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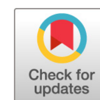
MECHANISMS OF RESISTANCE



Ceftazidime-Avibactam Resistance Mediated by the N346Y Substitution in Various AmpC β-Lactamases

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ABSTRACT Chromosomal and plasmid-borne AmpC cephalosporinases are a major resistance mechanism to β-lactams in *Enterobacteriaceae* and *Pseudomonas aeruginosa*. The new β-lactamase inhibitor avibactam effectively inhibits class C enzymes and can fully restore ceftazidime susceptibility. The conserved amino acid residue Asn346 of AmpC cephalosporinases directly interacts with the avibactam sulfonate. Disruption of this interaction caused by the N346Y amino acid substitution in *Citrobacter freundii* AmpC was previously shown to confer resistance to the ceftazidime-avibactam combination (CAZ-AVI). The aim of this study was to phenotypically and biochemically characterize the consequences of the N346Y substitution in various AmpC backgrounds. Introduction of N346Y into *Enterobacter cloacae* AmpC ($AmpC_{cloacae}$), plasmid-mediated DHA-1, and *P. aeruginosa* PDC-5 led to 270-, 12,000-, and 79-fold decreases in the inhibitory efficacy (k_2/K_i) of avibactam, respectively. The kinetic parameters of $AmpC_{cloacae}$ and DHA-1 for ceftazidime hydrolysis were moderately affected by the substitution. Accordingly, $AmpC_{cloacae}$ and DHA-1 harboring N346Y conferred CAZ-AVI resistance (MIC of ceftazidime of 16 μg/ml in the presence of 4 μg/ml of avibactam). In contrast, production of PDC-5 N346Y was associated with a lower MIC (4 μg/ml) since this β-lactamase retained a higher inactivation efficacy by avibactam in comparison to $AmpC_{cloacae}$ N346Y. For FOX-3, the I346Y substitution did not reduce the inactivation efficacy of avibactam and the substitution was highly deleterious for β-lactam hydrolysis, including ceftazidime, preventing CAZ-AVI resistance. Since $AmpC_{cloacae}$ and DHA-1 display substantial sequence diversity, our results suggest that loss of hydrogen interaction between Asn346 and avibactam could be a common mechanism of acquisition of CAZ-AVI resistance.

KEYWORDS avibactam, β-lactamase inhibitor, AmpC, cephalosporinase, ceftazidime, DHA, FOX, *Enterobacter cloacae*, *Pseudomonas aeruginosa*

Chromosomally encoded class C β-lactamases (cAmpCs) are ubiquitous in many clinically relevant Gram-negative bacteria such as *Enterobacter cloacae*, *Citrobacter freundii*, *Morganella morganii*, and *Pseudomonas aeruginosa* (1). These enzymes display broad substrate profiles, which include narrow-spectrum cephalosporins, cephamycins, and aminopenicillins (1). Derepression of cAmpC-encoding genes generally results in resistance to most β-lactams, except cefepime and carbapenems (1). Related members of the cAmpC family, designated extended-spectrum AmpC β-lactamases (ESACs), have evolved to include in their substrate profiles ceftazidime, other oxyimino-cephalosporins, and, in some instances, carbapenems (2–4). In the late 90s, the high diversity of class C β-lactamases further increased with the spread of AmpC-encoding genes by horizontal transfer of mobile genetic elements (plasmid-mediated AmpC or pAmpC) (5). These transfers have increased the burden of class C enzymes to include Gram-negative bacteria that are naturally devoid of any cAmpC, such as *Klebsiella pneumoniae* (5). Plasmid-born AmpCs are derivatives of the chromosomally encoded

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cephalosporinases from *Enterobacter cloacae* (ACT and MIR types), *Citrobacter freundii* (CMY-2, BIL, LAT, and CFE types), *Morganella morganii* (DHA type), *Hafnia alvei* (ACC type), and *Aeromonas* spp. (CMY-1, FOX, and MOX types). The expression of genes encoding pAmpCs is usually under the control of strong promoters, resulting in resistance levels similar to those found in cAmpC-derepressed isolates (6). The level of resistance to β-lactams conveyed by AmpC β-lactamases is determined by a complex combination of several factors, including the efficacy of drug hydrolysis, the level of AmpC production, and bacterial host factors such as porins, efflux pumps, and the drug targets, all of which may be mutationally altered under the selective pressure of β-lactams (7).

