmpC} genes were amplified by PCR with primers SeqAmpC-PA14-Fw1 (5′-TGGGGTCGAACCAATCTCTA-3′), SeqAmpC-PA14-Fw2 (5′-CAGATCCGCGACTACTACCG-3′), SeqAmpC-PA14-Rv1 (5′-ACGTCGAGGTGGGTCTGTT-3′), and SeqAmpC-PA14 Rv2 (5′-CTCATGGCACCATCATAGCC-3′). The amplicons were sequenced using the BigDye Terminator chemistry on an automated ABI 3730 sequencer (Applied Biosystems), and the resulting data were edited using BioEdit 7.1.9 software (Tom Hall, North Carolina State University, Raleigh, NC). For cloning purposes, the bla_{AmpC} genes were PCR amplified and cloned into plasmid vector pUCP24, as already described (23). After a DNA sequencing step ensuring that no mutations had been introduced during PCR amplification, the resulting pPDC plasmids were transferred by electroporation (Bio-Rad MicroPulser) into *P. aeruginosa* 4098. Transformants were selected on MH agar supplemented with gentamicin, the selection marker for pUCP24, and characterized as to their resistance levels to selected β-lactams. Plasmids pPDC-1 and pPDC-3 were further electroporated into porin OprD-deficient mutant 4098ΔoprD.

RT-qPCR experiments. For the reverse transcription-quantitative PCR (RT-qPCR) experiments, the bacterial strains were cultivated aerobically to the mid-log phase in drug-free Mueller-Hinton broth. Their total RNA content was extracted and reverse transcribed as described by Dumas et al. (24). The expression of gene bla_{AmpC} and housekeeping gene *uvrD* was then assessed in a Rotor Gene RG6000 instrument (Qiagen, Courtaboeuf, France) by using the intercalating dye Rotor-Gene SYBR green (Qiagen) and primer pairs AmpC1/AmpC2 and UvrD1/UvrD2, respectively (24, 25). Sequence alignment analysis confirmed the absence of base mismatch between the primers and the target genes, ensuring efficient PCR amplification. The mRNA levels of bla_{AmpC} were normalized with those of *uvrD* for each strain and expressed as a ratio (fold change) to that of wild-type strain PAO1, used as the reference. Mean gene expression values were calculated from two independent bacterial cultures, each assayed in duplicate.
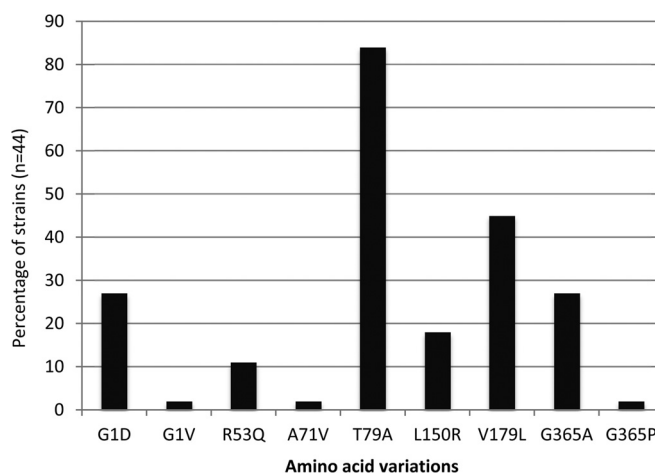

FIG 1 Amino acid sequence polymorphism of β-lactamase AmpC among nonredundant clinical (*n* = 39) and environmental (*n* = 5) antibiotic-susceptible strains of *P. aeruginosa*.

Structure-function analysis of AmpCs. The AmpC sequence, corresponding to the genomic locations 4594029 to 4595222 on strain PAO1, was extracted from the *Pseudomonas* Genome Database (http://www.pseudomonas.com/). A BLAST search was performed, choosing the Protein Data Bank (PDB) as the search database (http://blast.ncbi.nlm.nih.gov/), resulting in 43 PDB structures presenting an E value lower than 10^{-100} and 11 with an E value between 10^{-11} and 10^{-4}. Among them, those presenting a β-lactamase in complex with a molecule in its active site were selected and superposed on the structure of PAO1 AmpC (PDB code 2WZZ). In order to evaluate the possible effect of the different observed mutations, all of the corresponding positions were mutated using PyMOL on the 2WZZ structure. All the structural figures were drawn using Pymol software (PyMOL Molecular Graphics system [http://pymol.sourceforge.net/]).

