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.

**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 AmpC variants 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-3. 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 PAO1Δ*oprD* (19), which lacks the carbapenem-specific uptake porin OprD. The PBP4-deficient mutant PAO1Δ*acB* (6), which constitutively overproduces PDC-1, was used as a control in MIC experiments.
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), cefazidine (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 claxocillin 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, nusD, 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 primersSeqAmpC-PA14-Fw1 (5′-TGGGGTCAACAACTCTCTTA-3′), SeqAmpC-PA14-Fw2 (5′-CGATCTCGGACTTACC-3′), SeqAmpC-PA14-Fv1 (5′-AGCTCGAGGTGGTCGTTT-3′), and SeqAmpC-PA14-Fw2 (5′-CCATGGACACCATGACCC-3′). The amplifications were sequenced using the BigDye Terminator chemistry on an automated ABI 3730 sequencer (Applied Biosystems), and the resulting nucleotide sequences 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 pUCP24 plasmids were transferred by electroporation (Bio-Rad MicroPulse) 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 primers were designed and used for RT-qPCR experiments by Dumas et al. (24). The expression of gene *bla_{AmpC}* and housekeeping gene *uuvD* was then assayed in a Rotor Gene RG6000 instrument (Qiagen, Courtabœuf, France) 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 *uuvD* 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.

**Structure-function analysis of AmpCs.** The AmpC sequence, corresponding to the genomic locations 4594029 to 4595222 on strain PAO1, was extracted from the *Pseudomonas* Genomic 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^-10 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 2ZZZ). In order to evaluate the possible effect of the different observed mutations, all of the corresponding positions were mutated using PyMOL on the 2ZZZ structure. All the structural figures were drawn using PyMol software (PyMOL Molecular Graphics system [http://pymol.sourceforge.net/]).


**RESULTS AND DISCUSSION**

**Sequence polymorphism of *P. aeruginosa* AmpC.** Amino acid substitutions GI/D/V, A29T, R35Q, A71V, T79A, L150R, V179L, V330L, 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

<table>
<thead>
<tr>
<th>Strain(s)</th>
<th>Residue at amino acid position shown*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type AmpC</td>
<td></td>
</tr>
<tr>
<td>producers</td>
<td></td>
</tr>
<tr>
<td>PAO1</td>
<td>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</td>
</tr>
<tr>
<td>14.2628</td>
<td>PDC-3 A</td>
</tr>
<tr>
<td>13.1642</td>
<td>PDC-8 A R</td>
</tr>
<tr>
<td>13.1781</td>
<td>PDC-24 A</td>
</tr>
<tr>
<td>PA14</td>
<td>PDC-34 D T A R L</td>
</tr>
<tr>
<td>11.773</td>
<td>PDC-35 D V A L</td>
</tr>
<tr>
<td>ESAC producers</td>
<td></td>
</tr>
<tr>
<td>12.1227 and 12.1255</td>
<td>PDC-44 A</td>
</tr>
<tr>
<td>10.257</td>
<td>PDC-50 A</td>
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<tr>
<td>12.1285</td>
<td>PDC-73 A</td>
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<td>12.1111 and 13.1716</td>
<td>PDC-74 A R</td>
</tr>
<tr>
<td>12.1129 and 13.1727</td>
<td>PDC-75 A R I</td>
</tr>
<tr>
<td>13.1415</td>
<td>PDC-76 A</td>
</tr>
<tr>
<td>13.1404, 13.1696,</td>
<td>PDC-77 A</td>
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<td>and 13.1760</td>
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<tr>
<td>11.571</td>
<td>PDC-78 H</td>
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<tr>
<td>11.516</td>
<td>PDC-79 K</td>
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<tr>
<td>13.1737</td>
<td>PDC-80 G</td>
</tr>
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<td>13.1514</td>
<td>PDC-81 A L</td>
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<td>13.1482</td>
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<td>12.961</td>
<td>PDC-85 A I A</td>
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<tr>
<td>13.1755 and 14.1999</td>
<td>PDC-86 D A</td>
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<tr>
<td>11.698 and 13.1775</td>
<td>PDC-87 D A</td>
</tr>
<tr>
<td>13.1601</td>
<td>PDC-88 A L A</td>
</tr>
<tr>
<td>09.236</td>
<td>PDC-89 A L A</td>
</tr>
<tr>
<td>13.1562</td>
<td>PDC-90 A L A</td>
</tr>
<tr>
<td>11.813</td>
<td>PDC-91 A L A</td>
</tr>
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<tr>
<td>14.2036</td>
<td>PDC-93 D A L P</td>
</tr>
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</table>

