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CTX-M-33 Is a CTX-M-15 Derivative Conferring Reduced Susceptibility to Carbapenems

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ABSTRACT CTX-M-type extended-spectrum β-lactamases (ESBLs) are widespread among *Enterobacterales* strains worldwide. The most common variant is CTX-M-15, which hydrolyzes ceftazidime at a high rate but spares carbapenems. Here, we identified CTX-M-33, a point mutation derivative of CTX-M-15 (Asp to Ser substitution at Ambler position 109) that exhibited low carbapenemase activity. β-Lactamase CTX-M-33 was identified in a *Klebsiella pneumoniae* isolate, belonging to sequence type 405 and lacking the outer membrane protein OmpK36, that was resistant to broad-spectrum cephalosporins and β-lactam/β-lactamase inhibitor combinations and displayed decreased susceptibility to carbapenems. Comparative hydrolytic activity assays showed that CTX-M-33 hydrolyzed ceftazidime at a lower level than CTX-M-15 but significantly hydrolyzed meropenem. In addition, CTX-M-33 showed higher mutant prevention concentration values and a wider mutant selection window in the presence of meropenem, in accordance with its observed hydrolytic properties. Here, we identified the very first CTX-M enzyme possessing weak carbapenemase activity, which may correspond to an emerging phenomenon, considering its possible evolution from the widespread ESBL CTX-M-15.

KEYWORDS CTX-M-33, CTX-M-15, ESBL, carbapenems, meropenem

The widespread diffusion and emergence of CTX-M-type extended-spectrum β-lactamases (ESBLs) as a source of resistance to broad-spectrum cephalosporins in Gram-negative organisms represent a phenomenon that was identified 20 years ago (1). In contrast to the older ESBLs of the TEM and SHV types, which were identified mostly in nosocomial *Enterobacterales* species such as *Klebsiella pneumoniae*, the CTX-M enzymes emerged in community settings, mostly in *Escherichia coli*, and then were found in many other enterobacterial species identified in hospital settings (2, 3). CTX-M-1 was the first identified CTX-M enzyme and exhibited a "cefotaximase" property, hydrolyzing cefotaxime at high rates but almost sparing ceftazidime (4). Many CTX-M derivatives have since been identified, such as CTX-M-15, which is a point mutation derivative of CTX-M-3 that was first identified in enterobacterial isolates from India (5) and possesses the ability to hydrolyze ceftazidime very efficiently (6). CTX-M-15 rapidly became the most widespread ESBL determinant worldwide (4). One of the consequences of the spread of CTX-M enzymes is the increased use of carbapenems for treatment of infections caused by those ESBL producers, ultimately leading to the selection of carbapenem-resistant bacteria, including carbapenemase producers.

The increasing prevalence of carbapenemase producers worldwide represents a major threat, with enzymes such as KPC, NDM, and OXA-48 being almost endemic in many geographical areas (7, 8). The acquisition of the latter resistance determinants is

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often superimposed on the spread of CTX-M-type ESBLs, as exemplified by the very high rate of isolates producing both types of enzymes. Nevertheless, acquired resistance or decreased susceptibility to carbapenems has never been shown to be related to the CTX-M enzymes gaining carbapenemase activity, in contrast to the rarely identified GES-type ESBLs, among which variants such as β-lactamases GES-2 and GES-5 exhibit significant carbapenemase activity (9).

We recently performed a retrospective study aiming to evaluate the occurrence of carbapenemase-producing *Enterobacterales* in a hospital in Lisbon, Portugal, during a 6-year period (10). An increase in the occurrence of carbapenemase-producing *Enterobacterales* over time was observed, with KPC-3 being the predominant carbapenemase but OXA-181 also being found to be emerging. Among the carbapenem-nonsusceptible isolates recovered in that study, a single *K. pneumoniae* isolate that was negative for the known carbapenemases was recovered. Our aim here was to decipher the molecular and biochemical bases of resistance in that isolate.