Accession numbers. The sequences of bla_{AmpC} genes corresponding to the following PDCs have been deposited in GenBank under the accession numbers shown in parentheses: PDC-73 (KR057742), PDC-74 (KR057743), PDC-75 (KR057744), PDC-76 (KR057745), PDC-77 (KR057746), PDC-78 (KR057747), PDC-79 (KR057748), PDC-80 (KR057749), PDC-81 (KR057750), PDC-82 (KR057751), PDC-83 (KR057752), PDC-84 (KR057753), PDC-85 (KR057754), PDC-86 (KR057755), PDC-87 (KR057756), PDC-88 (KR057757), PDC-89 (KR057758), PDC-90 (KR057759), PDC-91 (KR057760), PDC-92 (KR057761), and PDC-93 (KR057762).

RESULTS AND DISCUSSION

Sequence polymorphism of *P. aeruginosa* AmpC. Amino acid substitutions G1D/V, A29T, R53Q, A71V, T79A, K82E, Q129R, L150R, V179L, V330I, and G365A have been reported to occur in AmpC enzymes produced by β-lactam-resistant strains of *P. aeruginosa*, compared with the mature (i.e., after cleavage of the 26-amino-acid-long signal peptide) protein from reference strain PAO1 (26, 27). In this study, analysis of the coding sequence of bla_{AmpC} genes from 44 antibiotic-susceptible strains isolated at the hospital (*n* = 39) or in natural environments (*n* = 5) showed the same amino acid variations described above at positions 1, 53, 71, 79, 150, 179, and 365 (Fig. 1). Given that most of these substitutions are present in the enzyme of wild-type reference strain PA14 (Table 1), collectively, these results support the notion that such a polymorphism has little or no impact on the enzymatic activity of AmpC (i.e., as it is found in both bacteria susceptible to and bac-

TABLE 1 Amino acid variations in the sequences of PDC enzymes

Strain(s)	PDC	1	19	29	71	79	100	121	129	150	154	175	179	213	216	221	223	290	291	292	293	294	295	330	347	365
Wild-type AmpC producers																										
PAO1[b]	PDC-1	**G**	V	**A**	**A**	**T**	R	F	**Q**	**L**	P	M	**V**	V	G	E	Y	T	P	M	A	L	Q	**V**	N	**G**
14.2028	PDC-3				A																					
13.1642	PDC-8				A				R																	
13.1781	PDC-24				A																					A
PA14[b]	PDC-34	D		T	A			R					L													A
11.773	PDC-35	D			V	A							L													A
ESAC producers																										
12.1227 and 12.1255	PDC-44					A															P					
10.257	PDC-50					A						A														
12.1285	PDC-73					A					L															
12.1111 and 13.1716	PDC-74					A									R											
12.1129 and 13.1727	PDC-75					A									R									I		
13.1415	PDC-76					A																			I	
13.1404, 13.1696, and 13.1760	PDC-77														R											
11.571	PDC-78						H								R											
11.516	PDC-79															K										
13.1737	PDC-80															G										
13.1514	PDC-81					A					L															A
13.1770	PDC-82					A		L			L															A
4836	PDC-83	A				A						A														A
13.1482	PDC-84	A				A						A													I	A
12.961	PDC-85					A											H									A
13.1755 and 14.1999	PDC-86	D				A							L			K								I		A
11.698 and 13.1775	PDC-87	D				A							L											I	I	A
13.1601	PDC-88	D				A							L					Δ	Δ					I		A
09.236	PDC-89	D				A							L					Δ	Δ	Δ						A
13.1562	PDC-90	D				A							L					Δ	Δ	Δ				I		A
11.813	PDC-91	D				A							L					Δ	Δ	Δ	Δ			I		A
12.1121 and 13.1389	PDC-92	D				A							L									Δ	Δ			A
14.2036	PDC-93	D				A							L									P				A

[a] The numbering of the amino acids refers to the mature protein from strain PAO1, after cleavage of the 26 N-terminal amino acid residues of the signal peptide. Variations highlighted in boldface are considered common polymorphisms. The role of V19A has not been investigated specifically in this study.
[b] Reference strain of *P. aeruginosa* (http://v2.pseudomonas.com).

