Residues Distal to the Active Site Contribute to Enhanced Catalytic Activity of Variant and Hybrid β-Lactamases Derived from CTX-M-14 and CTX-M-15

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A variety of CTX-M-type extended-spectrum β-lactamases (ESBLs), including hybrid ones, have been reported in China that are uncommon elsewhere. To better characterize the substrate profiles and enzymatic mechanisms of these enzymes, we performed comparative kinetic analyses of both parental and hybrid CTX-M enzymes, including CTX-M-15, -132, -123, -64, -14 and -55, that are known to confer variable levels of β-lactam resistance in the host strains. All tested enzymes were susceptible to serine β-lactamase inhibitors, with sulbactam exhibiting the weakest inhibitory effects. CTX-M-55, which differs from CTX-M-15 by one substitution, $A^{77}V$, displayed enhanced catalytic activity (k_{cat}/K_m) against expanded-spectrum cephalosporins (ESCs). CTX-M-55 exhibits higher structure stability, most likely by forming hydrophobic interactions between $A^{77}V$ and various key residues in different helices, thereby stabilizing the core architecture of the helix cluster, and indirectly contributes to a more stable active site conformation, which in turn shows higher catalytic efficiency and is more tolerant to temperature change. Analyses of the hybrids and their parental prototypes showed that evolution from CTX-M-15 to CTX-M-132, CTX-M-123, and CTX-M-64, characterized by gradual enhancement of catalytic activity to ESCs, was attributed to introduction of different substitutions to amino acids distal to the active site of CTX-M-15. Similarly, the increased hydrolytic activities against cephalosporins and sensitivity to β-lactamase inhibitors, clavulanic acid and sulbactam, of CTX-M-64 were partly due to the amino acids that were different from CTX-M-14 and located at both the C and N termini of CTX-M-64. These data indicate that residues distal to the active site of CTX-Ms contributed to their enhanced catalytic activities to ESCs.

The rapid dissemination of CTX-M-type extended-spectrum β-lactamases (ESBLs) in *Enterobacteriaceae* poses a huge threat to both human and animal health. To date, more than 150 different CTX-M-type ESBLs have been identified (http://www.lahey.org/studies/other.asp#table1). Based on the genetic relatedness, CTX-Ms can be divided into six clusters, including CTX-M-1, -2, -8, -9, and -25 and the KLUC groups (1). More than 95% identity is often observed within the same cluster, whereas less than 90% identity is detectable between clusters (2). Several hybrids of the CTX-M-1 and -9 groups, namely, CTX-M-64, CTX-M-123, CTX-M-137, and CTX-M-132, have also been identified recently (3–5). Formation of these hybrid CTX-M enzymes was suggested to be the result of recombination between $bla_{CTX-M-15}$ and $bla_{CTX-M-14}$, the two most dominant variants detectable worldwide (1, 6). However, the genetic environments of $bla_{CTX-M-123}$ and $bla_{CTX-M-64}$ were highly similar to that of $bla_{CTX-M-55}$ identified in pHN1122-1, the second-most prevalent bla_{CTX-M} variant in China (7). This suggests that recombination may take place between $bla_{CTX-M-14}$ and $bla_{CTX-M-55}$, producing various hybrid enzymes. Interestingly, all three published hybrids, CTX-M-64, CTX-M-123, and CTX-M-137, confer relatively high ceftazidime MICs compared to the majority of other CTX-M-type ESBLs (3–5). Conversely, the hydrolytic activities of CTX-M-55, CTX-M-123, and CTX-M-132 enzymes have not been described. Although the kinetic parameters of CTX-M-14, -15, and -64 have been reported, only a few substrates have been characterized (5, 8, 9). In addition, kinetic parameters of these enzymes for β-lactamase in-

hibitors and animal-use-only cephalosporins, namely, ceftiofur, are unknown. In this study, we performed comprehensive kinetic characterization of the major hybrids and their parental enzymes, including CTX-M-15, CTX-M-55, CTX-M-132, CTX-M-123, CTX-M-64, and CTX-M-14, with objectives of better understanding the mechanisms underlying the variable catalytic activities of these enzymes and providing insight into mechanisms of action of this class of β-lactamases.

MATERIALS AND METHODS

Antibiotics and chemicals. Cefuroxime, ceftriaxone, ceftazidime, and cefotaxime were purchased from Sigma Chemical Co. (St. Louis, MO, USA), while cephalothin, ceftiofur, tazobactam, clavulanic acid, and sulbactam

TABLE 1 Primers used to construct the CTX-M enzymes in this study

Primer	Sequence
CTX-M-14-F	CCGGGATCCATGGTGACAAAGAGAGTGC
CTX-M-14-R	CGCGAATTCTTACAGCCCTTCGGCG
CTX-M-15/55/64/123/132-F	CCGGGATCCATGGTTAAAAAATCACTGC
CTX-M-15/55/64/123/132-R	RCGCGAATTCTTACAAACCGTCGGTGACG
mCTX-M-14-F	CCGGGATCCCAGACGAGTGCGGTGCAGC
mCTX-M-15/55/64/123/132-F	CCGGGATCCCAAACGGCGGACGTACAGC

were purchased from Melonepharma Co. (Dalian, China). Ampicillin, kanamycin, and isopropyl-β-D-1-thiogalactopyranoside (IPTG) were purchased from IBI, Inc. (Boca Raton, FL, USA). Luria broth (LB) and nitrocefin were purchased from BD Co. (Franklin Lakes, NJ, USA). Mueller-Hinton broth (MHB) was purchased from Oxoid Co. (Hampshire, United Kingdom).

Bacterial strains and recombinant DNA. *Escherichia coli* strains AHC4, carrying $bla_{CTX-M-123}$ and $bla_{CTX-M-55}$, and AHC46, carrying $bla_{CTX-M-64}$, were isolated from chicken samples submitted to a veterinary diagnostic center in Anhui Province, China (4). *E. coli* strain LDH19 carrying $bla_{CTX-M-132}$ was obtained from a urine sample from a female patient at a community hospital in Guangzhou, China. Clinical *E. coli* strains carrying $bla_{CTX-M-14}$ and $bla_{CTX-M-15}$ described in our previous study were also used in this study (10, 11). The bla_{CTX-M} genes were amplified by PCR using primers listed in Table 1 and cloned into the pET28b vector (Novagen). Recombinant plasmids pET28-bla_{CTX-Ms} were transformed into *E. coli* BL21(DE3) for CTX-M expression. Two versions of bla_{CTX-Ms} were cloned, namely, those encoding the full-length precursor enzymes and enzymes without a signal peptide. *E. coli* carrying the full-length bla_{CTX-Ms} was used to determine the MICs, whereas *E. coli* carrying the truncated versions of the $bla_{H6-mCTX-M}$ genes (without signal peptide regions; m represents mature form of CTX-M) was used for protein expression and purification.