RESULTS

Susceptibility testing and molecular characterization. *K. pneumoniae* MAS9 was recovered from rectal screening of a patient hospitalized in Lisbon, Portugal, in 2015. No record of carbapenem-containing treatment was identified in the medical history of the patient. This isolate was resistant to several β-lactams, including broad-spectrum cephalosporins, and displayed decreased susceptibility to carbapenems (Table 1). It was also resistant to the β-lactam/β-lactamase inhibitor combinations amoxicillin-clavulanate and piperacillin-tazobactam, remaining susceptible to ceftazidime-avibactam and ceftolozane-tazobactam. In addition, this isolate was resistant to fluoroquinolones, kanamycin, tobramycin, co-trimoxazole, and tetracycline, remaining susceptible to amikacin, gentamicin, tigecycline, and colistin. A positive result was obtained with the Rapid ESBL NP test (with cefotaxime as the substrate [11]), whereas the Rapid Carba NP test (with imipenem as the substrate [12]) result remained negative. PCR-based tests identified a $bla_{CTX\text{-}M\text{-}like}$ gene, and sequencing of the corresponding amplicon identified the $bla_{CTX\text{-}M\text{-}33}$ gene. The corresponding enzyme, CTX-M-33, differed from CTX-M-15 by a single amino acid substitution, namely, a Ser residue replacing an Asn residue at position Ambler 109. In addition, the isolate possessed the $bla_{TEM\text{-}1}$ β-lactamase gene (data not shown).

Multilocus sequence typing (MLST) identified isolate MAS9 as belonging to sequence type 405 (ST405), and plasmid typing performed by PCR-based replicon typing (PBRT) (13) showed that the $bla_{CTX\text{-}M\text{-}33}$ gene was located on a plasmid belonging to incompatibility group IncFIB. PCR mapping of the surrounding sequences of the $bla_{CTX\text{-}M\text{-}33}$ gene also showed that, as usually observed for the $bla_{CTX\text{-}M\text{-}15}$ gene, the $bla_{CTX\text{-}M\text{-}33}$ gene was preceded by insertion sequence IS*Ecp1*, which was likely responsible for its acquisition on a plasmid and for its high-level expression (14, 15).

Cloning and expression of the ESBL gene. In order to compare the relative resistance patterns mediated by CTX-M-33 and CTX-M-15, MICs for the recombinant strains *E. coli* with pTOPO-CTX-M-33 and *E. coli* with pTOPO-CTX-M-15 were determined. Notably, the ceftazidime MIC was significantly lower for the CTX-M-33 producer, while paradoxically the ceftazidime-avibactam MIC was slightly higher for the latter (Table 1). In addition, the meropenem and imipenem MICs were slightly higher for the CTX-M-33 producer. This phenomenon was exacerbated when CTX-M-33 was produced in the porin-deficient *E. coli* HB4 strain, with significantly higher meropenem and imipenem MICs for the CTX-M-33 producer, compared to those for the CTX-M-15 producer in the same strain background (Table 1). In the wild-type *K. pneumoniae* CIP53153 strain, production of CTX-M-33 increased the carbapenem MICs only slightly (2-fold) (Table 1).

Notably, a peculiar phenomenon was observed when the ertapenem MICs for *E. coli* strain HB4 and its corresponding recombinants were measured. While MICs measured by Etest on Mueller-Hinton agar plates remained quite low and clear (neither double zones nor colonies growing in the inhibition zone), those measured by broth microdilution were found to be much higher (Table 1). Importantly, the ertapenem MIC observed by broth microdilution for the CTX-M-33 producer was $>$8-fold higher than

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TABLE 1 MICs of β-lactams for various *K. pneumoniae* and *E. coli* strains[a]