teria resistant to β-lactams). Consistent with this, the interchangeable amino acids appeared to be distant from the active site, with residues at positions 1, 79, 129, and 365 being located on the opposite surface and those at positions 29 and 179 being part of secondary structure elements close to the surface that can easily adapt the local mutations. Referring to the initial classification of AmpC variants (from PDC-1 to PDC-10 for *Pseudomonas*-derived cephalosporinases 1 to 10) established by Rodriguez-Martinez et al. (26), variant PDC-1 (identical to that of PAO1 by definition) was found to be produced by 7 of the 44 wild-type strains analyzed, PDC-3 by 6 strains, PDC-5 by 4 strains, PDC-7 by 2 strains, and PDC-8 by 8 strains, while the 17 remaining isolates harbored various allelic combinations not reported in this classification scheme. The K82E and V330I changes reported previously were not identified in the present collection (26).

Clinical strains with unusual resistance profiles. From 2009 to 2014, 2,040 clinical isolates of *P. aeruginosa* were referred to the French National Reference Center for Antibiotic Resistance to characterize their resistance mechanisms to antibiotics, mainly β-lactams. Among these strains, 506 (24.8%) and 471 (23.1%) were found to produce extended-spectrum β-lactamases (ESBLs) and carbapenemases, respectively, by phenotypic and molecular biology techniques (unpublished data). In addition, 810 (37.7%) strains recovering their susceptibility to penicillins and cephalosporins when tested by the disk diffusion method on Mueller-Hinton agar supplemented with class C inhibitor cloxacillin (2) at 1,000 μg/ml were considered AmpC-overproducing mutants. Unlike these typical AmpC overproducers (as illustrated in Table S1 in the supplemental material), 31 (1.5%) isolates that showed rather uncommon resistance profiles characterized by a higher resistance to ceftazidime than to piperacillin-tazobactam were investigated more extensively. None of them appeared to produce transferable β-lactamases (data not shown).

According to the current CLSI breakpoints, 12.9% ($n = 4$) and 87.1% ($n = 27$) of these strains were intermediate and resistant to ceftazidime, respectively, with MICs ranging from 16 to >512 μg/ml. Using the same breakpoints (8 μg/ml ≤ S and R ≥ 32 μg/ml), ceftolozane combined with 4 μg/ml tazobactam performed better, with 58.1% ($n = 18$), 12.9% ($n = 4$), and 29% ($n = 9$) of strains being susceptible, intermediate, and resistant to the

drug, respectively (MICs from 0.5 to >64 μg/ml). Categorization of bacteria as S, I, and R for the other β-lactams tested was as follows: ticarcillin, 6.4%, 9.7%, and 83.9%; piperacillin plus 4 μg/ml tazobactam, 48.4%, 35.5%, and 16.1%; aztreonam, 16.1%, 12.9%, and 71%; cefepime, 12.9%, 25.8%, and 61.3%; imipenem, 22.6%, 9.7%, and 67.7%; and meropenem, 19.4%, 29%, and 51.6%. In contrast to *in vitro* mutant PAO1Δ*dacB*, only 4 of the 31 strains recovered their susceptibility to ceftazidime (MIC, ≤8 μg/ml) in the presence of cloxacillin (see Table S1 in the supplemental material).

Identification of new AmpC variants. Sequencing of gene *bla*$_{AmpC}$ revealed the occurrence of 23 different AmpC variants (dubbed PDCs in the current nomenclature) among the selected bacteria, including 21 new variants with respect to the 72 PDCs listed previously (15, 26) (Table 1). Seventeen so far uncharacterized mutations at 15 amino acid positions were combined with 0 to 5 variations presumed to be common polymorphisms (described above): R100H, F121L, P154L, M175L, V213A, G216R, E221K/G, Y223H, ΔT290-ΔP291, ΔT290-ΔM292, ΔT290-ΔA293, ΔL294-ΔQ295, L294P, and N347I (Table 1). Of note, AmpC variant PDC-77 was produced by 3 strains, and PDC-44, PDC-74, PDC-75, PDC-86, PDC-87, and PDC-92 were each expressed by 2 isolates, while the 16 remaining variants were found in single isolates. RT-qPCR experiments showed that all of the selected strains constitutively overexpressed the *bla*$_{AmpC}$ gene, with relative values ranging from 4.4- to 2,895-fold (median 686-fold) compared with wild-type strain PAO1. As noted previously (27, 28), no correlation could be found between these expression levels and β-lactam MICs, suggesting a posttranscriptional regulation of AmpC production or the influence of undetermined resistance mechanisms in individual strains. Interestingly, the *bla*$_{AmpC}$ transcripts were quantitatively lower (median, 162-fold) in slow-growing bacteria isolated from cystic fibrosis (CF) patients than in bacteria from chronic obstructive pulmonary disease (COPD) patients (median 1,031-fold) or non-CF, non-COPD patients (median, 906-fold).