β-lactam-based first-generation β-lactamase inhibitors, clavulanic acid, tazobactam, and sulbactam, are generally not active against AmpC enzymes (1). Ceftazidime combined with avibactam, a second-generation inhibitor based on the diazabicyclooctane scaffold (8), has been proposed as a therapeutic option against AmpC-overproducing multidrug-resistant strains of Gram-negative bacteria (9). The AmpC residues that determine the efficacy of enzyme inhibition by avibactam and the risk of resistance acquisition by modification of these residues have not been extensively investigated. *In vitro* selection of ceftazidime-avibactam-resistant mutants of *P. aeruginosa* strains producing derepressed AmpC led to deletions of 5 to 19 amino acid residues of the Ω loop. Purification of a representative enzyme with a 5-residue deletion showed that resistance was due both to an increase in the efficacy of ceftazidime hydrolysis and to a reduction of the efficacy of inhibition by avibactam (10). One of the mutants harbored a $G^{183}D$ substitution located outside the Ω loop, but the corresponding enzyme was not biochemically characterized (10). In another study, also based on *in vitro* selection of mutants, resistance or decreased susceptibility to the ceftazidime-avibactam combination in derepressed AmpC-producing *Enterobacteriaceae* was found to result from the $Arg^{168}Pro/His$, $Gly^{176}Arg/Asp$, or $Asn^{366}Tyr$ substitution or from small deletions around positions 309 to 314 (11). These enzymes were not kinetically characterized. Crystallographic structures revealed that eight residues are in direct contact with avibactam, including Ser64 and Lys67 (Ambler amino-acid numbering scheme) from the SXXK motif, Tyr150 and Asn152 from the YXN motif, Lys315 and Thr316 from the KTG motif, Gln120, and Asn346 (10, 12, 13). Among these residues, Ser64 from the SXXK motif forms a covalent adduct with avibactam (carbamoyl-enzyme) leading to reversible enzyme inactivation (12, 13). Residues from the conserved SXXK, YXN, and KTG motifs are invariant in AmpC β-lactamases, whereas positions 120 (Gln or Phe) and 346 (Asn or Ile) display limited variations (See Fig. S1 in the supplemental material for a sequence alignment). In the latter position, an $N^{346}Y$ substitution in cAmpC was previously reported to confer resistance to the ceftazidime-avibactam combination in *C. freundii* (11, 13). This prompted us to investigate the impact of replacing asparagine or isoleucine at Ambler position 346 by tyrosine ($N^{346}Y$ and $I^{346}Y$ substitutions, respectively) in four highly divergent AmpC β-lactamases (See Fig. S2 for an evolutionary tree). This panel of four sequences included two chromosome-mediated cephalosporinases ($AmpC_{cloacae}$ from *Enterobacter cloacae* P99 and PDC-5 from *P. aeruginosa* ATCC 27853) and two plasmid-mediated cephalosporinases (DHA-1 and FOX-3, which derive from chromosomally encoded enzymes from *Morganella morganii* and *Aeromonas spp*, respectively). The phenotypic consequences of the substitutions were evaluated by constructing isogenic strains of *Escherichia coli* producing $AmpC_{cloacae}$, PDC-5, DHA-1, FOX-3, and derivatives of these four β-lactamases containing a Tyr residue at position 346. The impact of the substitutions was also evaluated by purifying soluble forms of the β-lactamases in order to compare efficacies of β-lactam hydrolysis and of inactivation by avibactam. In parallel, we investigated whether exposure of the recombinant *E. coli* strains producing wild-type $AmpC_{cloacae}$, PDC-5, DHA-1, or FOX-3 could lead to the selection of mutations resulting in the $N^{346}Y$ or $I^{346}Y$ substitution in these enzymes. We show that the $N^{346}Y$ substitution, but not $I^{346}Y$, is a likely route of acquisition of resistance to the ceftazidime-avibactam combination in AmpC β-lactamases as it drastically reduced the efficacy of enzyme carbamoylation by avibactam while preserv-

TABLE 1 MIC of β-lactams against *E. coli* TOP10 isogenic strains producing various AmpC β-lactamases

AmpC β-lactamase	MIC (μg/ml)[a]												
	CAZ	CAZ-AVI	AMX	AMC	PIP	ETP	MEM	CF	MA	FOX	CTX	ATM	FEP
None[b]	0.25	0.25	2	2	2	≤0.06	≤0.06	8	1	4	0.06	0.25	0.12
AmpC$_{cloacae}$	64	0.25	>256	>256	128	0.25	≤0.06	>256	256	128	64	32	1
AmpC$_{cloacae}$ N^{346}Y	128	16	>256	>256	16	≤0.06	≤0.06	128	128	16	32	32	0.5
PDC-5	16	0.5	>256	>256	256	≤0.06	≤0.06	>256	256	128	32	16	0.5
PDC-5 N^{346}Y	8	4	64	32	4	0.12	≤0.06	128	16	128	4	1	0.25
DHA-1	256	0.25	>256	>256	256	≤0.06	≤0.06	256	128	16	16	16	0.12
DHA-1 N^{346}Y	64	16	128	128	8	≤0.06	≤0.06	16	4	8	2	16	0.12
FOX-3	256	2	128	128	8	≤0.06	≤0.06	>256	256	256	16	8	0.5
FOX-3 I^{346}Y	16	1	32	32	1	≤0.06	≤0.06	32	1	8	0.25	0.5	0.12

[a]CAZ, ceftazidime; CAZ-AVI, ceftazidime combined with avibactam (4 μg/ml); AMX, amoxicillin; AMC, amoxicillin combined with clavulanate (2 μg/ml); PIP, piperacillin; ETP, ertapenem; MEM, meropenem; CF, cephalothin; MA, cefamandole; FOX, cefoxitin; CTX, cefotaxime; ATM, aztreonam; FEP, cefepime.
[b]None, control pTRC-99k vector without any *ampC*-encoding gene.

ing the efficacy of ceftazidime hydrolysis. This conclusion applies to various members of the AmpC family despite substantial sequence divergence.

RESULTS AND DISCUSSION

Resistance to ceftazidime and to the ceftazidime-avibactam combination mediated by the AmpC cephalosporinases. MICs were determined for isogenic strains of *E. coli* TOP10 producing AmpC$_{cloacae}$, PDC-5, DHA-1, and FOX-3 (Table 1). Production of these four β-lactamases conferred ceftazidime resistance to the *E. coli* host with 64- to 1,024-fold increases in the MIC of this antibiotic (from 0.25 μg/ml to 16 to 256 μg/ml). Avibactam fully restored the activity of ceftazidime against TOP10 derivatives producing AmpC$_{cloacae}$ and DHA-1 (MIC of ceftazidime = 0.25 μg/ml in the presence of 4 μg/ml of avibactam). Inhibition of PDC-5 and FOX-3 was almost complete (ceftazidime MIC in the presence of avibactam of 0.5 μg/ml and 2 μg/ml for PDC-5 and FOX-3, respectively). As expected, these results indicate that avibactam restored completely (AmpC$_{cloacae}$ and DHA-1) or partially (PDC-5 and FOX-3) the activity of ceftazidime against the four isogenic strains of *E. coli* producing AmpC β-lactamases.

