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In Vivo Evolution of CMY-2 to CMY-33 β-Lactamase in *Escherichia coli* Sequence Type 131: Characterization of an Acquired Extended-Spectrum AmpC Conferring Resistance to Cefepime

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Cefepime is frequently prescribed to treat infections caused by AmpC-producing Gram-negative bacteria. CMY-2 is the most common plasmid-mediated AmpC (pAmpC) β-lactamase. Unfortunately, CMY variants conferring enhanced cefepime resistance have been reported. Here, we describe the evolution of CMY-2 to an extended-spectrum AmpC (ESAC) in clonally identical *Escherichia coli* isolates obtained from a patient. The CMY-2-producing *E. coli* isolate (CMY-2-*Ec*) was isolated from a wound. Thirty days later, one CMY-33-producing *E. coli* isolate (CMY-33-*Ec*) was detected in a bronchoalveolar lavage fluid sample. Two weeks before the isolation of CMY-33-*Ec*, the patient received cefepime. CMY-33-*Ec* and CMY-2-*Ec* were identical by repetitive extragenic palindromic-PCR (rep-PCR), being of hyperepidemic sequence type 131 (ST131) but showing different β-lactam MICs (e.g., cefepime MIC, 16 and ≤0.5 μg/ml for CMY-33-*Ec* and CMY-2-*Ec*, respectively). Identical CMY-2-*Ec* isolates were also found in a rectal swab. CMY-33 differs from CMY-2 by a Leu293-Ala294 deletion. Expressed in *E. coli* strain DH10B, both CMYs conferred resistance to ceftazidime (≥256 μg/ml), but the cefepime MICs were higher for CMY-33 than CMY-2 (8 versus 0.25 μg/ml, respectively). The k_{cat}/K_m or inhibitor complex inactivation (k_{inact})/$K_{i\ app}$ ($\mu M^{-1}\ s^{-1}$) indicated that CMY-33 possesses an extended-spectrum β-lactamase (ESBL)-like spectrum compared to that of CMY-2 (e.g., cefoxitin, 0.2 versus 0.4; ceftazidime, 0.2 versus not measurable; cefepime, 0.2 versus not measurable; and tazobactam, 0.0018 versus 0.0009, respectively). Using molecular modeling, we show that a widened active site (~4-Å shift) may play a significant role in enhancing cefepime hydrolysis. This is the first *in vivo* demonstration of a pAmpC that under cephalosporin treatment expands its substrate spectrum, resembling an ESBL. The prevalence of CMY-2-*Ec* isolates is rapidly increasing worldwide; therefore, awareness that cefepime treatment may select for resistant isolates is critical.

Enterobacteriaceae can manifest resistance to third-generation cephalosporins as a result of the production of extended-spectrum β-lactamases (ESBLs), chromosomal AmpC (cAmpCs), or plasmid-mediated AmpCs (pAmpCs) (1, 2). In general, ESBLs are inhibited by the commercially available β-lactamase inhibitors but hydrolyze well the fourth-generation cephalosporin cefepime (FEP). On the other hand, AmpCs are not inhibited by inhibitors and do not hydrolyze FEP (1, 3–5). Therefore, FEP is suggested for the treatment of infections caused by AmpC producers (6–8).

In the past, AmpC variants with enhanced hydrolytic efficiency against FEP were sporadically reported in *Enterobacter* spp. (8–11), *Serratia marcescens* (12), and *Escherichia coli* (13–17). These chromosomal extended-spectrum AmpC β-lactamases (cESACs) possess specific amino acid insertions, deletions, duplications, or substitutions in the H-10 helix (also named the R2-loop) that allow better accommodation and hydrolysis of FEP in the serine active site (1, 4, 11, 14). More recently, plasmid-mediated ESACs (pESACs) derived from the most frequently detected pAmpC (CMY-2) were also identified (1). In particular, we previously reported the phenotypic characteristics of CMY-33- and CMY-44-producing *E. coli* isolates (18); further pESACs were described in *E. coli* (CMY-10 and CMY-94) and *Klebsiella pneumoniae* (CMY-19) (19–21). However, for these pESACs, structural information regarding the hydrolytic performance of different β-lactam substrates is still needed (20).