Impact of PDCs on β-lactam MICs. The *bla*$_{AmpC}$ genes of 20 PDC enzymes harboring the various mutations were amplified by PCR, cloned into the broad-host-range vector pUCP24, and overexpressed in AmpC-deficient *P. aeruginosa* mutant 4098. The same approach was used to clone the *bla*$_{AmpC}$ genes from PAO1 (PDC-1), PA14 (PDC-34), and 4 clinical strains presumed to overproduce wild-type AmpCs (PDC-3, PDC-8, PDC-24, and PDC-35) because of their greater resistance to piperacillin-tazobactam than to ceftazidime (Table 1; see Table S1 in the supplemental material). As expected, the resistance levels to β-lactams conferred by these latter AmpC variants and by PDC-34 were nearly identical to that provided by PDC-1, thus confirming the absence of impact of the polymorphic variations G1D, A29T, A71V, T79A, Q129R, L150R, V179L, and G365A individually or collectively on the enzyme activity (PDCs of group 0 in Table 2). Since T79A (T105A in the nonprocessed peptide) had previously been found to be prevalent in carbapenem-nonsusceptible clinical strains (29) and to weakly increase the catalytic activity of AmpC *in vitro* on carbapenems (26), we overexpressed enzymes PDC-1 (T79) and PDC-3 (A79) in mutant 4098Δ*oprD*, reasoning that the slow penetration of carbapenems into this porin OprD-negative strain would potentiate the AmpC action and thus reveal slight differences in resistance due to T79A. In agreement with other

results (28), the variations at position 79 did not affect the resistance of 4098Δ*oprD* to carbapenems (MICs of imipenem and meropenem were equal to 4 and 2 μg/ml, respectively, with both PDCs), clearly demonstrating that T79A does not broaden the substrate spectrum of AmpC at least *in vivo*.

Based on their location in the three-dimensional (3D) structure of AmpC and their effects on β-lactam MICs, the other mutations found in this study could be assigned to 4 distinct groups, designated I to IV.

Group I PDCs. Group I mutations (V213A, G216R, E221K, E221G, and Y223H) are located in the C-terminal region of the Ω loop and in the following α-helix (Fig. 2). Compared with PDC-1, they increased the MICs of ticarcillin, aztreonam (except for PDC-80, which contains E221G), ceftazidime, and ceftolozane-tazobactam, with variable effects on resistance levels to piperacillin-tazobactam and cefepime (Table 2). Remarkably, amino acid changes at position E221 (K/G) had the greatest impact on the ceftolozane-tazobactam MIC (≥32 μg/ml ceftolozane). Reminiscent of these results, AmpC-overexpressing, ceftolozane-tazobactam-resistant clones exhibiting the E221K mutation together with F121L, Q131R, and/or V330I were selected *in vitro* from a Δ*mutS* hypermutator PAO1 mutant exposed to increasing drug concentrations (23). The Ω-loop region, which is part of the active site of the enzyme and which accommodates the R1 side chain of the β-lactam nucleus, is a well-known hot spot for mutations able to extend the substrate specificity of chromosome- or plasmid-encoded class C β-lactamases (14). Interestingly, most of the mutated amino acid residues found in *P. aeruginosa* AmpC mapped at the same position or in the vicinity of residues previously reported as cause of ESACs in other organisms, such as *E. coli*, *Enterobacter* sp., *Serratia marcescens*, and *Acinetobacter baumannii* (see details in Table S2 in the supplemental material). It should be noted here that the R100H change, which coexists with G216R in PDC-78, slightly enhanced the enzyme activity against all the β-lactams tested (compare the MICs conferred by PDC-74, -75, and -78). No predictable explanation could be found by structural analysis as this residue is situated at the surface of the protein on the back side compared with the binding pocket (Fig. 2). With the exception of PDC-50 which carries the V213A mutation, group I PDCs (PDC-74, -75, -78, -79, -80, -84, -85, and -86) turned out to be poorly antagonized by cloxacillin at 1,000 μg/ml. This observation is of practical importance as tests using this AmpC inhibitor even at high doses may fail to recognize clinical strains overproducing such variants.

