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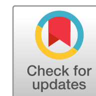

# CTX-M-190, a Novel $\beta$-Lactamase Resistant to Tazobactam and Sulbactam, Identified in an *Escherichia coli* Clinical Isolate

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**ABSTRACT** A novel $\beta$-lactamase, CTX-M-190, derived from CTX-M-55 by a single substitution of Ser for Thr at position 133 (Ser133Thr), was identified in a natural *Escherichia coli* clinical isolate. CTX-M-190 exhibited potent hydrolytic activity against cefotaxime, with a $k_{cat}/K_m$ ratio of 14.5 $\mu M^{-1}$ $s^{-1}$, and was highly resistant to inhibition by the $\beta$-lactamase inhibitors tazobactam and sulbactam, whose 50% inhibitory concentrations were 77- and 55-fold higher, respectively, for CTX-M-190 than for CTX-M-55. *bla*$_{CTX-M-190}$ was located within the genetic platform IS*Ecp1-bla*$_{CTX-M}$*-orf477*, which was harbored by a 70-kb IncI1 plasmid.

**KEYWORDS** *Escherichia coli*, inhibitor-resistant $\beta$-lactamase, CTX-M-190, tazobactam, sulbactam

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The high prevalence of CTX-M extended-spectrum $\beta$-lactamase (ESBL) genes in *Enterobacteriaceae*, particularly in *Escherichia coli* and *Klebsiella pneumoniae*, has been documented worldwide (1, 2). Since CTX-M-producing *Enterobacteriaceae* often confer resistance to many other antimicrobial agents, they constitute one of the most worrying problems in modern medical practice (2, 3). On the basis of the available evidence, $\beta$-lactam/$\beta$-lactamase inhibitors (BLBLIs), such as piperacillin-tazobactam, remain active *in vitro* against a high proportion of CTX-M-producing *Enterobacteriaceae* and may provide a reasonable carbapenem-sparing option for ESBL producers (4). A recent surveillance program conducted across China, which included 196 ESBL-producing *E. coli* and 124 ESBL-producing *K. pneumoniae* strains, found *bla*$_{CTX-M}$ occurrence in 99.5% of *E. coli* strains and 91.1% of *K. pneumoniae* strains and indicated resistance rates of 2.6% and 4.8% to piperacillin-tazobactam in these two kinds of ESBL producers (5). BLBLI resistance in CTX-M-producing *Enterobacteriaceae* is frequently associated with the coexistence of OXA-1 $\beta$-lactamases (6), whereas no natural CTX-M variants have been reported to confer resistance to BLBLIs.

**Isolation of a clinical *E. coli* strain resistant to piperacillin-tazobactam.** A clinical strain of *E. coli* HS37 was isolated from a urine specimen of a 56-year-old female outpatient with urinary tract infection in July 2015 at a university hospital in Shanghai, China. The Clinical and Laboratory Standards Institute (CLSI)-recommended double-disk synergy test confirmed the production of an ESBL by this isolate (7), while it displayed resistance to both piperacillin-tazobactam and ampicillin-sulbactam. The presence of a CTX-M-like $\beta$-lactamase in *E. coli* strain HS37 was confirmed by CTX-M-1 group-specific PCR and sequencing as previously described (8). The new CTX-M-1 group $\beta$-lactamase was derived from CTX-M-55 by a single substitution of Ser for Thr at position 133 (Ser133Thr) and was designated CTX-M-190 (Table 1).

**TABLE 1** Amino acid alterations of CTX-M-190 and $IC_{50}$s of tazobactam, sulbactam, and clavulanate for CTX-M-15, CTX-M-55, and CTX-M-190

| | Amino acid alteration (vs CTX-M-1 sequence) at position: | | | | | Mean $IC_{50}$ ± SD (μM)[a] | | |
|---|---|---|---|---|---|---|---|---|
| **β-Lactamase** | **80** | **117** | **133** | **242** | **289** | **Tazobactam** | **Sulbactam** | **Clavulanate** |
| CTX-M-1 | Val | Asp | Ser | Asp | Asn | ND[b] | ND | ND |
| CTX-M-3 | Ala | Asn | | | Asp | ND | ND | ND |
| CTX-M-15 | Ala | Asn | | Gly | Asp | 1.5 ± 0.4 | 5.8 ± 1.7 | 3.4 ± 0.7 |
| CTX-M-55 | | Asn | | Gly | Asp | 0.6 ± 0.2 | 1.4 ± 0.6 | 0.8 ± 0.3 |
| CTX-M-190 | | Asn | Thr | Gly | Asp | 46.2 ± 4.8 | 77.3 ± 5.2 | 0.5 ± 0.2 |

[a]$IC_{50}$, concentration of the β-lactamase inhibitor required to attain 50% enzyme inhibition. Data are the averages of the results obtained from three independent experiments.
[b]Not determined.