β-Lactam(s)[b]	**MIC (μg/ml)**										
	***K. pneumoniae* MAS9 (CTX-M-33)**	***E. coli* R1818 (CTX-M-15)**	***E. coli* TOP10 with CTX-M-33**	***E. coli* TOP10 with CTX-M-15**	***E. coli* TOP10**	***E. coli* HB4 with CTX-M-33**	***E. coli* HB4 with CTX-M-15**	***E. coli* HB4**	***K. pneumoniae* CIP53153 with CTX-M-33**	***K. pneumoniae* CIP53153 with CTX-M-15**	***K. pneumoniae* CIP53153**
Amoxicillin	>512	>512	>512	>512	4	>512	>512	16	>512	>512	512
Amoxicillin + CLA	>512	32	64	32	4	>512	32	16	256	256	4
Ticarcillin	>512	>512	>512	>512	8	>512	>512	16	>512	>512	256
Ticarcillin + CLA	>512	>512	256	512	1	>512	256	16	512	512	8
Piperacillin	>512	>512	>512	256	2	>512	>512	8	512	512	4
Piperacillin + TZB	>512	16	512	32	0.5	>512	>512	4	512	512	<0.25
Cefoxitin	64	8	8	8	2	64	64	64	2	2	1
Ceftazidime	16	32	32	512	0.25	32	>256	1	64	256	<0.25
Ceftazidime + AVI	0.5	0.125	0.75	0.5	<0.25	0.75	1	0.19	1	0.75	<0.25
Cefotaxime	>512	>512	>512	>512	0.06	>512	>512	0.5	256	256	<0.25
Cefepime	>512	128	512	512	0.5	>512	>512	0.5	64	128	<0.25
Ceftolozane	32	128	64	>512	0.25	64	>512	1	32	512	<0.25
Ceftolozane + TZB	8	1	8	32	<0.25	16	256	0.5	8	64	<0.25
Aztreonam	128	128	>256	>256	0.06	>256	>256	0.38	128	256	<0.25
Imipenem	4	0.19	1	0.5	0.06	2	0.25	0.12	1	0.5	0.5
Imipenem + CLA	1	0.12	0.25	0.25	0.06	1	0.125	0.12	0.25	0.25	0.25
Meropenem	6	0.032	0.25	0.12	0.03	8	1	0.25	0.19	0.06	0.02
Meropenem + CLA	4	<0.015	0.03	0.03	<0.015	2	0.125	0.12	0.03	0.03	<0.015
Meropenem + VAB	1.5	0.023	0.06	0.03	0.01	2	0.25	0.12	0.05	0.03	0.01
Ertapenem	32	0.032	0.25	0.25	0.015	>32/8[c]	4/0.38[c]	1/0.25[c]	0.5	0.25	<0.015
Ertapenem + CLA	32	<0.015	<0.015	<0.015	<0.015	16	0.5	0.5	0.03	<0.015	<0.015

[a]The strains included *K. pneumoniae* clinical isolate MAS9 producing CTX-M-33, *E. coli* clinical isolate R1818 producing CTX-M-15, *E. coli* TOP10 transformants producing CTX-M-15 and CTX-M-33, the *E. coli* TOP10 recipient strain, *E. coli* HB4 transformants producing CTX-M-15 and CTX-M-33, and the *E. coli* HB4 recipient strain.

[b]Clavulanic acid (CLA) was added at 2 μg/ml, tazobactam (TZB) was added at 4 μg/ml, avibactam (AVI) was added at 4 μg/ml, and vaborbactam (VAB) was added at 8 μg/ml.

[c]MIC data were determined by microdilution/Etest.