Antimicrobial susceptibility. MICs of *E. coli* strains were determined for 7 β-lactams (ampicillin, cephalothin, cefuroxime, ceftiofur, ceftriaxone, cefotaxime, and ceftazidime) alone or in combination with a fixed, 4-μg/ml concentration of β-lactamase inhibitors (tazobactam, clavulanic acid, or sulbactam) according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI) (12, 13). The strains were grown on a Mueller-Hinton agar (MHA) plate at 37°C overnight and transferred to MHB media supplemented with a serial concentration of selected β-lactams the next day, followed by incubation at 37°C for 16 to 20 h. The lowest concentrations of β-lactams that inhibited the growth of the *E. coli* cells were determined as the MICs. For *E. coli* BL21(DE3), the MICs toward different β-lactams were determined as aforementioned except in the presence of 1 mM IPTG in the MHB medium. Determination of MIC was repeated at least in triplicate for each antibiotic tested. *E. coli* strains (ATCC 25922 and ATCC 35218) were used as quality controls.

DNA and protein analyses. The bla_{CTX-M} genes and the phylogenetic tree were analyzed and constructed using SeqMan software, while the protein alignment was done using MegAlign software of Lasergene 7 (DNASTAR, Inc., WI, USA). The signal sequences of CTX-M enzymes were determined using the SignalP 4.1 server available at http://www.cbs.dtu.dk/services/SignalP/.

Overexpression and purification of CTX-M enzymes. Freshly cultured *E. coli* BL21(DE3) carrying pET28-$bla_{H6-mCTX-M}$, at a cell density of 0.6 at an optical density at 600 nm (OD_{600}) was induced with 1 mM IPTG and continuously incubated at 30°C for 5 h. The cells were recovered, resuspended in lysis buffer (10 mM Tris-HCl [pH 7.6], 0.3 M sucrose, 1% NP-40, 0.5% Triton X-100, and 0.5% Tween 20), and broken with a French press (Thermo Scientific, Inc., MA, USA) by 3 passes at 20,000
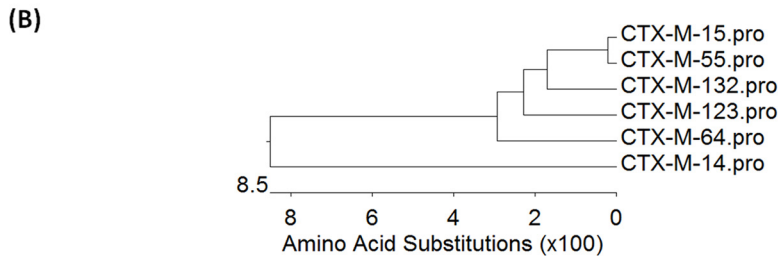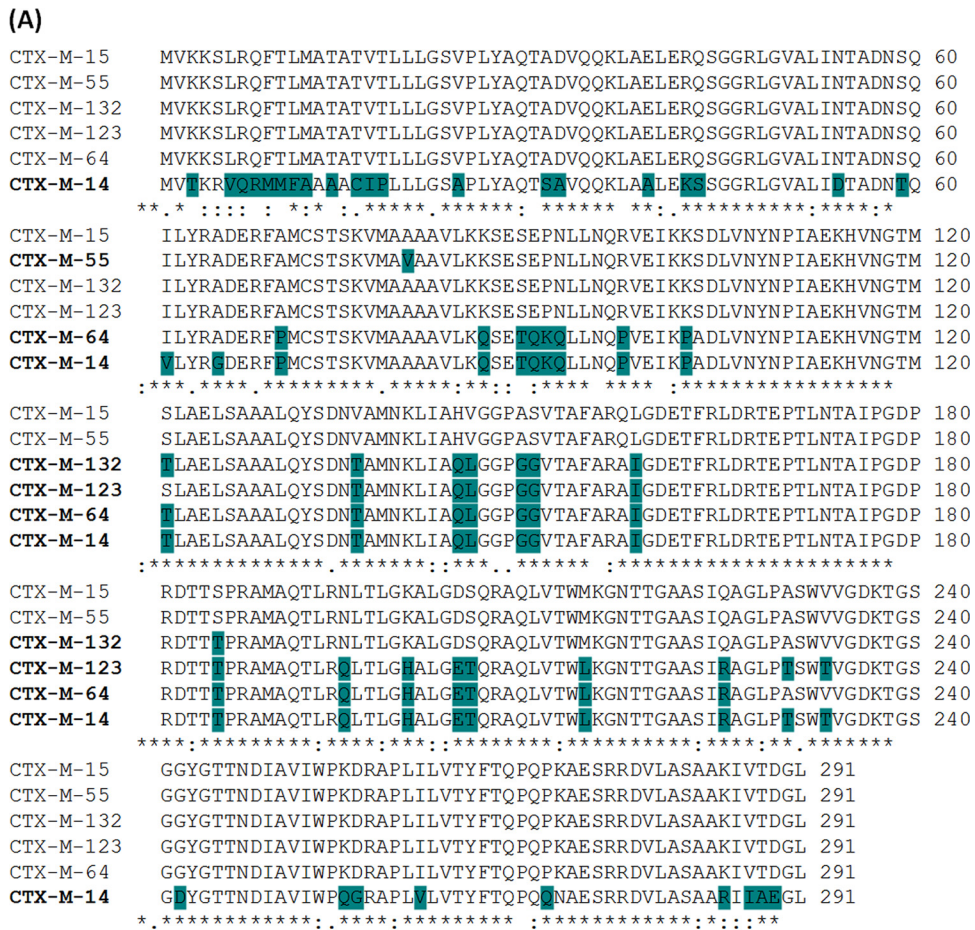
lb/in² with sample cooling in an ice-water bath for 1 min after each pass. The soluble fractions were loaded onto a Ni-nitrilotriacetic acid (NTA) column, washed, and eluted with a binding buffer of 20 mM Tris-HCl, pH 7.9, and 500 mM NaCl containing different concentrations of imidazole. Briefly, the Ni-NTA column was washed twice with binding buffers containing 10 mM and 20 mM imidazole. The His_6-tagged proteins were then eluted with binding buffer containing 100 mM imidazole, followed by concentration using an Amicon ultra-15 (nominal molecular weight limit, 10) centrifugal filter device, and the buffer was exchanged with 50 mM phosphate buffer, pH 7.4, during the process. The His_6 tag was removed by incubating the purified enzyme with thrombin at a ratio of 100 μg protein per unit of thrombin at room temperature for 1 h. The thrombin-treated proteins were further subjected to gel filtration chromatography on an Sephacryl S-200 column. Fractions containing the mCTX-M were pooled and concentrated using the Amicon ultra-15 centrifugal filter device. The purity of mCTX-M proteins was estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis and determined as at least 99% pure for all of the mCTX-M enzymes purified. The concentrations of enzymes were determined using Nanodrop Lite (Thermo Scientific, Inc., MA, USA). The yields for the six mCTX-M enzymes in this study ranged from 14 to 15.2 mg/500 ml.

