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Molecular and Biochemical Characterization of CTX-M-131, a Natural Asp240Gly Variant Derived from CTX-M-2, Produced by a *Providencia rettgeri* Clinical Strain in São Paulo, Brazil

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CTX-M-131 is a natural Asp240Gly variant from the CTX-M-2 group detected in a *Providencia rettgeri* clinical strain from Brazil. Molecular analysis showed that $bla_{CTX-M-131}$ was inserted in a complex class 1 integron harbored by a 112-kb plasmid, which has not been previously described as a platform for CTX-M-encoding genes with the Asp240Gly mutation. Steady-state kinetic parameters showed that the enzyme has a typical cefotaximase catalytic profile and an enhanced activity against ceftazidime.

Antimicrobial resistance, a global public health problem, is facilitated by the selective pressure of antimicrobial use in clinical infections and other activities. Point mutations lead to the emergence of new extended-spectrum β-lactamase (ESBL) variants (1, 2), and Asp240Gly substitution is involved in ceftazidime resistance phenotypes among CTX-M variants (3). CTX-M-131 is a natural Asp240Gly variant within the CTX-M-2 cluster that has been detected in FSP309/05, a clinical strain of *Providencia rettgeri* from a university hospital in São Paulo, Brazil. This study reports the molecular and biochemical characterization of CTX-M-131.

The presence of $bla_{CTX-M-131}$ in *P. rettgeri* strain FSP309/05 was confirmed by PCR and sequencing, and the new variant has been assigned as CTX-M-131 by the β-lactamase database site http://www.lahey.org/studies/. The association of $bla_{CTX-M-131}$ with complex class 1 integrons was assessed through amplification using *sul1* forward (5′-GGCGGAACTTCACGCGATC-3′) and *qacEΔ1* reverse (5′-AGCTTAGTAAAGCCCTCGC-3′) primers. Sequencing of the resulting 4.5-kb fragment confirmed the insertion of $bla_{CTX-M-131}$ into the 3′ end of a complex class 1 integron, flanked upstream by *sul1* and IS*CR1* and downstream by a sequence having high homology to the chromosome of *Kluyvera* spp. and by the *orf3*::*qacEΔ1* fusion gene. This structure has already been described for CTX-M-2-encoding genes (4, 5), and it has the ability to integrate resistance genes into its variable region and move itself through the IS*CR1* rolling-circle mechanism (6). To our knowledge, this is the first description of the association of a gene coding for an Asp240Gly variant of CTX-M β-lactamases in this type of integron. This finding could represent a clue regarding the evolution of these genes, suggesting that the mutation at position 240 may have occurred after the bla_{CTX-M} genes were recruited from *Kluyvera*'s chromosome by an IS*CR*-mediated mechanism (7), provided that all the described genes associated with this structure harbor the "native" Asp240.

Wild plasmids were electroporated into One Shot TOP10 Electrocomp *Escherichia coli* (Invitrogen, USA), and transformants were selected on Luria-Bertani agar containing 4 μg/ml cefotaxime, with a transformation efficiency of 10^3 CFU/μg. The S1–pulsed-field gel electrophoresis (PFGE) technique was used to verify the presence of large-molecular-size plasmids in both *P. rettgeri* FSP309/05 and the *E. coli* EC309 transformant (8). *Providencia rettgeri* harbored two plasmids, 112 kb and 145.5 kb in size, while EC309 harbored only a 112-kb plasmid, which was demonstrated to express CTX-M-131. The MICs of β-lactams for both FSP309/05 and EC309 were assessed using Etest strips (bioMérieux, France), in accordance with the manufacturer's instructions, and results were interpreted according to CLSI guidelines (9). The MICs were the same for both strains for cefotaxime (>256 μg/ml), ceftazidime (6 μg/ml), aztreonam (6 μg/ml), cefepime (64 μg/ml), and ertapenem (0.094 μg/ml), while decreased values were observed for EC309 (compared to FSP309/05) for cefoxitin (4 μg/ml versus 8 μg/ml, respectively), cefotetan (0.19 μg/ml versus 0.38 μg/ml, respectively), amoxicillin-clavulanate (8 μg/ml versus 128 μg/ml, respectively), meropenem (0.016 μg/ml versus 0.094 μg/ml, respectively), and imipenem (0.75 μg/ml versus 4 μg/ml, respectively), probably due to the fact that *P. rettgeri* is a natural AmpC β-lactamase producer and therefore the phenotypic behavior of this isolate is the sum of coexpression of both β-lactamases. Both strains were also submitted to PCR-based replicon typing (PBRT) to search for incompatibility groups (10), although the plasmids were nontypeable by this technique.