TABLE 2 Kinetic parameters of purified β-lactamases CTX-M-33 and CTX-M-15

	CTX-M-33[a]				CTX-M-15			
β-Lactam	K_{cat} (s^{-1})	K_m (μM)	K_{cat}/K_m (μM^{-1} s^{-1})	K_i (μM)	K_{cat} (s^{-1})	K_m (μM)	K_{cat}/K_m (μM^{-1} s^{-1})	K_i (μM)
Penicillin G	210	20	10		28	100	0.3	
Amoxicillin	215	160	1.35		15	90	0.15	
Ticarcillin	7.5	40	0.2		3.5	30	0.1	
Piperacillin	205	140	1.5		16	35	0.5	
Cephalothin	380	30	12.5		50	45	1	
Cefepime	70	100	0.7		20	480	0.03	
Cefotaxime	620	215	3		60	30	2.2	
Ceftazidime	0.35	1,500	0.0003		15	1,500	0.01	
Imipenem	<0.01	ND	ND	0.2	<0.01	ND	ND	0.6
Meropenem	0.13	90	0.0014	0.0024	<0.01	ND	ND	0.017
Ertapenem	<0.01	ND	ND	0.009	<0.01	ND	ND	0.007
Aztreonam	10	60	0.2		7	85	0.08	

[a]Standard deviations were below 15%. ND, not determined due to a low initial rate of hydrolysis; k_{cat}, catalytic efficiency; K_m, Michaelis constant; k_{cat}/K_m, specificity constant; K_i, relative; k_{off}/k_{on}, ratio.

that for the CTX-M-15 producer (>32 μg/ml versus 4 μg/ml) (Table 1), highlighting the original property of CTX-M-33.

Kinetic study. Measurements of kinetic parameters were performed using purified CTX-M-33 and CTX-M-15 enzymes. A significant rate of meropenem hydrolysis by CTX-M-33 was detected, whereas no hydrolysis could be detected with CTX-M-15 (Table 2). No hydrolysis could be detected with either enzyme with imipenem and ertapenem as the substrates, although increased imipenem and ertapenem MIC values were observed for the recombinant strains. Conversely, a 30-fold decreased rate of ceftazidime hydrolysis was measured with CTX-M-33, compared to CTX-M-15, an 8-fold decreased rate of amoxicillin hydrolysis, and a 3-fold decreased rate of piperacillin hydrolysis.

Sensitivity to inhibitors. The 50% inhibitory concentration (IC_{50}) values for CTX-M-33 versus CTX-M-15 showed significant decreases in the inhibitory capacities of the different β-lactamase inhibitors, as follows: clavulanic acid, 5-fold; tazobactam, 10-fold; non-β-lactam inhibitor avibactam, 5.5-fold (Table 3).

Reduced outer membrane permeability of isolate MAS9. Since the weak carbapenemase activity could not directly explain the elevated carbapenem MICs observed, particularly for ertapenem, it was suggested that isolate MAS9 might exhibit reduced outer membrane permeability. Outer membrane protein (OMP) profiles clearly showed that isolate MAS9 did not produce OmpK36, while OmpK35 and OmpK26 were present (see Fig. S1 in the supplemental material). Molecular investigations showed that isolate MAS9 possessed wild-type OmpK26 and OmpK35 porins, but the investigations identified a stop codon within the OmpK36 sequence (position 318 of the protein), confirming that the porin was deficient in that isolate (data not shown).

Impact on mutant prevention concentrations. In order to evaluate whether the differences observed in term of MIC values, which were found to be significant in *E. coli* HB4 but moderate in *E. coli* TOP10 and *K. pneumoniae* CIP53153, might play a role not only in susceptibility to carbapenems but also in selection of resistant mutants, mutant prevention concentration (MPC) determinations were performed using recombinant *E. coli* TOP10 strains. The study showed that the production of CTX-M-33 raised the MPC

TABLE 3 IC_{50} values for different β-lactamase inhibitors against CTX-M-33 and CTX-M-15

	IC_{50} (nM)[a]		
β-Lactamase	**Clavulanic acid**	**Tazobactam**	**Avibactam**
CTX-M-33	42 ± 0.7	20 ± 1.5	7.5 ± 1.3
CTX-M-15	8.5 ± 0.4	2 ± 0.2	1.5 ± 0.1

[a]IC_{50} represents the concentration of a drug that is required for 50% inhibition of the enzymatic activity. The indicated values correspond to the means of three independent measurements, and the standard deviations are indicated.