With regard to the possible factors leading to the generation of ESACs, treatment with β-lactams (especially third-generation cephalosporins and FEP) may permit the evolution of specific changes in the H-10 helix of the AmpC β-lactamase. However, in

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only three cases involving infections due to *Enterobacter* spp., the initial FEP-susceptible (FEP^s) isolates were available for comparison with subsequent isolates expressing the cESAC variants after FEP treatment (8, 9, 11). To our knowledge, similar clinical cases have not yet been reported for strains producing pESAC enzymes. Here, we report such a case to illustrate the dynamic nature of this process.

MATERIALS AND METHODS

Clinical case. A 71-year-old man underwent a radical cystectomy due to carcinoma of the urinary bladder. On day 14, an *E. coli* isolate (*Ec*-1) resistant to ceftriaxone (CRO) but susceptible to FEP was isolated in a swab taken from the surgical wound. One week later, the patient developed pneumonia that was empirically treated with CRO. After 2 days, the therapy was switched to FEP and continued for 6 days. Two weeks later, an *E. coli* isolate (*Ec*-2) resistant to both CRO and FEP was detected in a bronchoalveolar lavage fluid sample. A rectal swab also revealed that the patient was colonized with third-generation cephalosporin-resistant *E. coli* isolates; five colonies (named *Ec*-A to *Ec*-E) were randomly chosen from the selective plates for further investigations (see Text S1 in the supplemental material for a full description of the clinical case).

Phenotypic tests. Species identification was achieved using matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) (Bruker). The rectal swab was enriched overnight in lysogenic broth (LB) containing cefuroxime (3 μg/ml) and then plated on BLSE, chromID ESBL (bioMérieux), and Supercarba selective agars (22); a disk of FEP was also placed at the center of these plates to detect FEP-resistant (FEP^r) strains. MICs were obtained in cation-adjusted Mueller-Hinton II (MHII) broth (BBL) using microdilution ESB1F and GNX2F panels (Trek Diagnostics Systems) and interpreted according to the EUCAST criteria (23). The MICs for FEP were also measured using the Etest (bioMérieux) on MHII plates with (200 μg/ml) and without cloxacillin (Sigma) (1).

Characterization of resistance genes and clonality. The CT103XL microarray (Check-Points) was used to screen for *bla* genes. PCR and DNA sequencing for acquired *bla*, IS*Ecp1*, IS*CR1*, bla_{cAmpC} and its upstream region, and *ompF* and *ompC* porin genes were performed (18, 24–27). The results were compared to *E. coli* K-12 patterns (GenBank accession no. U00096). Genetic relatedness was studied using repetitive extragenic palindromic-PCR (rep-PCR) (28) and multilocus sequence typing (MLST) (http://mlst.warwick.ac.uk/mlst/dbs/Ecoli). Polymorphisms in the *fimH* and *fimA* genes of the type 1 fimbriae were also analyzed (29, 30).

Plasmid analysis. Plasmid replicon typing was performed using the PBRT kit (Diatheva) (31). Plasmid extraction was achieved using the PureYield plasmid midiprep system (Promega). Conjugation was performed using NEB 5-α competent *E. coli* (New England BioLabs), and cells were selected on LB plates containing ampicillin (20 μg/ml) (Sigma) (32). The plasmid DNA of the transconjugants was restricted with EcoRV and PstI enzymes (Bio-Concept) (33).

Cloning of bla_{CMY} genes, purification of proteins, and kinetic experiments. bla_{CMY-2} and bla_{CMY-33} were cloned into pBC SK(−) and electroporated into *E. coli* strain DH10B cells (18). The MICs were obtained with the agar dilution method (34). The purification of CMY-33 was performed as previously done for CMY-2 (25, 35).

Steady-state kinetic analyses were performed on an Agilent 8453 spectrophotometer; the maximum rate of metabolism (V_{max}) and K_m for nitrocefin (NCF), cephalothin, and cefoxitin (FOX) were obtained using Origin 7.5 (OriginLab) (25). For the "poor substrates" (k_{cat}, $<2\ s^{-1}$ or not measurable), cefotaxime (CTX), ceftazidime (CAZ), FEP, and aztreonam (ATM), the apparent K_m was obtained as a competitive inhibition constant ($K_{i\ app}$) in the presence of NCF (36). For sulbactam (SUL) and tazobactam (TAZ), the inhibitor complex inactivation (k_{inact}) in the presence of NCF was measured and the $K_{i\ app}$ determined (25).