Recombinant DNA methods were used to obtain clones expressing CTX-M-131 (11). PCR was carried out using primers C131-NheF (5′-GCTAGCCAGGCGAACAGCGTGCAA-3′) and C131-EcoRI (5′-GAATTCTCAGAAACCGTGGGTT-3′), containing NheI and EcoRI restriction sites (indicated by underlining), respectively, and the resulting 803-bp amplicon was purified and cloned using pTZ57R/T vector (Thermo Scientific, USA). The recombinant plasmid was then digested with NheI and EcoRI, and

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TABLE 1 Main kinetic parameters of CTX-M-131 β-lactamase[a]

β-Lactam substrate	k_{cat} (or k_{inact})[b] (s^{-1})	K_m (*or* K_i)[b] (μM)	k_{cat}/K_m ($\mu M^{-1}\ s^{-1}$) (or $K_{i,obs}$ [μM])[b]
Ampicillin	18 ± 1	37 ± 9	0.5 ± 0.15
Cephalothin	142 ± 6	23 ± 3	6.2 ± 0.9
Nitrocefin	49 ± 1	17 ± 4	2.9 ± 0.8
Cefuroxime	85 ± 4	33 ± 4	2.6 ± 0.4
Cefoxitin	9 ± 0.4	28 ± 1.2	0.3 ± 0.02
Cefotaxime	93 ± 4	70 ± 5	1.3 ± 0.1
Ceftazidime	7.5 ± 0.8	1,113 ± 116	0.007 ± 0.0008
Cefepime	30 ± 3	312 ± 50	0.096 ± 0.022
Aztreonam	22 ± 1.5	24 ± 2	0.9 ± 0.1
Imipenem	9 ± 0.6	102 ± 7	0.09 ± 0.01
Clavulanic acid (inhibitor)	0.031 ± 0.0045	0.64	5.10 ± 1.12
Tazobactam (inhibitor)	0.072 ± 0.005	0.18	1.47 ± 0.25

[a] Molar extinction coefficients and wavelengths are provided in parentheses for the indicated antibiotics: ampicillin (λ = 235 nm; $\Delta\varepsilon_{235} = -820\ M^{-1}\ cm^{-1}$), nitrocefin (λ = 482 nm; $\Delta\varepsilon_{482} = +15{,}000\ M^{-1}\ cm^{-1}$), cephalothin (λ = 273 nm; $\Delta\varepsilon_{273} = -6{,}300\ M^{-1}\ cm^{-1}$), cefuroxime (λ = 260 nm; $\Delta\varepsilon_{260} = -7{,}600\ M^{-1}\ cm^{-1}$), cefoxitin (λ = 260 nm; $\Delta\varepsilon_{260} = -6{,}600\ M^{-1}\ cm^{-1}$), ceftazidime (λ = 260 nm; $\Delta\varepsilon_{260} = -9{,}000\ M^{-1}\ cm^{-1}$), cefotaxime (λ = 260 nm; $\Delta\varepsilon_{260} = -7{,}500\ M^{-1}\ cm^{-1}$), cefepime (λ = 260 nm; $\Delta\varepsilon_{260} = -10{,}000\ M^{-1}\ cm^{-1}$), imipenem (λ = 300 nm; $\Delta\varepsilon_{300} = -9{,}000\ M^{-1}\ cm^{-1}$), and aztreonam (λ = 318 nm; $\Delta\varepsilon_{318} = -750\ M^{-1}\ cm^{-1}$).

[b] The parameters in parentheses apply to values for substrates indicated to be inhibitors. $K_{i,obs}$, observed K_i.

the released insert was ligated into an NheI-EcoRI-digested pET28a(+) expression vector. The ligation mixture was transformed into competent *E. coli* TOP10 F′ cells for selection and storage of recombinant clones, while a second transformation into competent *E. coli* BL21(DE3) cells was carried out and the resulting clones were used for CTX-M-131 production experiments. Constructions in both pTZ57R/T and pET28a(+) were double-strand sequenced to check the identity of CTX-M-131.