Western blot. The expression levels of CTX-M enzymes were verified by Western blotting. In brief, *E. coli* cultures carrying pET28-$bla_{H6-mCTX-M}$ were induced with 1 mM IPTG, as described previously. The cells were recovered and resuspended in B-per buffer (Thermo Scientific, Inc., MA, USA). The obtained total lysate were loaded onto an SDS-PAGE, followed by transfer to a nitrocellulose membrane. The membrane was probed with anti-H_6 antibody to check the expression levels of *E. coli* cultures carrying different CTX-M enzymes. Anti-GAPDH (glyceraldehyde-3-phosphate dehydrogenase) antibody was probed to ensure the equal loading.

Determination of kinetic constants and IC_{50}. The kinetic parameters of mCTX-Ms were determined by mixing the enzyme with different concentrations of β-lactams at 25°C in the assay buffer (50 mM phosphate buffer, pH 7.0) without preincubation. The initial velocities of substrate hydrolysis for ampicillin ($\Delta\varepsilon_{235} = -820$ M^{-1} cm^{-1}), nitrocefin ($\Delta\varepsilon_{482} = 15,000$ M^{-1} cm^{-1}), cephalothin ($\Delta\varepsilon_{260} = -6,500$ M^{-1} cm^{-1}), cefuroxime ($\Delta\varepsilon_{260} = -7,600$ M^{-1} cm^{-1}), ceftriaxone ($\Delta\varepsilon_{260} = -10,351$ M^{-1} cm^{-1}), cefotaxime ($\Delta\varepsilon_{260} = -7,500$ M^{-1} cm^{-1}), ceftiofur ($\Delta\varepsilon_{260} = -5,614$ M^{-1} cm^{-1}), and ceftazidime ($\Delta\varepsilon_{260} = -9,000$ M^{-1} cm^{-1}) were measured by monitoring the changes of absorbance in a 1-cm quartz cuvette by a spectrometer (PerkinElmer Lambda Bio20) for 5 min (14). The molar extinction coefficients for ceftriaxone and ceftiofur were determined in our lab, while the others were obtained from a previous study (data not shown) (14). The initial velocities obtained from the substrate range of 0.2 to $2 \times K_m$ were measured in at least triplicate and fitted to the nonlinear regression of the Michaelis-Menten equation using least-squares (ordinary) fit by GraphPad Prism5 (San Diego, CA, USA) to determine the K_m, k_{cat}, and standard errors. Bovine serum albumin (BSA) (100 μg/ml) was added to the diluted enzymes and the reaction mixture to prevent enzyme degradation. For ceftazidime, which was a poor substrate, the K_i values of mCTX-Ms enzymes were determined by a competitive inhibition assay using 100 μM nitrocefin as a reporter substrate (5, 15). The effect of temperature on the enzymatic activity was studied by determination of the kinetic constants of CTX-M-15 and CTX-M-55 toward cefotaxime using the same assay buffer and condition as aforementioned except at temperatures of 25°C, 40°C, and 60°C.

The 50% inhibitory concentration (IC_{50}) was determined as the concentration of β-lactamase inhibitors (clavulanic acid, tazobactam, and sulbactam) required to reduce the hydrolysis rate of nitrocefin by 50% when the enzyme was preincubated with various concentrations of the inhibitor for 5 min at 25°C prior to addition of substrate.

Circular dichroism. The thermal stability assay was performed by using 0.47 μM CTX-M-15 and CTX-M-55 in milli-Q water in a Jasco-

(A)

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CTX-M-15   MVKKSLRQFTLMATATVTLLLGSVPLYAQTADVQQKLAELERQSGGRLGVALINTADNSQ 60
CTX-M-55   MVKKSLRQFTLMATATVTLLLGSVPLYAQTADVQQKLAELERQSGGRLGVALINTADNSQ 60
CTX-M-132  MVKKSLRQFTLMATATVTLLLGSVPLYAQTADVQQKLAELERQSGGRLGVALINTADNSQ 60
CTX-M-123  MVKKSLRQFTLMATATVTLLLGSVPLYAQTADVQQKLAELERQSGGRLGVALINTADNSQ 60
CTX-M-64   MVKKSLRQFTLMATATVTLLLGSVPLYAQTADVQQKLAELERQSGGRLGVALINTADNSQ 60
CTX-M-14   MVTKRVQRMMFAAAACIFLLLGSAPLYAQTSAVQQKLAALEKSSGGRLGVALIDTADNTQ 60
           **.* :::: : *:* :.***** ******: ****** **: *********:****.*
CTX-M-15   ILYRADERFAMCSTSKVMAAAAVLKKSESEPNLLNQRVEIKKSDLVNYNPIAEKHVNGTM 120
CTX-M-55   ILYRADERFAMCSTSKVMAVAAVLKKSESEPNLLNQRVEIKKSDLVNYNPIAEKHVNGTM 120
CTX-M-132  ILYRADERFAMCSTSKVMAAAAVLKKSESEPNLLNQRVEIKKSDLVNYNPIAEKHVNGTM 120
CTX-M-123  ILYRADERFAMCSTSKVMAAAAVLKKSESEPNLLNQRVEIKKSDLVNYNPIAEKHVNGTM 120
CTX-M-64   ILYRADERFPMCSTSKVMAAAAVLKQSETQKQLLNQPVEIKPADLVNYNPIAEKHVNGTM 120
CTX-M-14   VLYRGDERFPMCSTSKVMAAAAVLKQSETQKQLLNQPVEIKPADLVNYNPIAEKHVNGTM 120
           :*** .**** .********.*****:**:: :**** **** :*****************
CTX-M-15   SLAELSAAALQYSDNVAMNKLIAHVGGPASVTAFARQLGDETFRLDRTEPTLNTAIPGDP 180
CTX-M-55   SLAELSAAALQYSDNVAMNKLIAHVGGPASVTAFARQLGDETFRLDRTEPTLNTAIPGDP 180
CTX-M-132  TLAELSAAALQYSDNTAMNKLIAQLGGPGGVTAFARAIGDETFRLDRTEPTLNTAIPGDP 180
CTX-M-123  SLAELSAAALQYSDNTAMNKLIAQLGGPGGVTAFARAIGDETFRLDRTEPTLNTAIPGDP 180
CTX-M-64   TLAELSAAALQYSDNTAMNKLIAQLGGPGGVTAFARAIGDETFRLDRTEPTLNTAIPGDP 180
CTX-M-14   TLAELSAAALQYSDNTAMNKLIAQLGGPGGVTAFARAIGDETFRLDRTEPTLNTAIPGDP 180
           :************* .*******::***.. ****** :*******************
CTX-M-15   RDTTSPRAMAQTLRNLTLGKALGDSQRAQLVTWMKGNTTGAASIQAGLPASWVVGDKTGS 240
CTX-M-55   RDTTSPRAMAQTLRNLTLGKALGDSQRAQLVTWMKGNTTGAASIQAGLPASWVVGDKTGS 240
CTX-M-132  RDTTTPRAMAQTLRNLTLGKALGDSQRAQLVTWMKGNTTGAASIQAGLPASWVVGDKTGS 240
CTX-M-123  RDTTTPRAMAQTLRQLTLGHALGETQRAQLVTWLKGNTTGAASIRAGLPTSWTVGDKTGS 240
CTX-M-64   RDTTTPRAMAQTLRQLTLGHALGETQRAQLVTWLKGNTTGAASIRAGLPTSWTVGDKTGS 240
CTX-M-14   RDTTTPRAMAQTLRQLTLGHALGETQRAQLVTWLKGNTTGAASIRAGLPTSWTVGDKTGS 240
           ****:*********:****.***::*********:*********:****:**.*******
CTX-M-15   GGYGTTNDIAVIWPKDRAPLILVTYFTQPQPKAESRRDVLASAAKIVTDGL 291
CTX-M-55   GGYGTTNDIAVIWPKDRAPLILVTYFTQPQPKAESRRDVLASAAKIVTDGL 291
CTX-M-132  GGYGTTNDIAVIWPKDRAPLILVTYFTQPQPKAESRRDVLASAAKIVTDGL 291
CTX-M-123  GGYGTTNDIAVIWPKDRAPLILVTYFTQPQPKAESRRDVLASAAKIVTDGL 291
CTX-M-64   GGYGTTNDIAVIWPKDRAPLILVTYFTQPQPKAESRRDVLASAAKIVTDGL 291
CTX-M-14   GDYGTTNDIAVIWPQGRAPLVLVTYFTQPQQNAESRRDVLASAARIIAEGL 291
           * .************: ****:********* :***********:**::**
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(B)