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TABLE 4 MPC values of two carbapenems for *E. coli* DH10B recombinant strains

	Imipenem			Meropenem		
β-Lactamase	MIC (μg/ml)	MPC (μg/ml)	MPC/MIC	MIC (μg/ml)	MPC (μg/ml)	MPC/MIC
CTX-M-33	0.75	18	24	0.125	0.667	5.3
CTX-M-15	0.25	6	24	0.064	0.167	2.6

values of imipenem and meropenem by 3- and 4-fold, respectively, compared to CTX-M-15 (Table 4). The mutant selection window, corresponding to the MPC/MIC ratio, was found to be 2 for meropenem (Table 4), while it remained the same for imipenem.

DISCUSSION

We showed here that decreased susceptibility to carbapenems may result from a combination of porin deficiency and production of a CTX-M enzyme, as suggested previously (16–18). We identified here a point mutation derivative of CTX-M-15 with some carbapenemase activity. We acknowledge that this carbapenemase activity remained relatively low; however, its impact was clearly seen with cloning in different strain backgrounds, particularly in a porin-deficient *E. coli* strain. In fact, by analyzing the OMP profile of isolate MAS9, we confirmed that this clinical isolate presented weaker permeability, since it lacked one OMP known to contribute significantly to the resistance to β-lactams when absent, as shown in different studies (19, 20).

This CTX-M-33 enzyme was identified previously in a single carbapenem-susceptible *E. coli* clinical isolate from Greece from 2002 (imipenem MIC of 1 μg/ml but meropenem MIC not determined) (21) and a single *Aeromonas dhakensis* isolate recovered from eel in South Korea in 2014 (no data on carbapenem susceptibility) (22). This report is another example among the rare ones of ESBLs that may evolve by point mutations toward carbapenemase activity, after the report of GES and SHV derivatives (23, 24). The carbapenemase activity reported here and occurring in a CTX-M-producing *K. pneumoniae* strain might be considered a serious cause for concern, due to global dissemination of the CTX-M producers.

It was demonstrated previously that pleiotropic effects may be encountered upon evolution of CTX-M β-lactamases (25–27). For instance, reduced sensitivity of CTX-M-1, CTX-M-2, or CTX-M-9-like enzymes to clavulanic acid or tazobactam (leading to increased resistance to amoxicillin-clavulanic acid or piperacillin-tazobactam combinations) was often accompanied by reduced hydrolytic activity toward broad-spectrum cephalosporins, leading to greater susceptibility to those antibiotics (27). Here, we showed that CTX-M-33, while gaining significant hydrolytic activity toward meropenem, conversely showed significant reduction of its sensitivity to inhibitors, including avibactam, compared to CTX-M-15. This finding highlights that the Asn109Ser substitution observed in CTX-M-33 led to decreased susceptibility to both carbapenems and β-lactam/β-lactamase inhibitor combinations. Conversely, this pleiotropic effect was observed in ceftazidime being less hydrolyzed by CTX-M-33 than by CTX-M-15.

Owing to the quite strong selective pressure with meropenem, which is used in Portuguese hospitals to treat infections caused by ESBL-producing *Enterobacterales* strains, and the spread of CTX-M-15 producers in this country (28), it is tempting to speculate that this carbapenem molecule might have been a driving force to select for the CTX-M-33 enzyme. Indeed, it is interesting to note that CTX-M-33 hydrolyzes meropenem, while it does not significantly hydrolyze imipenem and ertapenem.

By determining the K_i values of CTX-M-33 and CTX-M-15 for carbapenems, we showed that the affinity of CTX-M-33 for imipenem was higher, although no hydrolysis was detectable. Such effects of CTX-M enzymes lacking obvious carbapenemase activity against ertapenem or imipenem were already observed (16, 17), demonstrating that those enzymes actually possess excellent affinity for those substrates, to which they strongly bind.