An overnight culture of the BL21(DE3) clone at 37°C in LB broth containing 30 μg/ml kanamycin was diluted 1/20 in the same culture medium and grown at 37°C until an optical density of 0.9 at 600 nm was reached, and 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) was added in order to induce β-lactamase expression. After 20 h of incubation at 28°C, crude extracts were obtained through sonication, and after centrifugation, supernatants containing CTX-M-131 were dialyzed against buffer A (50 mM Tris, 200 mM NaCl [pH 8.0]) and purified by affinity chromatography using HisTrap columns (GE Healthcare, USA) by elution with a linear gradient of buffer B (buffer A plus 500 mM imidazole [pH 8.0]). A total of 7.88 mg of highly pure CTX-M-131 β-lactamase, with a molecular mass of 28,800 Da, was obtained after thrombin digestion for His tag removal, in agreement with the theoretical mass (28,764 Da).

The hydrolysis of 12 β-lactam compounds by CTX-M-131 was monitored by absorbance variation at the corresponding wavelengths (Table 1), in phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 8 mM Na_2HPO_4) at 24°C, using a T-80 spectrophotometer (PG Instruments Ltd., United Kingdom), and steady-state kinetic parameters (K_m, k_{cat}, and k_{cat}/K_m) were determined. For poor substrates and inactivators, apparent K_m values were determined by competitive inhibition (as K_i) by monitoring the residual activity of the enzyme in the presence of nitrocefin as the reporter substrate (12). The main kinetic parameters for CTX-M-131 are shown in Table 1. The highest catalytic efficiency (k_{cat}/K_m) of CTX-M-131 was observed toward first- and second-generation cephalosporins and cefotaxime. We compared the kinetic parameters of key substrates for both CTX-M-131 and CTX-M-2, produced and assayed under the same conditions. The catalytic efficiencies (k_{cat}/K_m) observed toward cephalothin, cefotaxime, and ceftazidime were higher for CTX-M-131 than for CTX-M-2. For cefotaxime, the k_{cat}/K_m remained almost invariant (1.08-fold increase), being 1.2 $\mu M^{-1}\ s^{-1}$ for CTX-M-2 (K_m = 19 ± 2 μM^{-1}; k_{cat} = 23 ± 0.7 s^{-1}). On the other hand, for ceftazidime, k_{cat}/K_m increased from 0.0004 $\mu M^{-1}\ s^{-1}$ for CTX-M-2 to 0.007 $\mu M^{-1}\ s^{-1}$ for CTX-M-131, representing a 17.5-fold increment. Even though the efficiency toward ceftazidime is significantly higher for CTX-M-131, it represents only 0.12% and 0.5% of the k_{cat}/K_m values for cephalothin and cefotaxime, respectively, suggesting that the enzyme has only weak activity toward ceftazidime, as observed for the other studied Asp240Gly variants within the CTX-M enzymes (3). Also, CTX-M-131 was well inhibited by clavulanic acid and tazobactam, as observed by the low values for the enzyme's maximum rate constant for inactivation (k_{inact}).

This study presents the characterization of the novel ESBL CTX-M-131, a naturally occurring Asp240Gly variant within the CTX-M-2 cluster, which shows a typical cefotaximase catalytic profile and an enhanced activity against ceftazidime.

Like other CTX-M-2-encoding genes, $bla_{CTX\text{-}M\text{-}131}$ is part of a complex class 1 integron harbored by an easily transferable large-molecular-size plasmid, not previously described as a platform for dissemination of CTX-M-encoding genes having the Asp240Gly mutation.

It is generally accepted that CTX-M variants harboring the Asp240Gly mutation have emerged as a consequence of intense and sustained use of ceftazidime in clinical settings (7, 13). If this is true, CTX-M-2 could indeed be a "primordial" enzyme, which was recruited from *Kluyvera* at an early stage, and upon dissemination among clinical pathogens that were exposed to ceftazidime, mutations at position 240 could have emerged, as observed in CTX-M-131. On the other hand, alternative paths have probably derived from CTX-M-2, such as the incorporation of an arginine residue at position 274 (generally serine in most CTX-M enzymes) in TOHO-1 (14), and lately, upon ceftazidime use, the Asp240Gly mutation emerged, as seems to have occurred for CTX-M-43 (15).

Considering these findings, we could propose a new evolutionary scenario regarding the CTX-M β-lactamases, and we describe in this study the first β-lactamase belonging to the CTX-M-2 cluster that could have emerged from ceftazidime usage, representing the first Asp240Gly β-lactamase encoded by a $bla_{CTX\text{-}M}$ gene associated with IS*CR*-type integrons.

Nucleotide sequence accession number. The CTX-M-131 β-lactamase gene sequence has been deposited in GenBank under accession number JN969893.

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