FIG 1 Sequence alignment of CTX-M-14, CTX-M-15, CTX-M-55, and various related hybrids. (A) The alignment of different CTX-M enzymes. The amino acids are numbered by the standard numbering scheme for the class A β-lactamases according to Ambler et al. (23). (B) Phylogenetic tree of the 6 CTX-M variants used in this study.

J810 spectropolarimeter (Easton, MD, USA) at 25°C, 40°C, and 60°C. Quartz cells with a 10-mm path length were used for all experiments. Circular dichroism (CD) spectra were obtained and recorded every 0.1 nm between λ_{190} and λ_{250} with a scan rate of 50 nm/min and a 2-s response time. Data were measured in triplicate, and the CD signals were expressed as molar ellipticity.

Structural modeling. Structures of CTX-M variants were generated using their amino acid sequences (GenBank accession numbers AEX93385.1, ACU87987.1, AGX00969.1, and JX313020 for CTX-M-55, -64, -123, and -132, respectively) and the comparative protein modeling Swiss-Model server available at http://swissmodel.expasy.org, employing 4HBT (for CTX-M-55, -123, and -132) and 1YLT (for CTX-M-64), of which both are CTX-M β-lactamases, as the templates. The Global Model Quality Estimation (GMQE) scores, the values used to estimate the accuracy of a model built with the template, were between 0.93 and 0.97 for the models built in this study. The protein structures and built models were analyzed by PyMOL.

RESULTS AND DISCUSSION

In this study, we aimed to characterize the catalytic activities and functional mechanisms of the two most prevalent CTX-M types of β-lactamases, CTX-M-14 and CTX-M-15, and the related hybrid enzymes reported recently, including CTX-M-132, CTX-M-123, and CTX-M-64. CTX-M-55 was also included in the characterization, since this variant exhibits a close relationship with CTX-M-15 and is generally regarded as the parental enzyme from which the hybrids evolved. Phylogenetic and sequence alignment analysis showed that CTX-M-14 and CTX-M-15 were the most divergent enzymes that comprised two distinct clusters, whereas CTX-M-55 differed from CTX-M-15 by only one residue, $V^{77}A$ (Fig. 1). Among the hybrids, CTX-M-132 was genetically closest to CTX-M-15, followed by CTX-M-123 and CTX-M-64, which were more closely related to CTX-M-14 (Fig. 1).

TABLE 2 MICs of *E. coli* isolates carrying various *bla*$_{CTX-Ms}$ toward different β-lactams

E. coli strain	*bla*$_{CTX-M}$	MIC (μg/ml)^a for:									
		AMP	CEP	CFX	CTO	CRO	CAZ	CTX	CTX + TZB^b	CTX + CLA^b	CTX + SUL^b
Parental strain	M-15	≥2,048	≥2,048	≥2,048	≥2,048	≥2,048	32	≥2,048	0.25	0.25	0.125
	M-55	≥2,048	≥2,048	≥2,048	≥2,048	≥2,048	64	≥2,048	0.5	0.5	2
	M-132	≥2,048	≥2,048	≥2,048	≥2,048	≥2,048	64	≥2,048	0.06	0.125	2
	M-123	≥2,048	≥2,048	≥2,048	≥2,048	≥2,048	32	≥2,048	0.125	0.125	4
	M-64	≥2,048	≥2,048	≥2,048	≥2,048	≥2,048	64	≥2,048	0.125	0.125	8
	M-14	≥2,048	1,024	1,024	512	1,024	4	256	0.06	0.06	0.125
BL21(DE3) carrying	M-15	≥2,048	256	≥2,048	512	256	2	128	<0.015	<0.015	0.06
pET28b-*bla*$_{CTX-M}$	M-55	≥2,048	256	≥2,048	512	512	16	256	<0.015	<0.015	0.06
	M-132	≥2,048	512	≥2,048	512	512	32	512	<0.015	<0.015	0.5
	M-123	≥2,048	512	≥2,048	512	512	32	512	<0.015	<0.015	0.125
	M-64	≥2,048	256	≥2,048	512	512	32	1024	<0.015	<0.015	0.125
	M-14	≥2,048	256	1,024	128	256	0.5	128	<0.015	<0.015	0.5
	M-14(D^{240}G)	≥2,048	256	1,024	128	256	0.5	256	<0.015	<0.015	0.5
E. coli BL21(DE3)		16	16	4	0.03	0.03	0.03	0.06			

^{a} AMP, ampicillin; CEP, cephalothin; CFX, cefuroxime; CTO, ceftiofur; CRO, ceftriaxone; CAZ, ceftazidime; CTX, cefotaxime; CLA, clavulanic acid; TZB, tazobactam; SUL, sulbactam.
^{b} TZB, CLA, and SUL were each at a fixed concentration of 4 μg/ml.