Impact of N^{346}Y and I^{346}Y on AmpC-mediated resistance to the ceftazidime-avibactam combination. In the AmpC$_{cloacae}$ and DHA-1 backgrounds, the N^{346}Y substitution was associated with a 64-fold increase in the MIC of ceftazidime in the presence of avibactam (from 0.25 μg/ml to 16 μg/ml) (Table 1). Introduction of N^{346}Y in PDC-5 produced a lower increase (8-fold) in the MIC of ceftazidime in the presence of avibactam (from 0.5 μg/ml to 4 μg/ml). For these three β-lactamases (AmpC$_{cloacae}$, DHA-1, and PDC-5), the substitution had a marginal impact on the MIC of ceftazidime alone. These results suggest that reduced activity of the ceftazidime-avibactam combination was due to impaired inhibition of these enzymes by avibactam but not to improved hydrolysis of ceftazidime.

The activity of the ceftazidime-avibactam combination was compromised by the introduction of a tyrosine residue at position 346 of AmpC cephalosporinases from various origins, AmpC$_{cloacae}$, DHA-1, and PDC-5, which contain an asparagine at this position (above). In contrast, for FOX-3, which contains an isoleucine at this position, tyrosine introduction did not lead to any increase in the MIC of ceftazidime in the presence of avibactam. The I^{346}Y substitution in FOX-3 impaired expression of ceftazidime resistance (a 16-fold decrease in the MIC of ceftazidime, from 256 to 16 μg/ml). Impaired hydrolysis of ceftazidime by FOX-3 I^{346}Y may therefore account for the lack of resistance to the ceftazidime-avibactam combination.

Impact of N^{346}Y and I^{346}Y on AmpC-mediated resistance to other β-lactams. The substitutions led to significant decreases of the MICs of certain β-lactams for the four AmpC cephalosporinases (Table 1). For AmpC$_{cloacae}$, the antibiotics most impacted were piperacillin, cephalotin, and cefoxitin with 8-, ≥4-, and 8-fold MIC decreases, respectively. For DHA-1, the MIC of amoxicillin, piperacillin, cephalotin, and cefotaxime were reduced ≥4-, 32-, 16-, and 8-fold, respectively. For PDC-5, resistance was impaired

for amoxicillin (≥8-fold), piperacillin (64-fold), cephalotin (≥4-fold), cefamandole (16-fold), aztreonam (16-fold), and cefotaxime (8-fold). For FOX-3, the $I^{346}Y$ substitution abolished resistance to piperacillin, cefamandole, cefoxitin, cefotaxime, and aztreonam. In conclusion, the $N^{346}Y$ substitutions in $AmpC_{cloacae}$, DHA-1, and PDC-5 impaired but did not abolish resistance to most β-lactams, while $I^{346}Y$ in FOX-3 was highly deleterious. As expected (7), clavulanate did not affect the MIC of amoxicillin against *E. coli* strains producing the four AmpC cephalosporinases or their derivatives with substitutions at position 346.

Substrate profile of purified wild-type AmpC cephalosporinases. Soluble forms of $AmpC_{cloacae}$, PDC-5, DHA-1, and FOX-3 that lacked the N-terminal signal peptides were produced in *E. coli* and purified by metal affinity and size-exclusion chromatography. The catalytic efficacy (k_{cat}/K_m) of the four AmpC cephalosporinases was high for amoxicillin ($>10^4$ M^{-1} s^{-1}), first-generation cephalosporins ($>10^7$ M^{-1} s^{-1}), and second-generation cephalosporins ($>10^5$ M^{-1} s^{-1}) (Table 2). For third-generation cephalosporins, hydrolysis efficacy was lower for ceftazidime (from 1.3×10^3 to 1.6×10^5 M^{-1} s^{-1}) than for cefotaxime (from 3.6×10^5 to 1.7×10^7 M^{-1} s^{-1}). Among cephalosporins, the lowest hydrolysis efficacy was observed for the fourth-generation drug cefepime (3.0×10^2 to 4.8×10^3 M^{-1} s^{-1}). Aztreonam was not a substrate of the purified enzymes.

Impact of $N^{346}Y$ and $I^{346}Y$ on the efficacy of AmpC cephalosporinases for hydrolysis of ceftazidime. The $N^{346}Y$ substitution had a moderate impact on the kinetic parameters for hydrolysis of ceftazidime by $AmpC_{cloacae}$ and PDC-5 (Table 2). This observation is in agreement with the phenotypic analysis, as the substitution did not affect (≤2-fold) the MIC of ceftazidime conveyed by these enzymes in *E. coli* Top10 (see above, Table 1). In contrast, $N^{346}Y$ was associated with a large (38-fold) decrease in the catalytic efficacy of DHA-1 due to a decrease in k_{cat} (220-fold) that was only partially compensated by a decrease in K_m (6-fold). The decrease in the catalytic efficacy of DHA-1 was associated with a 4-fold decrease in the MIC of ceftazidime (from 256 μg/ml to 64 μg/ml). Likewise, introduction of $I^{346}Y$ in FOX-3 was associated with decreases in the MIC of ceftazidime (16-fold) and in the k_{cat}/K_m ratio (20-fold) for hydrolysis of this drug. The latter difference resulted from a large reduction in k_{cat} (120-fold), which was only partially compensated by a 6-fold reduction in K_m. Together, these results indicate that impaired efficacy of *in vitro* ceftazidime hydrolysis due to the introduction of $N^{346}Y$ and $I^{346}Y$ in DHA-1 and FOX-3, respectively, was associated with impaired expression of ceftazidime resistance.