Group II PDCs. Group II relates to mutations, mainly deletions, that are located in the R2 domain of AmpC (ΔT290-ΔQ295, L294P), at or near positions already known to increase the hydrolytic activity of class C β-lactamases toward third- to fourth-generation cephalosporins (see Table S2 in the supplemental material). Indeed, alterations occurring in this region, which corresponds to H-10, the N terminus of the R2 loop, are able to widen the active site, making the enzyme more accessible to β-lactam molecules carrying a bulky R2 side chain (14). Consistent with this notion and with respect to PDC-1, group II PDCs (PDC-44, -88, -89, -90, -91, and -92) conferred higher resistance to ceftazidime, cefepime, and ceftolozane-tazobactam, with no or a negative impact on the MICs of penicillins and aztreonam (Table 2). Among all of the ESACs identified in this study, the group II AmpC variants were associated with the highest resistance to cefepime (>64 μg/ml).

TABLE 2 Susceptibility profiles of strain 4098 complemented with bla_{AmpC} genes

PDC-producing strain	MIC (µg/ml)[a]						Mutation(s)[b]
	TIC	TZP	ATM	CAZ	FEP	CZ/T	
4098(pUCP24)	16 (8)	4 (≤2)	4 (2)	1 (1)	1 (≤0.5)	0.5 (≤0.25)	
4098(pPDC-1)[c]	64 (16)	64 (≤2)	16 (2)	16 (1)	8 (1)	1 (0.5)	
Group 0							
4098(pPDC-3)	64 (16)	64 (4)	16 (2)	16 (2)	8 (1)	1 (0.5)	
4098(pPDC-8)	64 (16)	32 (4)	16 (2)	8 (1)	4 (1)	1 (0.5)	
4098(pPDC-24)	64 (8)	64 (≤2)	16 (2)	16 (1)	8 (1)	1 (0.5)	
4098(pPDC-34)	64 (16)	64 (≤2)	16 (2)	16 (1)	8 (1)	1 (0.5)	
4098(pPDC-35)	64 (16)	64 (4)	16 (2)	8 (1)	8 (1)	1 (0.5)	
Group I							
4098(pPDC-50)	128 (16)	64 (4)	32 (4)	**64** (2)	8 (1)	**4** (0.5)	V213A
4098(pPDC-74)	**256 (128)**	*8* (4)	**128 (32)**	**>64 (32)**	8 (4)	**8 (4)**	G216R
4098(pPDC-75)	**256 (128)**	*16* (8)	**128 (32)**	**>64 (64)**	8 (4)	**8 (4)**	G216R
4098(pPDC-78)	**>512 (512)**	32 (16)	**>128 (128)**	**>64 (>64)**	16 (16)	**16 (16)**	G216R, R100H
4098(pPDC-79)	**256 (128)**	*16* (16)	32 (16)	**>64 (64)**	16 (8)	**64 (32)**	E221K
4098(pPDC-80)	128 **(64)**	32 (16)	16 (8)	**64** (16)	4 (2)	**32 (8)**	E221G
4098(pPDC-85)	**256 (64)**	*16* (4)	32 (8)	32 (8)	8 (2)	**4 (4)**	Y223H
4098(pPDC-86)	**256 (128)**	*16* (16)	32 (32)	**>64 (>64)**	16 (8)	**>64 (64)**	E221K
Group II							
4098(pPDC-44)	32 (16)	*16* (4)	8 (2)	**64** (4)	**>64** (4)	2 (0.5)	L294P
4098(pPDC-88)	64 (16)	64 (4)	16 (2)	**64** (2)	**>64** (4)	4 (0.5)	Δ(T290-P291)
4098(pPDC-89)	32 (16)	32 **(8)**	16 (4)	**>64 (16)**	**>64 (32)**	**8 (2)**	Δ(T290-M292)
4098(pPDC-90)	*16* (16)	*16* (4)	8 (2)	**>64 (8)**	**>64 (16)**	4 (1)	Δ(T290-M292)
4098(pPDC-91)	32 (16)	64 **(8)**	32 **(8)**	**>64 (16)**	**>64 (64)**	**16 (2)**	Δ(T290-A293)
4098(pPDC-92)	32 (16)	32 (4)	16 (4)	**64 (8)**	**>64 (16)**	4 (1)	Δ(L294-Q295)
Group III							
4098(pPDC-73)	128 (16)	64 (≤2)	32 (2)	**64** (2)	16 (1)	**8** (1)	P154L
4098(pPDC-81)	128 (16)	64 (≤2)	16 (2)	**64** (2)	16 (2)	**8** (1)	P154L
4098(pPDC-82)	128 (32)	32 **(8)**	16 (4)	**64 (8)**	8 (2)	**16 (8)**	F121L, M175L
Group IV							
4098(pPDC-76)	32 (16)	*16* (4)	16 (4)	**>64 (16)**	16 (2)	**4** (1)	N347I
4098(pPDC-87)	64 (16)	32 **(8)**	32 (4)	**>64 (32)**	**32 (8)**	**8 (2)**	N347I
Groups I + IV							
4098(pPDC-84)	32 (16)	*16* (4)	**64 (16)**	**>64 (64)**	**32 (8)**	**16 (4)**	V213A, N347I