To directly compare the phenotypes of these 6 different CTX-Ms and rule out other factors that may contribute to resistance for β-lactams, MICs of *E. coli* strains producing the full-length CTX-Ms, including the clinical strains and the pET28b clones toward different β-lactams, were determined (Table 2). The levels of expression in the total cell lysate for NH-mCTX-Ms (where NH is the N-terminal His tag) in *E. coli* were determined by probing the total lysate with anti-His$_6$-tag antibody. As shown in Fig. 2, these 6 CTX-Ms showed similar expression levels. In terms of *in vivo* activities against different β-lactams tested, the order of

FIG 2 Protein expression levels of CTX-Ms by Western blotting. (A) The total lysate of *E. coli* carrying *bla*$_{H6-mCTX-Ms}$ was probed with anti-H$_6$ antibody as described in Materials and Methods. (B) The expression levels were normalized by quantifying the bands using AlphaEaseFC.

the levels of MICs to *E. coli* carrying these *bla*$_{CTX-Ms}$ were as follows: ampicillin, cefuroxime > cephalothin, ceftiofur, ceftriaxone, and cefotaxime > ceftazidime. These CTX-Ms were susceptible to all three serine-based β-lactamase inhibitors, clavulanic acid, tazobactam, and sulbactam; sulbactam was the weakest inhibitor (Table 2). Regarding the MICs of different β-lactams for *E. coli* producing different CTX-Ms, CTX-M-14 showed the lowest MICs to most of the β-lactams tested, except ampicillin, compared to other CTX-Ms (Table 2). Among the other 5 CTX-Ms, CTX-M-64 exhibited the highest MICs for cefotaxime, whereas CTX-M-64, CTX-M-132, CTX-M-123, and CTX-M-55 conferred higher MICs for ceftazidime and cefotaxime than those mediated by CTX-M-15. These data suggested that CTX-M-64 showed relatively higher MICs for different β-lactams than other enzymes, such as CTX-M-132, CTX-M-123, and CTX-M-55, which in turn conferred higher MICs than CTX-M-15 (Table 2). All three commercially available serine β-lactamase inhibitors, clavulanic acid, tazobactam, and sulbactam, were very effective when used in a combinatorial manner with cefotaxime, with the activity of cefotaxime exhibiting the highest degree of reduction (up to 70,000-fold) compared to cefotaxime alone.

Enzyme kinetic characterization of these CTX-Ms provided a clearer and better understanding of the differences between the catalytic activities of these enzymes than MICs, since one may doubt that the latter could be partially attributed to the high concentration of these enzymes in the periplasmic space. Similar to the MIC results, CTX-M-14 exhibited the lowest catalytic activity toward the β-lactams tested, except cephalothin. CTX-M-15 displayed slightly lower catalytic activities to most of the β-lactams tested, except ampicillin, compared to CTX-M-55 and the other three hybrids (Table 3). Consistent with the MIC data, CTX-M-55 exhibited similar catalytic activity as CTX-M-132 and CTX-M-123 for most of the β-lactams tested, except ampicillin, whereas CTX-M-64 exhibited the highest catalytic activity toward nitrocefin, cefuroxime, ceftiofur, ceftriaxone, and cefotaxime (Table 3). The IC$_{50}$ and K_i of clavulanic acid, tazobactam, and sulbactam
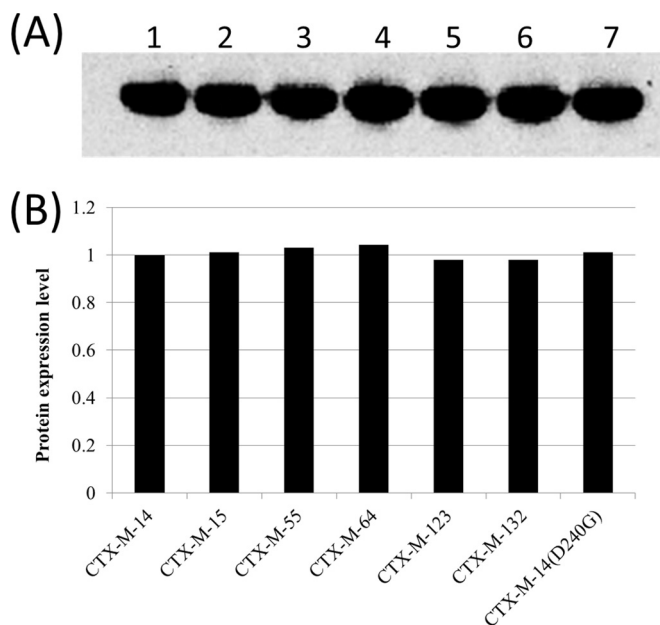
TABLE 3 Kinetic constants of six purified CTX-M enzymes

CTX-M enzyme	Constant	Value for:							
		AMP	NCF[a]	CEP	CFX	CTO	CRO	CTX	CAZ[b]
M-15	K_m (μM)	7 ± 0.7	11 ± 1	30 ± 4	22 ± 3	14 ± 2	15 ± 1	27 ± 2	972 ± 179
	k_{cat} (s^{-1})	25 ± 0.4	132 ± 11	34 ± 2	61 ± 2	43 ± 2	68 ± 1	60 ± 2	ND
	k_{cat}/K_m (μM^{-1} s^{-1})	3.5	12	1.1	2.8	3.1	4.5	2.2	ND
M-55	K_m (μM)	18 ± 2	6 ± 0.8	15 ± 2	15 ± 2	10 ± 2	8 ± 1	23 ± 3	592 ± 114
	k_{cat} (s^{-1})	23 ± 0.6	161 ± 9	216 ± 8	70 ± 3	54 ± 3	57 ± 3	73 ± 4	ND
	k_{cat}/K_m (μM^{-1} s^{-1})	1.3	26	14	4.7	5.4	7.1	3.2	ND
M-132	K_m (μM)	39 ± 3	10 ± 1	14 ± 2	10 ± 1	7 ± 1	8 ± 0.4	25 ± 4	649 ± 98
	k_{cat} (s^{-1})	42 ± 1	242 ± 17	226 ± 9	64 ± 2	43 ± 2	74 ± 1	83 ± 5	ND
	k_{cat}/K_m (μM^{-1} s^{-1})	1.1	24	16	6.4	6.1	9.3	3.3	ND
M-123	K_m (μM)	11 ± 0.7	9 ± 2	16 ± 2	19 ± 3	8 ± 0.7	12 ± 1	23 ± 2	1,218 ± 254
	k_{cat} (s^{-1})	428 ± 5	183 ± 23	214 ± 7	116 ± 5	69 ± 2	124 ± 5	95 ± 3	ND
	k_{cat}/K_m (μM^{-1} s^{-1})	39	20	13	6.1	8.6	10	4.1	ND
M-64	K_m (μM)	17 ± 2	10 ± 2	25 ± 3	31 ± 4	16 ± 2	10 ± 1	24 ± 4	1,516 ± 166
	k_{cat} (s^{-1})	107 ± 3	345 ± 34	91 ± 3	235 ± 12	233 ± 14	195 ± 7	174 ± 9	ND
	k_{cat}/K_m (μM^{-1} s^{-1})	6.3	35	3.6	7.6	15	20	7.3	ND
M-14	K_m (μM)	72 ± 11	11 ± 2	64 ± 6	22 ± 3	14 ± 0.5	17 ± 3	34 ± 5	>8,000
	k_{cat} (s^{-1})	25 ± 2	114 ± 12	357 ± 18	56 ± 3	16 ± 0.2	42 ± 4	37 ± 2	ND
	k_{cat}/K_m (μM^{-1} s^{-1})	0.35	10	5.6	2.5	1.1	2.5	1.1	ND
M-14(D^{240}G)	K_m (μM)	17 ± 2	2 ± 0.4	68 ± 21	28 ± 2	13 ± 2	14 ± 2	30 ± 3	>8,000
	k_{cat} (s^{-1})	25 ± 0.8	41 ± 3	265 ± 57	50 ± 2	17 ± 1	68 ± 4	54 ± 3	ND
	k_{cat}/K_m (μM^{-1} s^{-1})	1.5	21	3.9	1.8	1.3	4.9	1.8	ND