Molecular modeling and docking of cefepime. The CMY-33 model was generated by the SWISS-MODEL server (http://swissmodel.expasy.org) using the deposited CMY-2 (PDB code 1zc2). The CMY-33 model was optimized by energy minimization (Discovery Studio 3.1 software; Accelrys) and using steepest descent and conjugate gradient algorithms to reach the minimum convergence (0.02 kcal/mol · Å). The protein was immersed in a water box (7 Å from any face), and the solvation model was used with periodic boundary conditions. The force field parameters of CHARMM were used for minimization, and the particle mesh Ewald method addressed long-range electrostatics. The bonds that involved hydrogen atoms were constrained with the SHAKE algorithm. CMY-33 model and CMY-2 were used for constructing the acylation complexes of both β-lactamases with FEP (25).

RESULTS AND DISCUSSION

In this case, the two *E. coli* isolates were resistant to third-generation cephalosporins but susceptible to piperacillin-tazobactam, carbapenems, and non-β-lactam antibiotics. However, while *Ec*-1 was susceptible to FEP (MIC, ≤0.5 μg/ml), *Ec*-2 was resistant to the drug, and its MIC was significantly reduced (from 16 to 1 μg/ml) in the presence of cloxacillin (Table 1). Since this behavior was suggestive of ESAC production (1), we characterized both *E. coli* isolates.

Molecular characterization. *Ec*-1 possessed the bla_{CMY-2} gene (CMY-2-*Ec*), whereas *Ec*-2 harbored the bla_{CMY-33} gene (CMY-33-*Ec*); both bla_{CMY} genes were located downstream of a truncated IS*Ecp1* (ΔIS*Ecp*1-3′), an element commonly associated with this group of genes (24, 37, 38). Other acquired *bla* genes were not detected. *Ec*-1 and *Ec*-2 also possessed (i) an identical promoter region of the bla_{cAmpC} with specific mutations (+81A, −28A, −73T, and −118A) leading to overproduction of the enzymes (15, 24); (ii) a cAmpC protein with Ala8Thr, Lys40Arg, Gln191Lys, Pro209Ser, Thr263Ile, Ser298Ile, Ala316Pro, Asp367Thr, and Ala375Thr; (iii) OmpF with several substitutions (identical to GenBank accession no. HG941718); and (iv) disrupted OmpC (identical to GenBank accession no. HG941718).

Both *Ec*-1 and *Ec*-2 carried plasmid replicon types I1, FIB, and FII. Each NEB 5-α transconjugant possessing the corresponding bla_{CMY} (along with the replicon type IncI1) displayed phenotypic patterns consistent with the specific CMY produced (Table 1). Moreover, plasmid extracts from these transconjugants generated identical restriction patterns (see Fig. S1 in the supplemental material). Thus, we concluded that both bla_{CMY} variants were carried in the same IncI1 plasmid.

Remarkably, all five strains obtained from the rectal swab (*Ec*-A to *Ec*-E) were CMY-2-*Ec*, whereas CMY-33-*Ec* was not detected. As 80% of FEP is excreted intact in urine (3), selective pressure upon the intestinal flora may be limited, which might explain why we did not find CMY-33-*Ec* strains in the rectal specimen, even after the administration of FEP.

The prevalence of CMY-2-*Ec* is rapidly increasing worldwide in human, food animal, and food chain settings, as the bla_{CMY} genes are usually carried by epidemic conjugative plasmids (24, 38–41). In Switzerland, the rate of clinical specimens with CMY-2-*Ec* among those resistant to third-generation cephalosporins was 12.5% in 2011 (24). CMY-2-*Ec* pathogens can also be responsible for intestinal colonization in healthy people (42). Therefore, since Switzerland is among the major consumers of FEP in Europe, this situation may create in the near future the "perfect storm" to select more isolates producing pESAC variants of CMY-2 (43).