Impact of the $N^{346}Y$ and $I^{346}Y$ substitutions on the hydrolysis efficacy of other β-lactams. For $AmpC_{cloacae}$, $N^{346}Y$ was associated with reductions in k_{cat}/K_m (10-fold) and k_{cat} (20-fold) for hydrolysis of cefoxitin, whereas the kinetic parameters for hydrolysis of other β-lactams were only marginally affected (Table 2). For PDC-5, $N^{346}Y$ was associated with decreases in the catalytic efficacy of amoxicillin (16-fold) and cefamandole (14-fold) hydrolysis, mainly due to increases in K_m (15- and 95-fold, respectively). The efficacy of hydrolysis of all tested β-lactams except cefepime was affected by introduction of $N^{346}Y$ in DHA-1 (10- to 230-fold decreases in k_{cat}/K_m) due to increases in K_m and decreases in k_{cat} alone or in combination. For FOX-3, $I^{346}Y$ did not impair the catalytic activity. On the contrary, hydrolysis of amoxicillin was improved with a 17-fold increase in the k_{cat}/K_m ratio resulting from a 21-fold increase in k_{cat}. Thus, $I^{346}Y$ in FOX-3 specifically impaired ceftazidime hydrolysis.

Overall, decreases in the catalytic efficacy of the cephalosporinases were correlated with decreases in the MICs of β-lactams—with two major discrepancies. First, introduction of $I^{346}Y$ in FOX-3 was associated with large decreases in the MICs of most β-lactams, although kinetic parameters were marginally affected. This observation suggests that the $I^{346}Y$ substitution may affect the stability or export of FOX-3. Second, hydrolysis of aztreonam was not detected for any of the AmpC cephalosporinases, although production of these enzymes conferred significant levels of resistance (MICs ranging from 8 to 32 μg/ml) corresponding to 32- to 128-fold increases in comparison with the host strain devoid of any β-lactamase (MIC = 0.25 μg/ml). Data from the literature suggest a possible explanation for

TABLE 2 Impact of the $N^{346}Y$ and $I^{346}Y$ substitutions on the hydrolysis of β-lactams by $AmpC_{cloacae}$, PDC-5, DHA-1, and FOX-3