[a] NCF, nitrocefin.
[b] The values were determined as K_i in the competition assay using nitrocefin as the reporter substrate. ND, the kinetic constants could not be determined due to low activity of the enzymes toward ceftazidime.

showed that all these three inhibitors displayed nanomolar levels of inhibition of these CTX-Ms, with sulbactam exhibiting higher IC$_{50}$ and K_i than clavulanic acid, which was in turn slightly higher than tazobactam (Table 4). Such findings were consistent with the MIC data and previous studies (8, 13).

Structural analysis was then performed on these CTX-Ms to investigate the mechanisms responsible for different catalytic activities observed among the enzymes. Structures of CTX-M-14 (1YLT) and CTX-M-15 (4HBT) were adopted from the Protein Data Bank, while structures of other CTX-Ms were modeled by Swiss-Model, with CTX-M-14 and CTX-M-15 as templates. CTX-M-55 harbored only one substitution compared to CTX-M-15. This A^{77}V substitution in CTX-M-55 could be associated with

TABLE 4 IC$_{50}$ and K_i of β-lactamase inhibitors[a]

Enzyme	Clavulanic acid		Tazobactam		Sulbactam	
	IC$_{50}$ (nM)	K_i (nM)	IC$_{50}$ (nM)	K_i (nM)	IC$_{50}$ (nM)	K_i (nM)
CTX-M-15	23.2	5.40 ± 1.24	7.61	0.92 ± 0.37	50.4	6.23 ± 2.51
CTX-M-55	13.9	2.37 ± 0.80	6.88	0.86 ± 0.15	50.7	7.59 ± 1.17
CTX-M-132	16.7	4.60 ± 0.51	3.70	1.07 ± 0.16	30.8	17.3 ± 1.73
CTX-M-123	17.6	3.37 ± 0.34	1.72	0.52 ± 0.11	59.4	11.1 ± 1.55
CTX-M-64	22.3	5.87 ± 1.28	5.75	1.44 ± 0.22	45.4	14.9 ± 1.79
CTX-M-14	60.1	24.3 ± 2.40	7.28	1.62 ± 0.25	403	119 ± 31.3

[a] The IC$_{50}$ and K_i values in the competition experiments were calculated using nitrocefin as the reporter substrate.
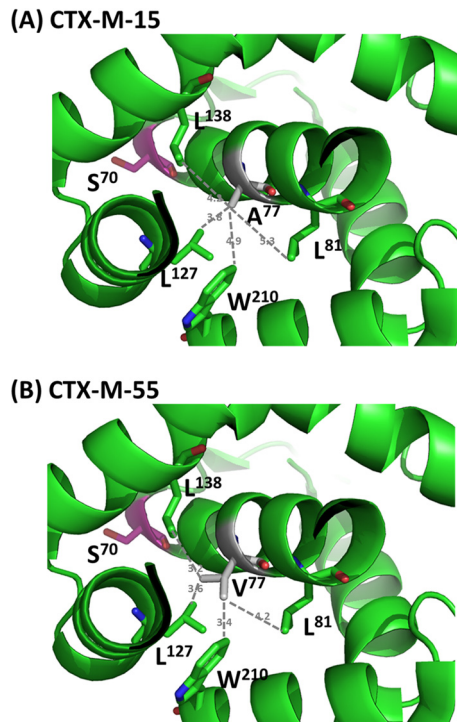
slightly higher catalytic activity of cephalosporins than CTX-M-15, with the highest effect an ~13-fold increase toward cephalothin (Table 3). Structural analysis showed that both A^{77} and V^{77} were located in the center of globular proteins of CTX-M-15 and CTX-M-55. This location suggested that these residues would not contribute to the recognition of substrate but may have an impact on the maintenance of stability of the protein structure. Residue A^{77} in CTX-M-15 was located in the center of a helix cluster that forms the major part of the enzyme. The loops connecting these helixes contribute to the formation of the active site of CTX-M enzyme. Due to the short side chain of A^{77}, this residue may not be able to form strong hydrophobic interactions with adjacent residues in other helices, limiting its ability to stabilize the helix cluster (Fig. 3A). Upon substitution with V^{77}, however, this residue may be able to form stronger hydrophobic interactions with the key residues, such as L^{81}, L^{127}, L^{138}, and W^{210}, in different helices, reflecting a shorter distance between these residues (Fig. 3B). The structure analysis of A^{77}/V^{77} of CTX-M-15/CTX-M-55 was supported by results of the thermal stability assay using far-UV CD; CTX-M-15 and CTX-M-55 showed highly similar spectra at 25°C. CTX-M-55 showed similar secondary structure spectra at 25°C and 40°C and showed subtle loss of helicity throughout 200 to 230 nm at 60°C (Fig. 4A). In contrast with CTX-M-55, CTX-M-15 underwent loss of helicity at both 40°C and 60°C, with more loss of helicity at the higher temperature (Fig. 4B). In addition, the activity assay at different temperatures indicated that CTX-M-55 hydrolyzed cefotaxime with slightly reduced activity efficiency, by

(A) CTX-M-15

(B) CTX-M-55

FIG 3 Interaction between A^{77}/V^{77} and other residues in the adjacent helixes of CTX-M-15 and CTX-M-55. (A) A^{77} in CTX-M-15 interacting with residues L^{81}, L^{127}, L^{138}, and W^{210}: the distances between A^{77} and L^{81}, L^{127}, L^{138}, and W^{210} were 5.3 Å, 3.8 Å, 4.2 Å, and 4.9 Å, respectively; (B) V^{77} in CTX-M-15 interacting with residues L^{81}, L^{127}, L^{138}, and W^{210}: the distances between V^{77} and L^{81}, L^{127}, L^{138}, and W^{210} were 4.2 Å, 3.6 Å, 3.2 Å, and 3.4 Å, respectively.