TABLE 1 Phenotypic characterization of clinical isolates, transconjugants, and transformants producing the CMY-2 or CMY-33 plasmid-mediated AmpC β-lactamases

Antibiotic	MIC (μg/ml) for *E. coli*[a]: *Ec*-1 isolate from wound (bla_{CMY-2})	*Ec*-2 isolate from BAL fluid (bla_{CMY-33})	NEB5α with IncI1 plasmid of *Ec*-1 (bla_{CMY-2})	NEB5α with IncI1 plasmid of *Ec*-2 (bla_{CMY-33})	DH10B pBC SK(−)/bla_{CMY-2}[b]	DH10B pBC SK(−)/bla_{CMY-33}[b]	DH10B[b]
Ampicillin	≥32, R	≥32, R	≥32, R	≥32, R	≥512	128	4
Ampicillin-sulbactam	NT	NT	NT	NT	32	0.125	4
Piperacillin	NT	NT	NT	NT	≥512	32	2
Piperacillin-tazobactam	≤2, S	8, S	≤2, S	16, S	4	8	2
Ticarcillin-clavulanate	≤8, S	≥256, R	≤8, S	≥256, R	NT	NT	NT
Cephalothin	≥32, NA	≥32, NA	≥32, NA	≥32, NA	≥512	128	4
Cefoxitin	64, NA	32, NA	64, NA	≥128, NA	64	16	4
Ceftriaxone	8, R	128, R	16, R	≥256, R	NT	NT	NT
Cefotaxime	4, R	64, R	16, R	64, R	8	16	≤0.06
Cefotaxime-clavulanate	4, NA	32, NA	8, NA	32, NA	NT	NT	
Ceftazidime	8, R	≥256, R	32, R	≥256, R	256	≥512	≤0.06
Ceftazidime-clavulanate	4, NA	≥256, NA	16, NA	≥256, NA	NT	NT	NT
Cefpodoxime	≥64, R	≥64, R	≥64, R	≥64, R	NT	NT	NT
Cefepime	≤0.5, S (0.125/≤0.016)	16, R (16/1)	≤0.5, S	≥32, R	0.25	8	≤0.06
Aztreonam	4, I	≥32, R	8, I	≥32, R	8	16	≤0.06
Imipenem	≤0.25, S	≤0.25, S	≤0.25, S	≤0.25, S	≤0.5	≤0.5	≤0.5
Meropenem	≤0.5, S	≤0.5, S	≤0.5, S	≤0.5, S	≤0.06	≤0.06	≤0.06
Ertapenem	≤0.125, S	≤0.125, S	≤0.125, S	≤0.125, S	NT	NT	NT
Gentamicin	≤0.5, S	≤0.5, S	≤0.5, S	≤0.5, S	NT	NT	NT
Tobramycin	≤0.5, S	≤0.5, S	≤0.5, S	≤0.5, S	NT	NT	NT
Amikacin	≤2, S	≤2, S	≤2, S	≤2, S	NT	NT	NT
Ciprofloxacin	≤0.125, S	≤0.125, S	≤0.125, S	≤0.125, S	NT	NT	NT
Doxycycline	≤1, NA	≤1, NA	≤1, NA	≤1, NA	NT	NT	NT
Tigecycline	≤0.125, S	≤0.125, S	≤0.125, S	≤0.125, S	NT	NT	NT
Co-trimoxazole	≤0.25, S	≤0.25, S	≤0.25, S	≤0.25, S	NT	NT	NT
Colistin	≤0.125, S	≤0.125, S	≤0.125, S	≤0.125, S	NT	NT	NT

[a] BAL, bronchoalveolar lavage; R, resistant; NT, not tested; S, susceptible; NA, not available; I, intermediate. The MICs were obtained with microdilution Trek panels and interpreted according to the EUCAST criteria (23). The tests were repeated three times. Values in parentheses indicate MICs obtained with the Etest method on plates without/with cloxacillin (200 μg/ml).

[b] MICs for *E. coli* DH10B transformants containing pBC SK(−) were achieved with the agar dilution method. Consistent results were also previously obtained with the Etest method (18).