*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).

bacteria 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
identification of new AmpC variants. Sequencing of gene bla

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Identification of new AmpC variants. Sequencing of gene bla
Table 2: Susceptibility profiles of strain 4098 complemented with bla<sub>AmpC</sub> genes

<table>
<thead>
<tr>
<th>PDC-producing strain</th>
<th>TIC (μg/ml)</th>
<th>TZP</th>
<th>ATM</th>
<th>CAZ</th>
<th>FEP</th>
<th>CZ/T</th>
<th>Mutation(s)&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>4098(pUCP24)</td>
<td>16 (8)</td>
<td>4 (≤2)</td>
<td>4 (2)</td>
<td>1 (1)</td>
<td>1 (≤0.5)</td>
<td>0.5 (≤0.25)</td>
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</tr>
<tr>
<td>4098(pPDC-1)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>64 (16)</td>
<td>64 (≤2)</td>
<td>16 (2)</td>
<td>16 (1)</td>
<td>8 (1)</td>
<td>1 (0.5)</td>
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Group 0

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<th>ATM</th>
<th>CAZ</th>
<th>FEP</th>
<th>CZ/T</th>
<th>Mutation(s)&lt;sup&gt;b&lt;/sup&gt;</th>
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<tbody>
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<td>4098(pPDC-3)</td>
<td>64 (16)</td>
<td>64 (≤2)</td>
<td>16 (2)</td>
<td>16 (2)</td>
<td>8 (1)</td>
<td>1 (0.5)</td>
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</tr>
<tr>
<td>4098(pPDC-8)</td>
<td>64 (16)</td>
<td>32 (4)</td>
<td>16 (2)</td>
<td>8 (1)</td>
<td>4 (1)</td>
<td>1 (0.5)</td>
<td></td>
</tr>
<tr>
<td>4098(pPDC-24)</td>
<td>64 (8)</td>
<td>64 (≤2)</td>
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<td>8 (1)</td>
<td>1 (0.5)</td>
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<td>64 (≤2)</td>
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<td>1 (0.5)</td>
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Group I

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<th>TIC (μg/ml)</th>
<th>TZP</th>
<th>ATM</th>
<th>CAZ</th>
<th>FEP</th>
<th>CZ/T</th>
<th>Mutation(s)&lt;sup&gt;b&lt;/sup&gt;</th>
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<td>4098(pPDC-50)</td>
<td>128 (16)</td>
<td>64 (4)</td>
<td>32 (4)</td>
<td>64 (2)</td>
<td>8 (1)</td>
<td>4 (0.5)</td>
<td>V213A</td>
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<td>4098(pPDC-74)</td>
<td>256 (128)</td>
<td>8 (4)</td>
<td>128 (32)</td>
<td>&gt;64 (32)</td>
<td>8 (4)</td>
<td>8 (4)</td>
<td>G216R</td>
</tr>
<tr>
<td>4098(pPDC-75)</td>
<td>256 (128)</td>
<td>16 (8)</td>
<td>128 (32)</td>
<td>&gt;64 (64)</td>
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<tr>
<td>4098(pPDC-78)</td>
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<td>16 (16)</td>
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<td>256 (128)</td>
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<td>16 (8)</td>
<td>64 (32)</td>
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</tr>
<tr>
<td>4098(pPDC-80)</td>
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<td>4 (2)</td>
<td>32 (8)</td>
<td>E221G</td>
</tr>
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<td>4098(pPDC-85)</td>
<td>256 (64)</td>
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<td>4 (4)</td>
<td>Y223H</td>
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<tr>
<td>4098(pPDC-86)</td>
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<td>16 (16)</td>
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<td>16 (8)</td>
<td>&gt;64 (64)</td>
<td>E221K</td>
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Group II