K. pneumoniae MAS9, producing CTX-M-33, belonged to ST405, which is a background previously identified in different parts of the world. It was particularly identified

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in *K. pneumoniae* isolates from Spain that coproduced CTX-M-15 and OXA-244 or OXA-245 carbapenemases (29, 30). Considering the wide dissemination of this CTX-M-15-producing ST405 clone in Spain, this finding supports the hypothesis that this clone might also be widespread in the neighboring country Portugal, with which there are many patient exchanges.

Further work will evaluate the possible dissemination of the $bla_{CTX\text{-}M\text{-}33}$ gene, particularly in the Iberic peninsula. Such findings underscore the value of sequencing the entire resistance gene complement (rather than performing only PCR-based analysis) when performing epidemiological surveys, taking into account the fact that CTX-M-33 and CTX-M-15 differ by only a single amino acid substitution. It remains to be determined whether the selection of CTX-M-33 is just a preliminary step toward the selection of CTX-M enzymes with higher carbapenemase activity. Taking into account the large and community-located reservoir of CTX-M producers worldwide, such selection would significantly compromise the efficacy of all β-lactam-containing antibiotic regimens.

Finally, CTX-M-33 conferred decreased susceptibility to carbapenems and β-lactamase inhibitors. This phenomenon that we observed here with CTX-M-33 is the opposite of findings observed with several KPC-type enzymes, with some variants reported to confer resistance to ceftazidime-avibactam showing decreased ability to hydrolyze carbapenems (31–34).

The biochemical properties of CTX-M-33 suggest that its selection may be the result of (i) the use of carbapenems, leading to an ESBL possessing some carbapenemase activity, or (ii) the use of ceftazidime-avibactam, leading to the selection of an ESBL conferring resistance to those inhibitors. From a clinical point of view, these are important considerations, since treatments of infections due to ESBL producers rely mainly on either carbapenems or β-lactam/β-lactamase inhibitor combinations.

MATERIALS AND METHODS

Bacterial strains and plasmids. *K. pneumoniae* MAS9, producing CTX-M-33, and *E. coli* R1818, producing CTX-M-15, were clinical isolates. Wild-type *E. coli* TOP10, *E. coli* HB4 (permeability defects due to the loss of porins OmpC and OmpF), and wild-type *K. pneumoniae* CIP53153 were used as recipient strains (35). Plasmid pTOPO (Invitrogen, Illkirch, France) was used as the shuttle vector for cloning and expression in *E. coli* and *K. pneumoniae* recipient strains.

Susceptibility testing and β-lactamase content. Susceptibility testing was performed first by the disk diffusion assay (Sanofi-Diagnostic Pasteur, Marnes-la-Coquette, France). Results were interpreted according to the CLSI guidelines (36). The MICs were then determined by Etest (AB bioMérieux; Solna, Sweden) on Mueller-Hinton agar plates at 37°C. The antimicrobial agents were obtained from standard laboratory powders and were used immediately after their solubilization. The agents and their sources were as follows: amoxicillin, piperacillin, cefepime, cephalothin, ceftazidime, and clavulanic acid, Sigma (Saint-Quentin Falavier, France); ticarcillin and cefoxitin, ROTH (Arlesheim, Switzerland); benzylpenicillin, Abcam (Cambridge, UK); cefotaxime, kanamycin, and aztreonam, Acros Organic (Geel, Belgium); imipenem and meropenem, Carbosynth (Berkshire, UK); avibactam, MedChem Express (Lucerne, Switzerland). MICs were determined by an agar dilution technique on Mueller-Hinton agar (Bio-Rad, Marnes-la-Coquette, France), with an inoculum of 10^4 CFU. All plates were incubated at 37°C for 18 h. MICs of β-lactams were determined alone or in combination with a fixed concentration of clavulanic acid (2 μg/ml), tazobactam (4 μg/ml), or avibactam (4 μg/ml), following the most recent EUCAST recommendations (www.eucast.org). Susceptibility to colistin was evaluated by broth microdilution (http://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/Breakpoint_tables/v_9.0_Breakpoint_Tables.pdf). In order to rapidly evaluate whether the *K. pneumoniae* clinical isolate produced an ESBL and/or a carbapenemase, the Rapid ESBL NP and Rapid Carba NP tests were performed (11, 12).