Genetic relatedness of *E. coli* isolates. *Ec*-1, *Ec*-2, and the five CMY-2-*Ec* strains from the rectal swab had identical rep-PCR profiles (see Fig. S2 in the supplemental material). Moreover, all strains were of ST131 and possessed the *fimH22*/*fimA7* type (30). Therefore, under selective pressure with CRO and mainly FEP, CMY-33 evolved from CMY-2 in the same *E. coli* host (the strain was colonizing the intestinal tract of the patient). *E. coli* ST131 is a hyperepidemic clone that has driven the worldwide spread of clinically important ESBLs (e.g., CTX-M-15) in both hospital and community settings (37). Consequently, the finding of a ST131 CMY-2-*Ec* (FEP^s) that can evolve to those producing CMY-33 (FEP^r) is a matter of serious clinical concern.

Biochemical characterization of CMY-2 and CMY-33. Both CMYs expressed in *E. coli* DH10B cells conferred resistance to third-generation cephalosporins. However, cells producing CMY-33 had a phenotype resembling that of an ESBL producer because they had higher MICs for FEP but lower MICs for FOX and ampicillin-SUL than those producing CMY-2 (Table 1).

CMY-33 (GenBank accession no. EU496816) differs from CMY-2 (GenBank accession no. X91840) by a Leu293-Ala294 deletion in the H-10 helix. Electrospray ionization-mass spectrom-

TABLE 2 Steady-state kinetic parameters of purified CMY-2 and CMY-33 plasmid-mediated AmpC β-lactamases[a]

β-Lactam[b]	K_m or $K_{i\ app}$ (μM)[c]		k_{cat} or k_{inact} for inhibitors (s^{-1})		k_{cat}/K_m or $k_{inact}/K_{i\ app}$ for inhibitors ($\mu M^{-1}\ s^{-1}$)	
	CMY-2	CMY-33	CMY-2	CMY-33	CMY-2	CMY-33
NCF	11.2	3.4	534.8	3.1	47.6	0.9
CEF	7.8	1.7	140.0	0.8	17.9	0.5
FOX	17.9	22.9	6.8	4.7	0.4	0.2
CAZ[c]	NM[d]	20.0	NM	3.2	NM	0.2
CTX[c]	1.8	3.9	NM	3.2	NM	0.8
FEP[c]	108.1	18.3	NM	3.0	NM	0.2
ATM[c]	0.12	1.5	NM	4.2	NM	2.8
SUL	101.3	35.0	0.025	0.025	0.0002	0.0007
TAZ	50.0	16.6	0.045	0.028	0.0009	0.0018

[a] Experimental error was ±10%.

[b] NCF, nitrocefin; CEF, cephalothin; FOX, cefoxitin; CAZ, ceftazidime; CTX, cefotaxime; FEP, cefepime; ATM, aztreonam; SUL, sulbactam; TAZ, tazobactam.

[c] $K_{i\ app}$ measured in the presence of NCF. The data regarding CMY-2 are from Endimiani et al. (25).

[d] NM, not measurable.

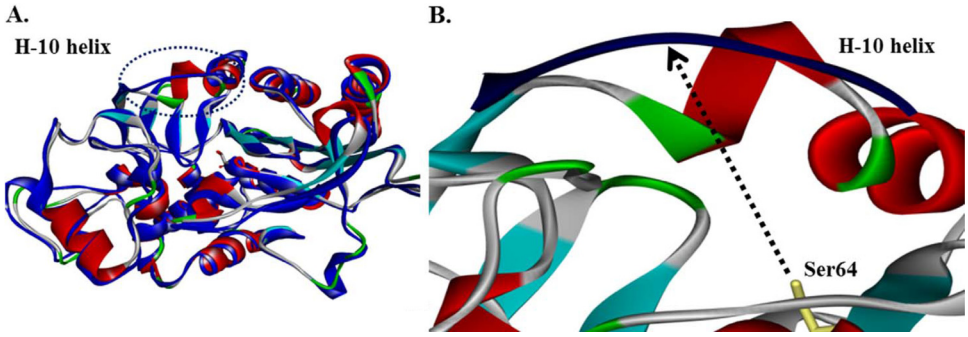

FIG 1 (A) CMY-2 and CMY-33 model (blue) molecular structure superimposition. (B) Magnification of the H-10 helix in CMY-2 and CMY-33. The deletion (Leu293-Ala294) in the CMY-33 enzyme increases the distance from Ser64 to the H-10 helix by ~4 Å (from 7.8 to 11.6 Å), changing the shape, size, and possibly the flexibility of the active site.