	Values of kinetic parameters (mean ± SD) for indicated β-lactamases							
Antibiotic and parameter	**$AmpC_{cloacae}$**	**$AmpC_{cloacae}$ $N^{346}Y$**	**PDC-5**	**PDC-5 $N^{346}Y$**	**DHA-1**	**DHA-1 $N^{346}Y$**	**FOX-3**	**FOX-3 $I^{346}Y$**
Nitrocefin								
K_m (μM)	50 ± 10	18 ± 3	15 ± 4	5 ± 3	11 ± 1	1.7 ± 0.4	43 ± 8	23 ± 6
k_{cat} (s^{-1})	790 ± 50	290 ± 10	240 ± 20	93 ± 8	240 ± 10	7.1 ± 0.6	(1.3 ± 0.1) × 10^3	580 ± 40
k_{cat}/K_m (M^{-1} s^{-1})	(1.6 ± 0.4) × 10^7	(1.6 ± 0.3) × 10^7	(1.6 ± 0.6) × 10^7	(1.9 ± 1.3) × 10^7	(2.3 ± 0.3) × 10^7	(4.1 ± 1.6) × 10^6	(3.1 ± 0.8) × 10^7	(2.5 ± 0.6) × 10^7
Amoxicillin								
K_m (μM)	2.4 ± 0.9[a]	6.9 ± 1.3[a]	4.4 ± 1.4[a]	67 ± 19	0.32 ± 0.10[a]	7.4 ± 5.4[a]	9.7 ± 2.7[a]	0.47 ± 0.03[a]
k_{cat} (s^{-1})	0.40 ± 0.04	0.10 ± 0.01	2.2 ± 0.3	2.1 ± 0.1	2.0 ± 0.2	0.26 ± 0.01	0.22 ± 0.02	0.19 ± 0.01
k_{cat}/K_m (M^{-1} s^{-1})	(1.6 ± 0.8) × 10^5	(1.4 ± 0.4) × 10^4	(4.9 ± 2.3) × 10^5	(3.1 ± 1.0) × 10^4	(6.2 ± 2.6) × 10^6	(3.5 ± 2.7) × 10^4	(2.3 ± 0.8) × 10^4	(4.0 ± 0.5) × 10^5
Cephalothin								
K_m (μM)	23 ± 7	5.5 ± 1.3[a]	12 ± 4	47 ± 12	8.7 ± 3.6[a]	2.0 ± 1.0[a]	48 ± 16	40 ± 11
k_{cat} (s^{-1})	370 ± 60	83 ± 10	180 ± 10	230 ± 20	170 ± 20	4.3 ± 0.2	(1.9 ± 0.2) × 10^3	(2.5 ± 0.2) × 10^3
k_{cat}/K_m (M^{-1} s^{-1})	(1.6 ± 0.8) × 10^7	(1.5 ± 0.5) × 10^7	(1.5 ± 0.6) × 10^7	(4.9 ± 1.6) × 10^6	(2.0 ± 1.0) × 10^7	(2.1 ± 1.2) × 10^6	(4.0 ± 1.8) × 10^7	(6.3 ± 2.3) × 10^7
Cefamandole								
K_m (μM)	3.1 ± 1.1[a]	1.6 ± 0.3[a]	0.6 ± 0.2[a]	57 ± 13	1.2 ± 0.4[a]	2.3 ± 1.4[a]	41 ± 7	17 ± 2
k_{cat} (s^{-1})	33 ± 1	4.1 ± 0.2	4.9 ± 0.5	32 ± 2	12 ± 1	0.50 ± 0.03	500 ± 20	110 ± 10
k_{cat}/K_m (M^{-1} s^{-1})	(1.1 ± 0.4) × 10^7	(2.6 ± 0.6) × 10^6	(8.1 ± 3.6) × 10^6	(5.6 ± 1.6) × 10^5	(1.0 ± 0.4) × 10^7	(2.2 ± 1.5) × 10^5	(1.2 ± 0.3) × 10^7	(6.8 ± 1.2) × 10^6
Cefoxitin								
K_m (μM)	0.04 ± 0.01[a]	0.022 ± 0.006[a]	0.10 ± 0.03[a]	0.20 ± 0.10[a]	0.027 ± 0.003[a]	0.20 ± 0.10[a]	0.20 ± 0.06[a]	0.022 ± 0.008[a]
k_{cat} (s^{-1})	0.050 ± 0.001	0.0025 ± 0.0005	0.050 ± 0.010	0.13 ± 0.01	0.026 ± 0.001	(2.5 ± 0.1) × 10^{-3}	1.8 ± 0.1	0.064 ± 0.004
k_{cat}/K_m (M^{-1} s^{-1})	(1.2 ± 0.3) × 10^6	(1.1 ± 0.5) × 10^5	(5.0 ± 2.5) × 10^5	(6.5 ± 3.7) × 10^5	(9.6 ± 1.4) × 10^5	(1.3 ± 0.7) × 10^4	(9.0 ± 3.2) × 10^6	(2.9 ± 1.2) × 10^6
Cefotaxime								
K_m (μM)	0.050 ± 0.010[a]	0.11 ± 0.02[a]	0.14 ± 0.01[a]	1.2 ± 0.7[a]	0.0030 ± 0.0010[a]	0.40 ± 0.20[a]	0.019 ± 0.006[a]	0.014 ± 0.007[a]
k_{cat} (s^{-1})	0.018 ± 0.002	0.050 ± 0.010	0.070 ± 0.020	0.20 ± 0.01	0.051 ± 0.002	0.029 ± 0.001	0.024 ± 0.002	0.016 ± 0.001
k_{cat}/K_m (M^{-1} s^{-1})	(3.6 ± 1.1) × 10^5	(4.5 ± 1.7) × 10^5	(5.0 ± 1.8) × 10^5	(1.7 ± 1.1) × 10^5	(1.7 ± 0.6) × 10^7	(7.3 ± 3.9) × 10^4	(1.3 ± 0.5) × 10^6	(1.1 ± 0.6) × 10^6
Ceftazidime								
K_m (μM)	4.8 ± 1.8[a]	3.6 ± 0.7[a]	7.3 ± 2.2[a]	19 ± 9[a]	32 ± 10	5.4 ± 2.8[a]	2.2 ± 0.9[a]	0.35 ± 0.25[a]
k_{cat} (s^{-1})	0.011 ± 0.001	0.048 ± 0.005	0.010 ± 0.001	0.06 ± 0.01	0.80 ± 0.10	(3.7 ± 0.3) × 10^{-3}	0.35 ± 0.05	0.0029 ± 0.0004
k_{cat}/K_m (M^{-1} s^{-1})	(2.3 ± 1.1) × 10^3	(1.3 ± 0.4) × 10^4	(1.3 ± 0.5) × 10^3	(3.2 ± 2.0) × 10^3	(2.6 ± 1.1) × 10^4	(6.9 ± 4.1) × 10^2	(1.6 ± 0.9) × 10^5	(8.3 ± 7.1) × 10^3
Aztreonam[b]								
K_m (μM)	NA	NA	NA	NA	NA	NA	NA	NA
k_{cat} (s^{-1})	NA	NA	NA	NA	NA	NA	NA	NA
k_{cat}/K_m (M^{-1} s^{-1})	$<4.2 \times 10^{-3}$ s^{-1}	$<4.2 \times 10^{-3}$ s^{-1}	$<4.2 \times 10^{-3}$ s^{-1}	$<4.2 \times 10^{-3}$ s^{-1}	$<4.2 \times 10^{-3}$ s^{-1}	$<4.2 \times 10^{-3}$ s^{-1}	$<4.2 \times 10^{-3}$ s^{-1}	$<4.2 \times 10^{-3}$ s^{-1}
Cefepime								
K_m (μM)	>290	>175	>250	>300	>260	>220	>250	>170
k_{cat} (s^{-1})	>0.5	>0.7	>0.15	>0.17	>0.07	>0.14	>1.1	>2.1
k_{cat}/K_m (M^{-1} s^{-1})	(1.7 ± 0.1) × 10^3	(4.0 ± 0.2) × 10^3	(6.0 ± 0.1) × 10^2	(5.5 ± 0.1) × 10^2	(3.0 ± 0.2) × 10^2	(5.0 ± 0.5) × 10^2	(4.8 ± 0.3) × 10^3	(1.2 ± 0.1) × 10^4

[a]For these drugs, the K_m and k_{cat} kinetic parameters were determined in independent experiments based on (i) hydrolysis of the tested drugs at saturating substrate concentrations for k_{cat} and (ii) hydrolysis of nitrocefin in the presence of various concentrations of the tested drugs for K_m (16).

[b]NA, not applicable, as hydrolysis of aztreonam (500 μM) could not be detected at the highest β-lactamase concentration tested (10 μM). Under our experimental conditions, the lower limit of detection corresponds to a turnover of 4.2×10^{-3} s^{-1}.