Analysis of OMPs. OMP profiles were determined by SDS-PAGE analysis, as performed previously (19). *K. pneumoniae* strain CIP53153 (wild-type phenotype) was used as a negative control, and *K. pneumoniae* strain 11978 (lacking the OmpK36 OMP) was used as a positive control (19).

In parallel, molecular investigations were performed in order to characterize the genetic bases of the OMP profiles. PCR amplifications of genes encoding OmpK36, OmpK35, and OmpK26 were performed using primers OmpK36-Fw (5′-GCAGTGGCATAATAAAAGGCA-3′) and OmpK36-Rv (5′-ACTGGTAAACCAGGCCCAG-3′), OmpK35-Fw (5′-AACTTATTGACGGCAGTGGC-3′) and OmpK35-Rv (5′-TTGGTAAACGATACCCACGG-3′), and OmpK26-Fw (5′-ATGTTAAAACGCTCTCTGGT-3′) and OmpK26-Rv (5′-TCAGAACGAGTAGGCCAC-3′), respectively, followed by sequencing of the corresponding amplicons.

Determination of MPCs. For MPC measurements, *E. coli* TOP10 recombinant strains producing CTX-M-33 or CTX-M-15 were inoculated into 100 ml of Mueller-Hinton broth (MHB) and then incubated overnight at 37°C. After incubation, cultures were estimated to have concentrations of $\geq 3 \times 10^8$ CFU/ml. Cultures were then centrifuged at 5,000 × *g* for 30 min and resuspended in 6 ml of MHB. Meropenem-

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or imipenem-supplemented agar plates were inoculated with 200-μl aliquots of the respective cultures, corresponding to bacterial amounts of $\geq 10^{10}$ CFU. Inoculated plates were incubated for 48 h at 37°C. MPC was recorded as the lowest antibiotic concentration for which no growth was observed, as described (37).

Molecular characterization and cloning experiments. Whole-cell DNA was extracted from *K. pneumoniae* MAS9 using the QIAamp minikit (Qiagen, Courtaboeuf, France), according to the manufacturer's recommendations, and the corresponding DNA was used as the template for detection of several β-lactamase genes using specific primers (38). Sequencing of the amplicons was performed by the Sanger technique (Microsynth, Balgach, Switzerland). MLST was performed as recommended (https://cge.cbs.dtu.dk/services/MLST). In order to evaluate and to compare the spectrum of hydrolysis of CTX-M-33 to that of CTX-M-15, cloning of the $bla_{CTX-M-33}$ and $bla_{CTX-M-15}$ genes was performed using the high-copy pTOPO vector (Invitrogen), followed by expression in the same *E. coli* backgrounds (either *E. coli* TOP10 or *E. coli* HB4) or *K. pneumoniae* CIP53153.

Selection was based on plates containing ampicillin (100 μg/ml) and kanamycin (30 μg/ml) or cefotaxime (1 μg/ml) and kanamycin (30 μg/ml) for *E. coli* and *K. pneumoniae*, respectively. The PCR amplicons encompassing the entire sequences of the bla_{CTX-M} genes used for cloning were obtained by using primers CTX-M-Fw (5′-GAGCTCGCTCTGTGGATAACTTGCAG-3′) and CTX-M-Rv (5′-GGATCCTTACAA ACCGTCGGTGACGA-3′).