[a] Abbreviations: TIC, ticarcillin; TZP, piperacillin plus tazobactam at a fixed concentration of 4 µg/ml; ATM, aztreonam; CAZ, ceftazidime; FEP, cefepime; CZ/T, ceftolozane plus tazobactam at a fixed concentration of 4 µg/ml. Shown are values from at least two independent experiments. Values in parentheses correspond to the MICs for bacteria grown in the presence of 1,000 µg/ml cloxacillin. MIC values in boldface are at least 4-fold higher and those in italics are at least 4-fold lower than those for 4098(pPDC-1). MIC values of imipenem and meropenem were equal to 0.5 and 0.25 µg/ml, respectively, for all strains tested.

[b] Shown are mutations different from common polymorphisms G1D, A29T, A71V, T79A, Q129R, L150R, V179L, V330I, and G365A.

[c] bla_{AmpC} genes cloned on broad-spectrum plasmid pUCP24.

Group III PDCs. P154 and F121, which are situated near the conserved YSN loop (at the extremity of H-5 and in loop α3-α4, respectively) are predicted to interact by making a stacking involving their aromatic side chains. According to this model, mutations P154L and F121L that occur in PDC-73, -81, or -82 are expected to abrogate this interaction, forcing loop α3-α4 to move away, enlarging the substrate binding pocket, with consequent modification of AmpC substrate specificity (Fig. 2). Supporting this hypothesis, both mutations resulted in similar effects on β-lactam MICs, with a strong increase in resistance of strain 4098 to ceftazidime and ceftolozane-tazobactam relative to PDC-1 (Table 2). As mentioned above, F121L was already found to coexist with group I mutation E221K in a ceftolozane-tazobactam-resistant mutant selected in vitro; however, its contribution to the whole

resistance of the mutant was not investigated further (23). M175L, concurrent with F121L in PDC-82, is localized in H-6 sided to H-6″ involved in the substrate-binding site at the C terminus of the Ω loop. Its mutation into leucine would require a small rearrangement of the surrounding region, but without clear knowledge on the necessary movements. To our knowledge, mutations at positions corresponding to P154 and F121 in *P. aeruginosa* AmpC have not been identified in other class C β-lactamases.