etry (ESI-MS) analysis indicated that the molecular mass of CMY-33 (39,671 Da) is less than that of CMY-2 (39,854 Da) due to the Leu293-Ala294 deletion (see Fig. S3 in the supplemental material). Consistent with the MIC determinations for the *E. coli* DH10B transformants, the steady-state kinetic parameters revealed that CMY-33 possesses significantly less catalytic efficiency (k_{cat}/K_m) for NCF and narrow-spectrum cephalosporins (cephalothin and FOX), but it is more inhibited (due to lower $K_{i\ app}$ values of approximately 30 to 35%) by SUL and TAZ than by CMY-2. On the other hand, CMY-33 had improved hydrolytic activity against ATM, CTX, CAZ, and FEP, substrates for which detectable hydrolysis was not recorded for CMY-2 (Table 2) (25). Overall, these kinetic parameters support again that CMY-33 is a pESAC with a phenotype very reminiscent of classic ESBLs (5).

Molecular modeling and acylation complexes with FEP. In our model, the deletion in the H-10 helix increases the distance from Ser64 to the H-10 helix by about 4 Å, altering the conformation, size, and possibly the flexibility of the active site of CMY-33 (Fig. 1). This may account for the differences in kinetic parameters. Previous hypotheses were raised stating that disruptions in the H-10 helix of cAmpCs (involving positions 282 to 296) are responsible for the ESBL phenotype. In particular, increased resistance to FEP, CAZ, CTX, and ATM was constantly observed in all previous cESACs (4, 11, 14); however, this is the first time that this phenomenon has been documented for a pESAC.

Our model suggests that the reason for increased hydrolysis of these substrates in CMY-33 is the ready formation of a Michaelis complex (k_{cat} is greater, K_m is lower). In particular, docking of FEP indicated that at least two different conformations are possible in the widened active site of CMY-33 rather than only one in CMY-2 (Fig. 2).

Conclusions. We describe here the first clinical case in which a pAmpC (CMY-2) evolved to a pESAC (CMY-33) under FEP treatment. In particular, a ST131 FEP^s CMY-2-*Ec* isolate rapidly became FEP^r due to a double amino acid deletion in the H-10 helix of the protein. CMY-33 is an atypical pAmpC that mimics an ESBL (i.e., is relatively susceptible to standard inhibitors and FOX but resistant to oxyiminocephalosporins) and therefore classifiable as pESAC. Given that the prevalence of CMY-2-*Ec* isolates is rapidly increasing worldwide, one should be aware that the standard FEP treatment may select for resistant isolates *in vivo*.

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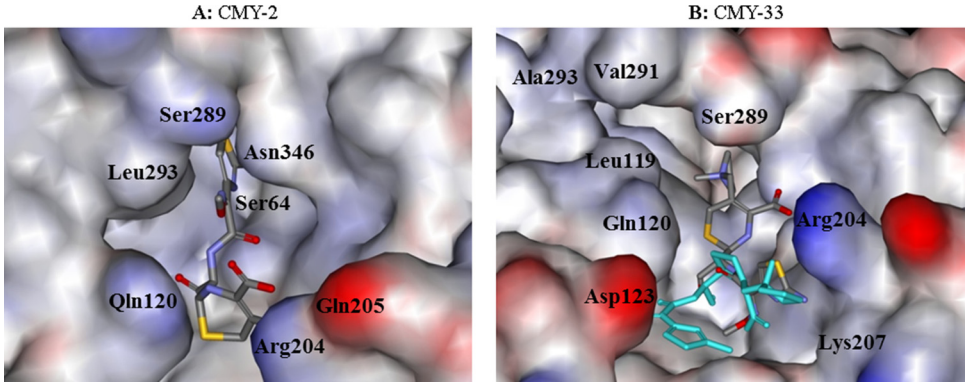

FIG 2 Molecular representation of the acyl-enzyme complex of CMY-2 (A) and CMY-33 (B) as Connolly surface, with FEP docked in the active site. The model suggests that the reason for increased drug hydrolysis is the ready formation of Michaelis complex (k_{cat} is greater, K_m is lower). In particular, two different possible conformations were seen in the widened active site of CMY-33, suggesting the structural basis for resistance in CMY-33 versus CMY-2.

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