TABLE 3 Kinetic parameters for β-lactamase inhibition by avibactam

β-lactamase	**Inhibition parameters**	
	k_2/K_i (M^{-1} s^{-1})	**k_{-2} (s^{-1})**
$AmpC_{cloacae}$	$(1.8 \pm 0.1) \times 10^4$	$(1.8 \pm 0.2) \times 10^{-3}$
$AmpC_{cloacae}$ $N^{346}Y$	66 ± 8	$(2.2 \pm 0.5) \times 10^{-3}$
PDC-5	$(6.3 \pm 0.5) \times 10^4$	$(2.4 \pm 0.3) \times 10^{-3}$
PDC-5 $N^{346}Y$	$(8.0 \pm 2.4) \times 10^2$	$(3.1 \pm 1.3) \times 10^{-3}$
DHA-1	$(1.7 \pm 0.1) \times 10^5$	$(2.3 \pm 0.3) \times 10^{-3}$
DHA-1 $N^{346}Y$	14 ± 1	$(1.2 \pm 0.1) \times 10^{-3}$
FOX-3	$(4.2 \pm 0.3) \times 10^3$	$(1.5 \pm 0.5) \times 10^{-3}$
FOX-3 $I^{346}Y$	$(2.0 \pm 0.1) \times 10^3$	$(2.7 \pm 0.4) \times 10^{-3}$

this discrepancy, as it has been reported that β-lactamases act by trapping aztreonam and thereby reducing the periplasmic drug concentration and preventing inactivation of penicillin-binding protein (PBP) targets (14).

Efficacy of AmpC cephalosporinase inhibition by avibactam. The four AmpC enzymes were inhibited by avibactam albeit with different efficacies (DHA-1 > PDC-5 > $AmpC_{cloacae}$ > FOX-3 with k_2/K_i ratios of 170,000, 63,000, 18,000, and 4,200 M^{-1} s^{-1}, respectively) (Table 3). The $N^{346}Y$ substitution impaired inhibition of $AmpC_{cloacae}$, DHA-1, and PDC-5 with fold decreases in the k_2/K_i efficacy parameter of 270, 12,000, and 79, respectively. In contrast, the $I^{346}Y$ substitution had only a modest impact on the inhibition efficacy of FOX-3 by avibactam (k_2/K_i of 4,200 versus 2,000 M^{-1} s^{-1} for the wild-type and $I^{346}Y$ variant, respectively). The k_{-2} rate constants were low for all enzymes, indicating that modification of the decarbamoylation efficacy is not a key element in defining the phenotypes under study (Table 3).

Selection of mutants resistant to the ceftazidime-avibactam combination. We investigated the acquisition of ceftazidime-avibactam resistance by *E. coli* TOP10 derivatives expressing the four *ampC* genes. Selection was performed on agar plates containing a fixed concentration of avibactam (4 μg/ml) and 2-fold increasing concentrations of ceftazidime (0.5 to 256 μg/ml). For the $AmpC_{cloacae}$-producing *E. coli* TOP10 strain, a single mutant was obtained on agar plates containing 4 μg/ml of ceftazidime and 4 μg/ml of avibactam (frequency of ca. 5×10^{-10}). Attempts to obtain additional mutants in two additional experiments were unsuccessful. Sequencing of the *$ampC_{cloacae}$* gene of the mutant revealed an A to T transversion at position 1096 leading to an asparagine-to-tyrosine substitution at Ambler position 346. Thus, the $N^{346}Y$ substitution characterized in this study is a mechanism of acquisition of ceftazidime-avibactam resistance under the selective pressure of the drug, although its frequency is very low.

For PDC-5, the $N^{346}Y$ substitution was not obtained in four independent experiments. This negative result may be accounted for by the modest (8-fold) increase in the MIC of ceftazidime in the presence of avibactam associated with the introduction of $N^{346}Y$ in PDC-5 (from 0.5 to 4 μg/ml) (Table 1). In comparison, a 64-fold increase was observed for introduction of $N^{346}Y$ in $AmpC_{cloacae}$ (from 0.25 to 16 μg/ml). The kinetic parameters that correlate with this difference between PDC-5 and $AmpC_{cloacae}$ are the higher efficacy of PDC-5 $N^{346}Y$ inhibition by avibactam in comparison to $AmpC_{cloacae}$ $N^{346}Y$ (k_2/K_i ratios of 800 ± 240 versus 66 ± 8 M^{-1} s^{-1}; Table 3) and the higher K_m of ceftazidime (19 ± 9 versus 1.9 ± 0.7 μM; Table 2). Since avibactam and ceftazidime competitively bind to the β-lactamase active sites, differences in the k_2/K_i and K_m kinetic constants are both expected to contribute to the higher efficacy of ceftazidime hydrolysis by PDC-5 $N^{346}Y$ in the periplasm.

Although the $N^{346}Y$ substitution was not obtained for PDC-5, two other modifications of this β-lactamase were selected by ceftazidime combined to avibactam. First, deletion of 21 nucleotides (^{711}ACGGGTCGGTCCCGGCCCGCT^{731}), leading to a deletion of seven amino acids in PDC-5 (^{238}RVGPGPL^{244}), was obtained in three out of four independent selection experiments (mean frequency of 3×10^{-10}). Second, a guanine to adenine transition at position 739, resulting in the $E^{247}K$ substitution, occurred in a