Group IV PDCs. Group IV is characterized by the N347I substitution as found in PDC-76, -84 (in combination with V213A), and -87. Reminiscent of the phenotype associated with these AmpC variants, N347I was reported to increase the activity of plasmid-determined enzyme CMY-2 and *Enterobacter* AmpC on cephalosporins ceftazidime and cefepime (30, 31) (see Table S2 in
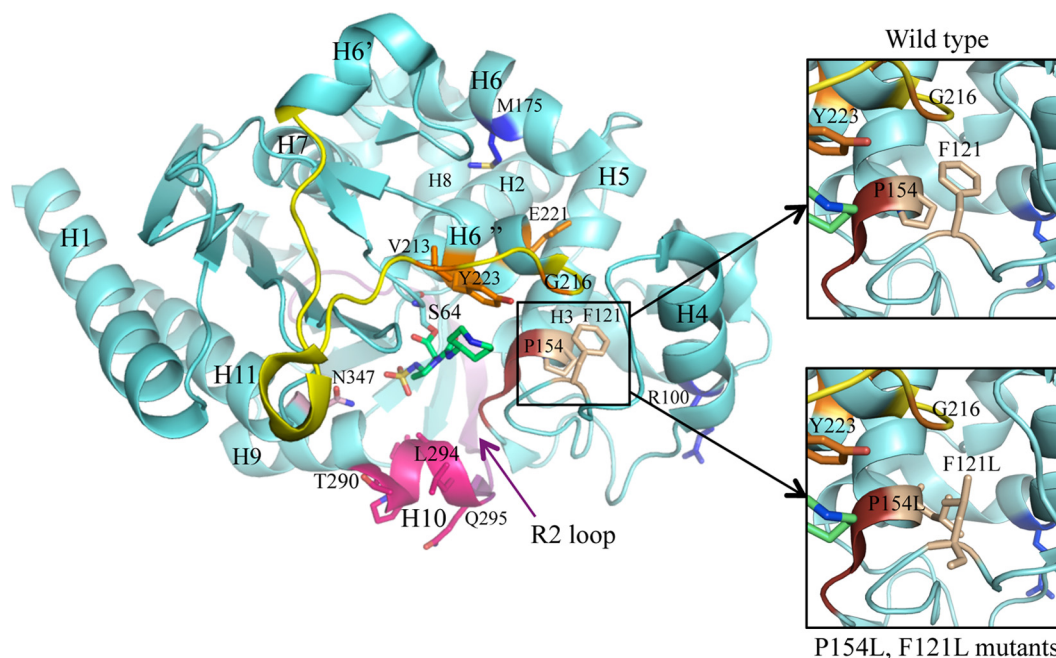
FIG 2 Representation of the AmpC β-lactamase structure from the *P. aeruginosa* PAO1 strain (light blue) in complex with an inhibitor (green) (PDB code 2WZZ) (41). The different structural regions lining the binding site are colored as follows: omega loop, yellow; helix H-10, pink; R2 loop, purple; and YSN, brick. The amino acids highlighted in this study as playing an important role in ESAC emergence are colored in terms of the function of their pertaining group (see the text): group I, orange; group II, pink; group III, wheat; and group IV, light pink. The two other mutants (M175L and R100H) found in combination with some from the other groups are colored in blue.

the supplemental material). According to the results of site-directed mutagenesis experiments and structural studies, the highly conserved N347 (346 in *E. coli* AmpC) is part of the C_3/C_4 carboxylate recognition region that directly contributes to substrate positioning in the catalytic site of class C enzymes through hydrogen bonding (32). How N347I affects this positioning and accounts for the phenotype associated with group IV PDCs is unclear so far.

Because of the structural plasticity of class C β-lactamases, enabling them to accommodate new substrates when mutated in specific regions, one could assume that coexistence of mutations in several of these regions would further increase the activity of AmpC enzymes toward newer cephalosporins and/or carbapenems. Actually, this was not verified here, as the occurrence of two ESAC-associated mutations in the same enzyme (PDC-84), namely, V213A (group I) and N347I (group IV), did not result in significant additive effects on the levels of resistance to cephalosporins and did not impact carbapenem MICs (Table 2).

Finally, the data presented above confirmed that the common polymorphism, which also includes V330I in *P. aeruginosa*, has no or negligible impact on AmpC activity when combined with ESAC-associated mutations (compare PDC-74 and PDC-75, PDC-79 and PDC-86, PDC-89 and PDC-90, PDC-73 and PDC-81, and PDC-76 and PDC-87 in Table 2).

Strain genotyping. The clonal relatedness of the 31 ESAC-producing *P. aeruginosa* strains was investigated by MLST. The bacteria could be grouped into 14 different sequence types (STs), some of which correspond to widespread clonal complexes or clones, such as ST175 (*n* = 5 isolates), ST235 (*n* = 3), ST260 (*n* = 1), ST298 (*n* = 1), ST308 (*n* = 4), ST309 (*n* = 1), and ST313 (*n* = 2) (33) (see Table S1 in the supplemental material). Interestingly, most of the strains isolated in the context of cystic fibrosis ap-