TABLE 5 Kinetic constants of CTX-M-55 and CTX-M-15 toward cefotaxime at different temperatures

CTX-M enzyme and kinetic constant	Value at a temp of:		
	25°C	40°C	60°C
CTX-M-55			
K_m (μM)	23 ± 3	45 ± 5	53 ± 6
k_{cat} (s^{-1})	73 ± 4	95 ± 6	101 ± 6
k_{cat}/K_m (μM^{-1} s^{-1})	3.2	2.1	1.9
CTX-M-15			
K_m (μM)	27 ± 2	56 ± 10	87 ± 12
k_{cat} (s^{-1})	60 ± 2	32 ± 3	39 ± 4
k_{cat}/K_m (μM^{-1} s^{-1})	2.2	0.6	0.4

M-55, compared to CTX-M-15, may contribute to a more stable active site conformation and, hence, better substrate recognition and catalysis, thereby accounting for the higher catalytic activity of CTX-M-55. Our results were consistent with previous findings showing that $A^{77}V$ played an important role in the phenotype of expanded-spectrum cephalosporin resistance, in which $A^{77}V$ increased resistance to cefotaxime and ceftazidime when associated with P^{167} and G^{240} in the background of CTX-M-10 ([16]). Collectively, these data suggested that, albeit being located distal to the active site, $A^{77}V$ plays an important role on the catalytic activity of CTX-Ms through stabilizing the structure of the enzymes, which has also been reported in other β-lactamases ([17]).

Through sequence alignment of CTX-Ms in Fig. 1, we could see that, if CTX-M-15 was used as parental enzyme template, different hybrids could be formed by introduction of different substitutions from CTX-M-14. Compared to CTX-M-15, CTX-M-132 harbored substitutions at 9 different residues, including $S^{118}T$, $V^{133}T$, $H^{141}Q$, $V^{142}L$, $A^{146}G$, $S^{147}G$, $Q^{154}A$, $L^{155}I$, and $S^{182}T$, which were located at the opposite side of the active site (Fig. 1A and 5A). The enhancement of catalytic activity, determined as k_{cat}/K_m, of CTX-M-132 by approximately 2-fold toward nitrocefin, cefuroxime, ceftiofur, and ceftriaxone, and by 13.6-fold for cephalo-
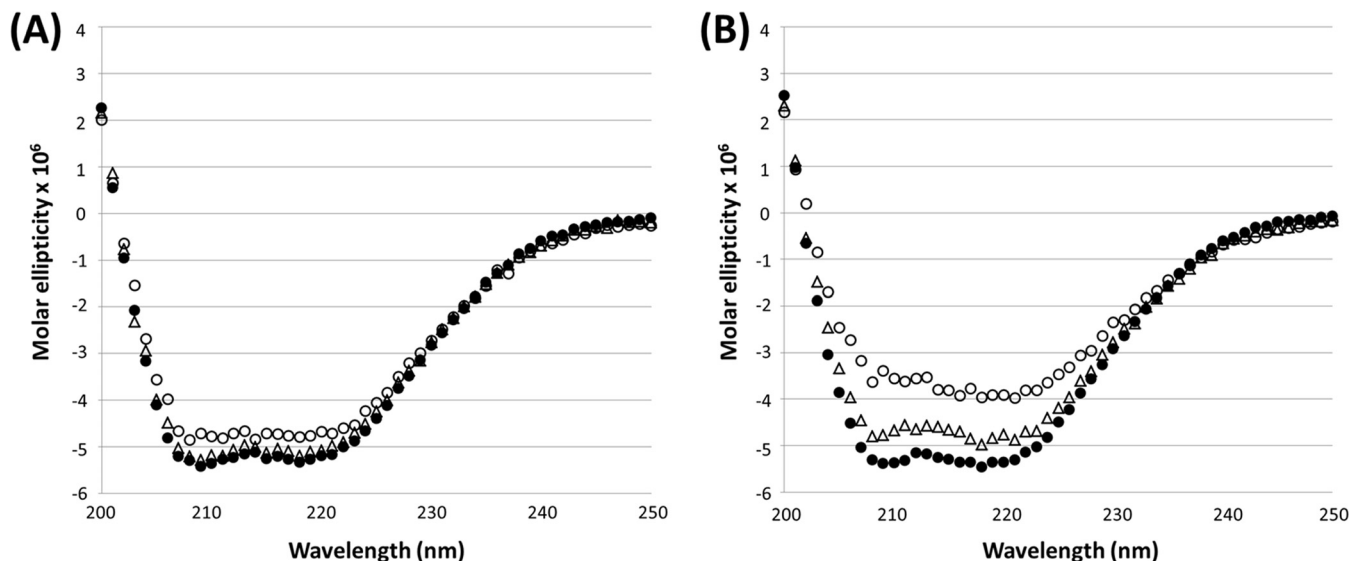
1.7-fold, at 40°C and 60°C, whereas CTX-M-15 showed more obviously attenuated k_{cat}/K_m, by 3.9- and 6.6-fold at 40°C and 60°C, respectively, compared with that at 25°C. Taken together, these results suggested that CTX-M-55 harbored higher structure stability and was more tolerant to the temperature change (Table 5 and Fig. 4). The higher stability of the protein structure of CTX-

FIG 4 Determination of secondary structures of CTX-M-55 (A) and CTX-M-15 (B) at 25°C (●), 40°C (△), and 60°C (○) by circular dichroism spectrum.
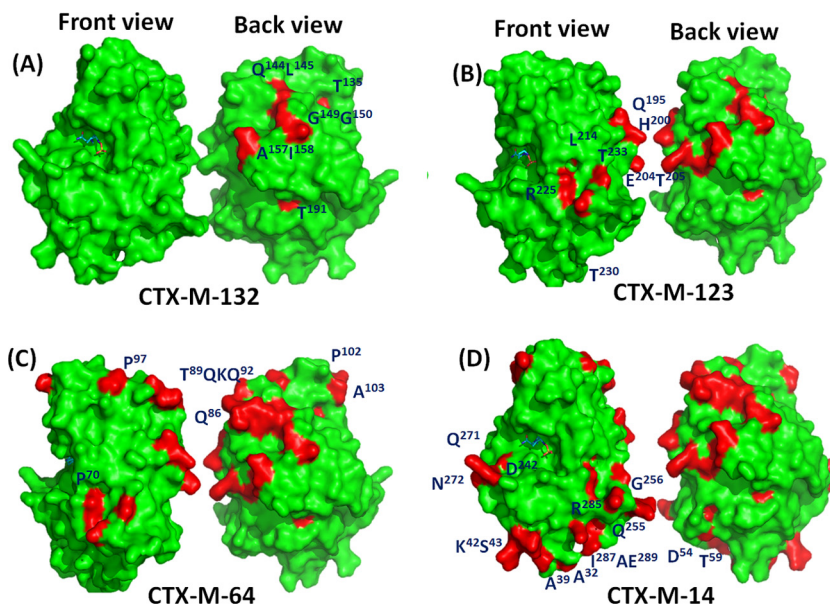
FIG 5 Structural models depicting the locations of divergent residues. Front and back views of the proteins are shown. The front view shows the active site side of protein, while the back view is distal to the active site. The active site is shown as the region with a compound bound. The structure of CTX-M-132, where the labeled residues highlighted in red are different from those in CTX-M-15 (A); the structure of CTX-M-123, where the labeled residues in red are different from those in CTX-M-132 and all the residues highlighted in red are different from those in CTX-M-15 (B); the structure of CTX-M-64, where the labeled residues in red are different from those in CTX-M-123 and all the residues highlighted in red are different from those in CTX-M-15 (C); and the structure of CTX-M-14, where the labeled residues in red are different from those in CTX-M-64 and all the residues highlighted in red are different from those in CTX-M-15 (D).