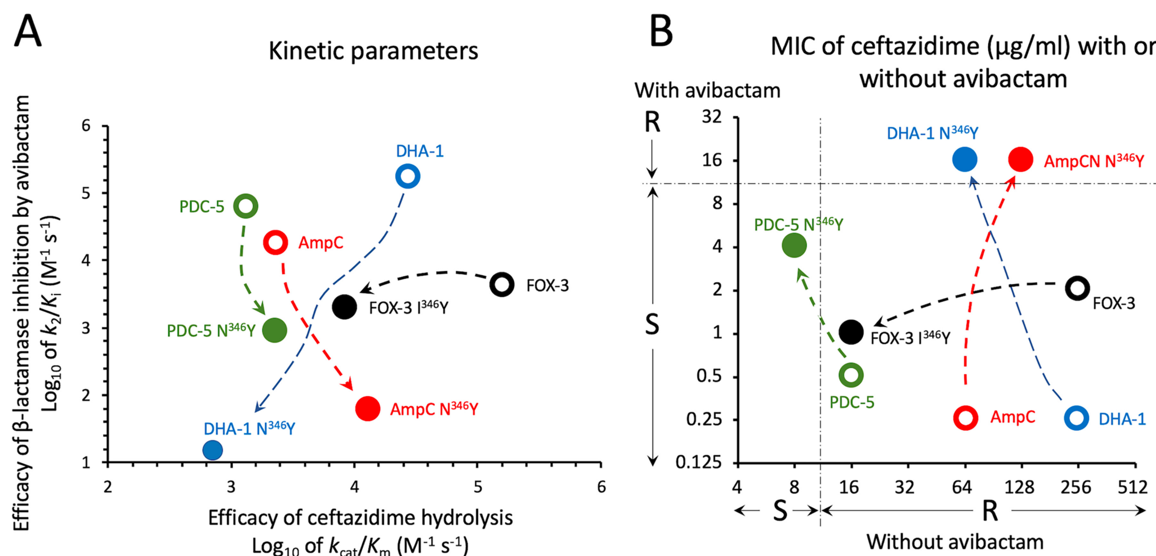

FIG 1 Impact of critical amino acid substitutions on the kinetic parameters for ceftazidime hydrolysis and β-lactamase inhibition by avibactam (A) and consequences of these modifications on the MICs of ceftazidime alone or in combination with avibactam (B). For AmpC$_{cloacae}$ (AmpC), a modest increase in the efficacy of ceftazidime hydrolysis results in a modest increase in the MIC of ceftazidime. In combination with a large decrease in the efficacy of avibactam, this leads to resistance to the ceftazidime-avibactam combination (MIC = 16 μg/ml). The N^{346}I substitution in DHA-1 is associated with the same level of resistance to the combination, as a reduction in the efficacy of ceftazidime hydrolysis is compensated by a very large decrease in the efficacy of avibactam. For PDC-5, the reduction in the efficacy of avibactam is relatively modest. This leads to an increase in the level of resistance to ceftazidime-avibactam but resistance to the combination is not achieved, despite a relatively low efficacy of ceftazidime hydrolysis. For Fox-3, the I^{346}Y substitution impairs the efficacy of ceftazidime hydrolysis, leading to a large decrease in the ceftazidime MIC. However, strains producing FOX-3 and FOX-3 I^{346}Y remain susceptible to the ceftazidime-avibactam combination since the substitution has only a moderate impact on the efficacy of avibactam. S, I, and R, susceptible, intermediary, and resistant according to EUCAST breakpoint values for *P. aeruginosa*.

single selection experiment (frequency of 5×10^{-10}). Both modifications occurred in the Ω loop of the PDC-5 *P. aeruginosa* cAmpC cephalosporinase (Fig. S1).

For DHA-1 and FOX-3, no mutant was recovered from three independent selection experiments with combinations of ceftazidime and avibactam (a frequency of $<10^{-10}$). The absence of mutants with the I^{346}Y substitution in FOX-3 was expected since the plasmids encoding FOX-3 I^{346}Y and wild-type FOX-3 conferred similar levels of resistance to ceftazidime in the presence of avibactam (MIC = 2 μg/ml versus 1 μg/ml, respectively; Table 1). In contrast, the mutation leading to the N^{346}Y substitution in DHA-1 produced a large increase in the MIC of ceftazidime in the presence of avibactam (from 0.25 μg/ml to 16 μg/ml; Table 1). However, selection of the corresponding mutation was not obtained.

Role of Asn^{346} in avibactam binding. Crystal structures have revealed that the carboxamide of Asn^{346} is in hydrogen interaction with the avibactam sulfonate (13). According to these structural data, loss of this interaction, possibly reinforced by steric hindrance due to the bulky Tyr side chain (13), is likely to account for the large decreases in carbamoylation efficacy caused by the N^{346}Y substitution in AmpC$_{cloacae}$, PDC-5, and DHA-1. Interestingly, FOX-3 harbors an isoleucine at position 346, a residue that cannot form any significant hydrogen interaction with the avibactam sulfonate but is similar to Asn with respect to size. For FOX-3, the carbamoylation efficacy was lower than for the N^{346}-containing AmpC$_{cloacae}$, PDC-5, and DHA-1 cephalosporinases (Table 3), possibly reflecting the absence of the carboxamide-Asn^{346} hydrogen interaction. Introduction of I^{346}Y in FOX-3 was associated with a moderate (2-fold) decrease in the carbamoylation efficacy, indicating that introduction of a Tyr residue at position 346 of FOX-3 was not associated with any adverse effect involving steric hindrance.

Conclusion. Here, we show that introduction of the N^{346}Y substitution in the AmpC$_{cloacae}$, PDC-5, and DHA-1 cephalosporinases leads to drastic reductions in the efficacy of β-lactamase inactivation by avibactam but largely preserves the hydrolysis efficacy of ceftazidime (Fig. 1A). These two features are essential for the acquisition of

resistance to the ceftazidime-avibactam combination, as shown by a graphical analysis of the consequences of changes in kinetics parameters on the expression of resistance (Fig. 1B). Since $AmpC_{cloacae}$, PDC-5, and DHA-1 display substantial sequence diversity, our results suggest that loss of hydrogen interaction between Asn^{346} and the avibactam sulfonate could be a common mechanism of acquisition of resistance to the ceftazidime-avibactam combination by mutational alteration of the corresponding codon. However, the low frequency of this mutational event may limit its emergence under treatment. The relevance of Asn^{346} for acquisition of resistance to the combination arises from its critical role for efficacious cephalosporinase inactivation by avibactam but not for hydrolysis of ceftazidime and, to a certain extent, of other β-lactams. This appears to be a remarkable property of Asn^{346} in AmpC cephalosporinases, as analyses of class A β-lactamases have revealed that amino acid substitutions reducing carbamoylation efficacy often lead to large decreases in hydrolysis efficacy and, in certain instances, to hyper-susceptibility to certain β-lactams (15, 16).