peared to belong to clones already found associated with this pathology, such as ST170 (*n* = 1), ST242 (*n* = 2), ST282 (*n* = 2), and ST274 (*n* = 4) (34–36). Our genotypic analysis also revealed the occurrence of clonally related isolates producing PDC-44 (*n* = 2), PDC-75 (*n* = 2), PDC-87 (*n* = 2), and PDC-92 (*n* = 2) in distinct institutions. With the exception of PDC-77 found in ST175 isolates from 3 distant cities (i.e., which suggests a geographical diffusion of a single clone), our results provide evidence that the ESACs emerged locally in genotypically distinct and rather prevalent *P. aeruginosa* clones (see Table S1). Since 15 (48.4%) ESAC-producing isolates came from chronically infected patients with cystic fibrosis (*n* = 10) or chronic obstructive pulmonary disease (COPD [*n* = 5]), we asked whether hypermutability, which is common in these diseases (37), was required for the emergence of ESAC-associated mutations, as suggested elsewhere (23, 38). Actually, only 1 (CF isolate 12.1111) out of 10 randomly selected ESAC strains (the other 9 strains including 5 CF, 1 COPD, and 3 non-CF, non-COPD isolates) turned out to give rise to rifampin-resistant mutants at rates significantly higher ($\geq$20-fold) than those of reference strain PAO1 (10^{-8}) and to fit with the definition of hypermutator (39; data not shown).

Conclusions. Despite differences in their amino acid sequences, class C β-lactamases share the same general structure and conserved sequence motifs near the active-site serine (14). So, it is not surprising *per se* that mutations occurring in specific regions of the substrate binding pocket, such as the Ω loop, the R2 domain, or the C-terminal domain, result in conformational changes prone to enhance the catalytic efficiencies of these enzymes toward poor substrates. Like in other Gram-negative species, these mutations increase the hydrolytic activity of *P. aeruginosa* AmpC on cephalosporins (ceftazidime, cefepime, and/or

ceftolozane), with mitigated effects on penicillins and/or aztreonam (14). Our assumption that P154L and F121L (which apparently belong to a new group of mutations) prevent the interaction between P154 and F121, with consequent remodeling of the substrate cavity, needs to be substantiated by crystallography experiments. In this work, none of the ESACs studied could increase the resistance of *P. aeruginosa* to carbapenems. Furthermore, confirming other data (28), we could not demonstrate any effect of the T79A substitution on carbapenem MICs even in the low-outer-membrane-permeability mutant 4098Δ*oprD*, consistent with T79 being distant from the active site, at the surface of the protein, in a hydrophobic environment showing no evidence of negative consequence of replacing a threonine by an alanine.

The prevalence of ESAC-producing strains among clinical *P. aeruginosa* isolates is still largely unknown. In the collection of the French NRC, mainly composed of β-lactam-resistant bacteria, 31 of 2,040 isolates (1.5%) were characterized as ESAC producers. However, sequencing of gene *bla*$_{AmpC}$ was not systematic and only applied to those strains exhibiting uncommon phenotypes (higher resistance to ceftazidime than to piperacillin-tazobactam) and devoid of secondary β-lactamases (ESBLs or carbapenemases), so we cannot rule out the possibility that the use of specific phenotypic criteria to screen for ESACs may miss bacteria in which these variants are phenotypically masked by other resistance mechanisms (e.g., active efflux pumps, penicillin-binding protein [PBP] alterations). Alternatively, some ESACs may well be responsible for phenotypes that are different from those reported here. Interestingly, we examined the recently released AmpC sequences of 531 *P. aeruginosa* isolates collected in 25 different countries and associated with multiple clinical situations (15). This search revealed the presence of 9 strains (1.7%) harboring several of the mutations described in this study (including 7 strains with V231A, 1 with L294P, and 1 with N347I). Therefore, if one considers that still unreported ESAC-associated mutations are likely to occur in the clinical setting, the prevalence of AmpC variants should be higher than 2%. That AmpC variants conferring high resistance to ceftolozane (in combination with tazobactam) preexist the introduction of this AmpC-stable molecule into clinical practice is of particular interest (40). Although clinical data were not available in this study, one can assume that older antipseudomonal cephalosporins (ceftazidime and cefepime) cross-selected resistance to this new antibiotic. Therefore, in the future it will be necessary to pay a special attention to the emergence of ceftolozane-resistant mutants under treatment since AmpC does not necessarily require hypermutability to give rise to ESACs in *P. aeruginosa*. The use of cloxacillin as an AmpC inhibitor may fail to characterize these variants, which as shown here, can potentially emerge in epidemic clones.

ACKNOWLEDGMENTS

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