***β*-Lactamase purification.** Cultures of *E. coli* TOP10 harboring plasmid pTOPO-CTX-M-33 or pTOPO-CTX-M-15 were grown overnight at 37°C in 1 liter of brain heart infusion broth with amoxicillin (100 μg/ml). The bacterial suspension was pelleted, resuspended in 10 ml of 100 mM phosphate buffer (pH 7), and disrupted by sonification. This suspension was dialyzed overnight against 20 mM 2-(*N*-morpholino)ethanesulfonic acid (MES) (pH 6) at 4°C. The enzyme extract was loaded onto a preequilibrated HiTrap SP XL column (GE Healthcare) with the same buffer. Proteins were then eluted with a linear NaCl gradient (from 0 to 0.5 M). The fractions showing the highest β-lactamase activity were pooled and dialyzed against 100 mM phosphate buffer (pH 7.0), prior to 10-fold concentration with a Vivaspin 20 concentrator (GE Healthcare). The purified β-lactamase extract was immediately used for enzymatic determinations.

Determination of *β*-lactamase relative molecular mass. The relative purity of β-lactamase CTX-M-33 was estimated by SDS-12% PAGE analysis. Enzyme extracts and marker proteins were boiled for 10 min in a 1% SDS-3% β-mercaptoethanol solution and then were subjected to electrophoresis at room temperature.

Kinetic measurements. Purified β-lactamase was used for kinetic measurements performed at 30°C in 100 mM sodium phosphate (pH 7.0). The initial rates of hydrolysis were determined with a Genesys 10S UV-visible spectrophotometer (Thermo Scientific). The following wavelengths and absorption coefficients were used: benzylpenicillin, 232 nm and Λ_ε of $-1{,}100$ M^{-1} cm^{-1}; amoxicillin, 240 nm and Λ_ε of $-1{,}100$ M^{-1} cm^{-1}; ticarcillin, 235 nm and Λ_ε of $-1{,}050$ M^{-1} cm^{-1}; piperacillin, 235 nm and Λ_ε of $-1{,}070$ M^{-1} cm^{-1}; cephalothin, 262 nm and Λ_ε of $-7{,}960$ M^{-1} cm^{-1}; cefoxitin, 265 nm and Λ_ε of $-7{,}380$ M^{-1} cm^{-1}; ceftazidime, 260 nm and Λ_ε of $-8{,}660$ M^{-1} cm^{-1}; cefepime, 264 nm and Λ_ε of $-8{,}240$ M^{-1} cm^{-1}; cefotaxime, 265 nm and Λ_ε of $-6{,}260$ M^{-1} cm^{-1}; imipenem, 297 nm and Λ_ε of $-9{,}210$ M^{-1} cm^{-1}; meropenem, 297 nm and Λ_ε of $-9{,}210$ M^{-1} cm^{-1}; aztreonam, 318 nm and Λ_ε of -640 M^{-1} cm^{-1}.

The K_i values were determined by direct competition assays using 100 μM nitrocefin. Inverse initial steady-state velocities ($1/V_0$) were plotted against the inhibitor concentration ([I]) to obtain a straight line. The plots were linear and provided *y* intercept and slope values used for K_i determinations. K_i was determined by dividing the value for the *y* intercept by the slope of the line and then was corrected by taking into account the nitrocefin affinity, using the following equation: K_i (corrected) = K_i (observed)/(1 + [S]/K_m), where [S] is the concentration of nitrocefin (100 μM) used in the assay and K_m is the Michaelis constant determined for nitrocefin (8.5 μM for CTX-M-33 and 15.4 μM for CTX-M-15).

Sensitivity to *β*-lactamase inhibitors. IC_{50} values were determined for clavulanic acid, tazobactam, and avibactam. Various concentrations of these inhibitors were preincubated with the purified enzymes CTX-M-15 and CTX-M-33 for 5 min at 30°C, to determine the concentrations that reduced the hydrolysis rate with 100 μM cephalothin by 50%. The results are expressed in nanomolar units. The total protein content was measured by using a Bradford assay.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AAC.01515-19.

SUPPLEMENTAL FILE 1, PDF file, 0.9 MB.

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