MATERIALS AND METHODS

Construction of recombinant plasmids. The four highly divergent class C β-lactamases evaluated in this study included cAmpC from *E. cloacae* P99 (designated $AmpC_{cloacae}$), cAmpC PDC-5 from *P. aeruginosa* ATCC 27853, and the pAmpCs DHA-1 and FOX-3, which derive from chromosomally encoded enzymes from *Morganella morganii* and *Aeromonas* spp. For phenotypic analyses, the genes were amplified using primers depicted in Table S1 and cloned under the control of the isopropyl β-D-1-thiogalactopyranoside (IPTG)-inducible promoter of the plasmid vector pTRC-99k (16). Recombinant plasmids were introduced by electroporation into *E. coli* TOP10 with selection for resistance to kanamycin (50 μg/ml) conveyed by the vector pTRC-99k. For protein purification, gene fragments encoding soluble forms of the enzymes were amplified (see Table S1 for the sequence of the primers) and cloned under the control of the IPTG-inducible promoter of the plasmid vector pET-TEV (16). Site-directed mutagenesis was performed using the mutagenic primers depicted in Table S1, except for $AmpC_{cloacae}$ that was obtained by selection of a spontaneous mutant of pTRC-99kΩ$ampC_{cloacae}$ on agar containing ceftazidime (4 μg/ml) and avibactam (4 μg/ml). The sequences of the cloned genes were verified by double-strand Sanger sequencing (Eurofins Genomics).

MIC determinations. MICs of amoxicillin, amoxicillin in the presence of clavulanate (2 μg/ml), piperacillin, cephalothin, cefamandole, cefoxitin, cefotaxime, ceftazidime, ceftazidime in the presence of avibactam (4 μg/ml; Sigma-Aldrich), cefepime, ertapenem, meropenem, and aztreonam were determined by the microdilution method in Mueller-Hinton broth (MHB) (Difco), according to the Clinical and Laboratory Standards Institute (CLSI) recommendations (17). The inoculum was prepared by growing bacteria in MHB containing IPTG (0.5 mM) for induction of the β-lactamase genes and kanamycin (50 μg/ml) for plasmid maintenance. IPTG but not kanamycin was added to MHB in the 96-well plates used for MIC determination. The experiments were performed in triplicate and the data are the medians of three experiments. Breakpoints for the susceptible, intermediary, and resistant phenotypes were those recommended by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (version 9.0) (http://www.eucast.org/clinical_breakpoints/).

Purification of β-lactamases. Production and purification of AmpC β-lactamases was performed as previously described (16). Briefly, fragments of β-lactamase genes, including soluble fragments of $AmpC_{cloacae}$ (residues 21 to 381), PDC-5 (residues 27 to 397), DHA-1 (residues 25 to 37), and FOX-3 (residues 24 to 382) were translationally fused to the sequence of the pET-TEV vector coding for an N-terminal 6×His tag followed by a cleavage site for the TEV protease (GSSHHHHHHSSGENLYFQG). The β-lactamases were produced in *E. coli* BL21(DE3) and purified from clarified lysates by affinity chromatography (Ni-nitrilotriacetic acid agarose; Sigma-Aldrich) and size-exclusion chromatography in 25 mM Tris-HCl (pH 7.5) containing 300 mM NaCl (Superdex 200 HL26/60; Amersham Pharmacia Biotech). The β-lactamases were stored at −20°C in the same buffer. Protein concentration was determined by the Bio-Rad protein assay using bovine serum albumin as a standard.

Kinetic analyses. Hydrolysis kinetics were performed at 20°C in 2-(*N*-morpholino)ethanesulfonic acid (MES; 100 mM, pH 6.4). Steady-state kinetic parameters for the hydrolysis of nitrocefin, amoxicillin, cephalothin, cefamandole, cefoxitin, cefotaxime, ceftazidime, cefepime, and aztreonam (k_{cat}, K_m, and k_{cat}/K_m) were determined by measuring initial enzymatic reaction rates in a Cary 300 spectrophotometer (Agilent) (15, 18). Table S2 reports the wavelength and molar extinction coefficient that were used for each antibiotic. Inhibition parameters (k_2/K_i and k_{-2}) were determined by measuring hydrolysis of nitrocefin (100 μM) in the presence of various concentrations of avibactam, as previously reported (8, 15). Inactivation of AmpC β-lactamases by avibactam was considered to proceed according to the two-step reversible reaction mechanism depicted in Fig. 2, in which E and I represent the β-lactamase and avibactam, respectively, and EI and EI* the noncovalent and covalent β-lactamase-avibactam adducts, respectively.

Equation 1 was fitted to data to determine the value of the rate constant (k_{obs}) for various concentrations of avibactam. In equation 1, [P] represents the concentration of hydrolyzed nitrocefin, v_i the initial velocity, v_s the steady-state velocity, and t the time.

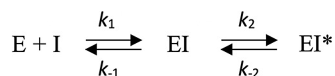

FIG 2 Two-step reversible reaction mechanism. E and I represent the β-lactamase and avibactam, respectively, and EI and EI* the noncovalent and covalent β-lactamase-avibactam adducts, respectively.

$$[P] = v_s t + \frac{(v_i - v_s)(1 - e^{-k_{obs}t})}{k_{obs}} \quad (1)$$

The carbamoylation efficacy (k_2/K_i) was determined by plotting the values of k_{obs} as a function of the concentration of avibactam and fitting equation 2 to the data, in which [S] represents the initial concentration of nitrocefin, K_m the Michaelis constant for hydrolysis of nitrocefin by the β-lactamase, and [I] the concentration of avibactam.

$$k_{obs} = k_{-2} + \frac{k_2}{K_i} \frac{[I]}{1 + \frac{[S]}{K_m}} \quad (2)$$

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.4 MB.

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We declare no conflicts of interest.

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