thin, was probably due to the introduction of these substitutions. The localization of these residues on the opposite surface to the active site suggested that surface residues distal to the active site could also contribute to the catalytic activity of CTX-Ms.

Further substitution of another set of 9 residues, $T^{118}S$, $N^{192}Q$, $K^{197}H$, $D^{201}E$, $S^{202}T$, $M^{211}L$, $Q^{222}R$, $A^{227}T$, and $V^{230}T$, in CTX-M-132 formed another new hybrid of CTX-M, CTX-M-123 (Fig. 1A and 5B). The catalytic activity of CTX-M-132 and CTX-M-123 toward different β-lactams was similar except toward ampicillin, suggesting these residues showed either a neutral effect or an antagonistic effect between some of them, such as the case of $P^{167}S$ and $D^{240}G$ reported by Novais et al., on the catalytic activity of these two CTX-Ms (16). Nonetheless, it is almost impossible to identify which ones within these 9 residues play important roles on the catalytic activity unless individual mutations are introduced into the background of CTX-M-132. Further substitution of several residues at the N termini, namely, $A^{67}P$, $K^{83}Q$, $S^{86}T$, $E^{87}Q$, $P^{88}K$, $N^{89}Q$, $R^{94}P$, $K^{99}P$, $S^{100}A$, $S^{118}T$, $T^{227}A$, and $T^{230}V$ in CTX-M-123, formed CTX-M-64 (Fig. 5C). It may be too speculative to assume these residues play critical roles in enhancing the cephalosporinase activity of CTX-M-64, considering the MIC results were similar for these two CTX-Ms. Nevertheless, the catalytic activity of CTX-M-64 toward several β-lactams, such as nitrocefin, ceftiofur, and ceftriaxone, were indeed higher than those of CTX-M-123, implying that some of these residues, including P^{67}, Q^{83}, T^{86}, Q^{87}, K^{88}, Q^{89}, P^{94}, P^{99}, A^{100}, T^{118}, A^{227}, and V^{230}, which are distal to the active site, may contribute to the catalytic activity of CTX-M-64 although the effects may not be crucial enough to alter the phenotypes of MICs (Tables 2 and 3). Further experiments with the individual mutation for each of the residues will be required to clarify their roles on the catalytic activity of CTX-M-64.

It is well accepted that CTX-M-64 is a hybrid between CTX-M-14 and CTX-M-15 and the closest CTX-M hybrid to CTX-M-14. Sequence analysis of CTX-M-14 and CTX-M-64 showed that these two CTX-Ms differed mainly at both C and N termini. Within these residues, the substitutions of $A^{27}S$, $D^{28}A$, $E^{35}A$, $R^{38}K$, $Q^{39}S$, $N^{50}D$, $S^{56}T$, $I^{58}V$, and $A^{62}G$ at the N termini and $A^{227}T$, $V^{230}T$, $G^{240}D$, $K^{254}Q$, $D^{255}G$, $I^{260}V$, $P^{270}Q$, and $K^{271}N$ at the C termini probably were the most important residues contributing to the dramatically decreased activity of CTX-M-14. Compared to CTX-M-14, these substitutions of CTX-M-64 extended the hydrolytic activities toward ampicillin, nitrocefin, cefuroxime, ceftiofur, ceftriaxone, and cefotaxime but resulted in increased sensitivity to clavulanic acid and sulbactam (Tables 2 to 4). Intriguingly, these residues were mostly located at the lower edge of the CTX-M-64 with the exception of one residue, D^{240}, which is close to the active site of CTX-M-64 (Fig. 5D). $D^{240}G$ has been reported to be associated with a higher MIC toward ceftazidime but reduced resistance to cefotaxime and/or cefepime due to an increased mobility of the B3 β strand and flexibility of the active site pocket, allowing the bulky ceftazidime to more easily accommodate the catalytic cavity (18, 19). To determine whether this residue contributed to the reduction in the catalytic activity observed in CTX-M-14, CTX-M-14($D^{240}G$) was generated by site-directed mutagenesis. The MICs of *E. coli* cells carrying CTX-M-14($D^{240}G$) were similar to that of CTX-M-14 toward all of the β-lactams tested (Table 2). The kinetic constants of CTX-M-14($D^{240}G$) showed that this substitution showed a limited effect on cephalosporin hydrolysis, with 2-fold differences, while slightly increased the k_{cat}/K_m toward ampicillin and nitrocefin by 4.3- and 2.1-fold, respectively, in our hands (Table 3). These results were somehow inconsistent with the finding that the single substitution of $D^{240}G$ in CTX-M-14 to become CTX-M-27 con-

ferred a higher MIC toward ceftazidime, yet this mutation also decreased the hydrolytic activity against other good substrates, such as cefuroxime and cefotaxime, compared to CTX-M-14 in a study by Bonnet et al. (20). The inconsistency may be due to differences in the methods and conditions used to determine the kinetic parameters in our approach and theirs, which used a computerized microacidimetric method (20).

Comparative analyses of these data suggested that, by acquisition of the middle part of CTX-M-14, the catalytic activity of CTX-M-15 could be enhanced through formation of highly efficient hybrids, such as CTX-M-132, CTX-M-123, and CTX-M-64. However, further acquisition of N and C termini of CTX-M-14 would dramatically decrease the catalytic activity of CTX-M-15. From the evolution point of view, it is likely that the formation of hybrids is through homologous recombination between CTX-M-14 and CTX-M-15, driven by the evolutionary pressure favoring selection of expanded-spectrum cephalosporins. Epidemiological findings that the hybrid CTX-Ms are more commonly reported from *E. coli* strains in regions where the use of expanded-spectrum cephalosporins is commonplace than in regions where expanded-spectrum cephalosporins are used less, support this hypothesis (21, 22).

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