<table>
<thead>
<tr>
<th>PDC-producing strain</th>
<th>TIC (μg/ml)</th>
<th>TZP</th>
<th>ATM</th>
<th>CAZ</th>
<th>FEP</th>
<th>CZ/T</th>
<th>Mutation(s)&lt;sup&gt;b&lt;/sup&gt;</th>
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<tbody>
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<td>4098(pPDC-44)</td>
<td>32 (16)</td>
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<td>2 (0.5)</td>
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<tr>
<td>4098(pPDC-88)</td>
<td>64 (16)</td>
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<td>4 (0.5)</td>
<td>Δ(T290-P291)</td>
</tr>
<tr>
<td>4098(pPDC-89)</td>
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<td>32 (8)</td>
<td>16 (4)</td>
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<td>&gt;64 (32)</td>
<td>8 (2)</td>
<td>Δ(T290-M292)</td>
</tr>
<tr>
<td>4098(pPDC-90)</td>
<td>16 (16)</td>
<td>16 (4)</td>
<td>8 (2)</td>
<td>&gt;64 (8)</td>
<td>&gt;64 (16)</td>
<td>4 (1)</td>
<td>Δ(T290-M292)</td>
</tr>
<tr>
<td>4098(pPDC-91)</td>
<td>32 (16)</td>
<td>64 (8)</td>
<td>32 (8)</td>
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<td>&gt;64 (64)</td>
<td>16 (2)</td>
<td>Δ(T290-A293)</td>
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<tr>
<td>4098(pPDC-92)</td>
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<td>16 (4)</td>
<td>64 (8)</td>
<td>&gt;64 (16)</td>
<td>4 (1)</td>
<td>Δ(L294-Q295)</td>
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Group III

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<tr>
<th>PDC-producing strain</th>
<th>TIC (μg/ml)</th>
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<th>ATM</th>
<th>CAZ</th>
<th>FEP</th>
<th>CZ/T</th>
<th>Mutation(s)&lt;sup&gt;b&lt;/sup&gt;</th>
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<tbody>
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<td>8 (1)</td>
<td>P154L</td>
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<tr>
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<td>64 (≤2)</td>
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<td>64 (2)</td>
<td>16 (2)</td>
<td>8 (1)</td>
<td>P154L</td>
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<tr>
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<td>16 (4)</td>
<td>64 (8)</td>
<td>8 (2)</td>
<td>16 (8)</td>
<td>F121L, M175L</td>
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Group IV

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<th>PDC-producing strain</th>
<th>TIC (μg/ml)</th>
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<th>ATM</th>
<th>CAZ</th>
<th>FEP</th>
<th>CZ/T</th>
<th>Mutation(s)&lt;sup&gt;b&lt;/sup&gt;</th>
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<td>&gt;64 (64)</td>
<td>16 (2)</td>
<td>4 (1)</td>
<td>N347I</td>
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<tr>
<td>4098(pPDC-87)</td>
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<td>32 (8)</td>
<td>32 (4)</td>
<td>&gt;64 (32)</td>
<td>32 (8)</td>
<td>8 (2)</td>
<td>N347I</td>
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Groups I + IV

<table>
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<th>PDC-producing strain</th>
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<th>TZP</th>
<th>ATM</th>
<th>CAZ</th>
<th>FEP</th>
<th>CZ/T</th>
<th>Mutation(s)&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
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<td>4098(pPDC-84)</td>
<td>32 (16)</td>
<td>16 (4)</td>
<td>64 (16)</td>
<td>&gt;64 (64)</td>
<td>32 (8)</td>
<td>16 (4)</td>
<td>V213A, N347I</td>
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</tbody>
</table>

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 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 side to H-6<sup>6</sup> involved in the substrate-binding site at the C terminus of the β 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 supplementary material).
Interestingly, PDC-79 and PDC-86, PDC-89 and PDC-90, PDC-73 and PDC-74 (group I) and PDC-75 (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 appeared 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 (≥20-fold) than those of reference strain PAO1 (10^{-6}) 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-outermembrane-permeability mutant 4098ΔoprD, 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 aeruginosa hydrophobic environment showing no evidence of negative consequence 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 L294F, 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 (ceftazidine 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 because AmpC does not necessarily require hypermutability to give rise to ESACs in P. aeruginosa. The use of claxocillin as an AmpC inhibitor may fail to characterize these variants, which as shown here, can potentially emerge in epidemic clones.

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