**Cloning of *bla*$_{\text{CTX-M-190}}$.** Plasmid DNA was extracted from HS37 with the Qiagen plasmid midikit (Qiagen, Hilden, Germany) and was introduced by electroporation into *E. coli* strain DH5α (Tiangen, Beijing, China). Transformants harboring the plasmid with *bla*$_{\text{CTX-M-190}}$ (pHS37) were selected on LB agar containing 50 μg/ml of ampicillin through specific PCR screening. *E. coli* DH5α clones producing CTX-M-190, CTX-M-55, and CTX-M-15 were obtained using the cloning vector pHSG396 (TaKaRa, Dalian, China), with PCR carried out using primers C1-BamHI-F (5′-CGGGATCCATGGTTAAAAA ATCACTGCG-3′) and C1-EcoRI-R (5′-CGGAATTCTTACAAACCGTCGGTGACGAT-3′), containing BamHI and EcoRI restriction sites and protective bases (indicated by underlining). PCR was performed with HS37 and other *E. coli* isolates that had been confirmed to produce CTX-M-55 or CTX-M-15. PCR products were purified with the TIANquick mini-purification kit (TIAGEN, Beijing, China), digested with BamHI and EcoRI (TaKaRa, Dalian, China), and then ligated into a BamHI-EcoRI-digested pHSG396 vector, which was then introduced by electroporation into *E. coli* DH5α. Transformants harboring the recombinant plasmids were selected on LB agar containing 50 μg/ml of chloramphenicol and were confirmed through PCR screening with the pair of cloning primers mentioned above.

Antimicrobial susceptibility testing for clinical isolate HS37, the corresponding DH5α transformant, and CTX-M-producing DH5α clones was performed by the CLSI reference broth microdilution method (7). The MICs are presented in Table 2. *E. coli* HS37, the corresponding pHS37 transformant, and CTX-M-190- and CTX-M-55-producing DH5α clones all were resistant to penicillins (ampicillin, amoxicillin, and piperacillin) and

**TABLE 2** MICs for the clinical isolate HS37, the corresponding DH5α transformant, and CTX-M-producing DH5α clones

| | MIC (μg/ml)[a] | | | | | | CLSI resistance breakpoint |
|---|---|---|---|---|---|---|---|
| **Antibiotics** | **HS37** | **DH5α(pHS37)[b]** | **DH5α(pCTX-M-190)** | **DH5α(pCTX-M-55)** | **DH5α(pCTX-M-15)** | **DH5α** | |
| Ampicillin | >1,024 | >1,024 | 256 | >1,024 | 1,024 | 8 | ≥32 |
| Ampicillin-sulbactam | 64 | 32 | 64 | 8 | 8 | 2 | ≥32/16 |
| Piperacillin | >1,024 | 512 | 512 | 512 | 512 | 2 | ≥128 |
| Piperacillin-tazobactam | 128 | 128 | 256 | 4 | 4 | 1 | ≥128/4 |
| Amoxicillin | >1,024 | >1,024 | 512 | >1,024 | >1,024 | 2 | NA[c] |
| Amoxicillin-clavulanate | 8 | 4 | 2 | 4 | 4 | 1 | ≥32/16 |
| Ceftazidime | 64 | 32 | 32 | 32 | 16 | 0.125 | ≥16 |
| Ceftazidime-clavulanate | 0.125 | 0.125 | 0.25 | 0.125 | 0.125 | 0.125 | NA |
| Cefotaxime | 128 | 128 | 128 | 256 | 256 | 0.125 | ≥4 |
| Cefotaxime-clavulanate | 0.25 | 0.125 | 0.125 | 0.125 | 0.125 | ≤0.06 | NA |
| Aztreonam | 128 | 8 | 4 | 32 | 16 | ≤0.06 | ≥16 |
| Cefepime | 64 | 32 | 32 | 4 | 2 | ≤0.06 | ≥16 |
| Cefoxitin | 16 | 2 | 2 | 2 | 2 | 2 | ≥32 |
| Imipenem | 0.125 | ≤0.06 | ≤0.06 | ≤0.06 | ≤0.06 | ≤0.06 | ≥4 |
| Ertapenem | 0.5 | 0.125 | 0.125 | 0.125 | 0.125 | ≤0.06 | ≥2 |

[a]For amoxicillin-clavulanate and ampicillin-sulbactam, the combinations were tested with concentrations at a 2:1 ratio (antibiotic: inhibitor). For piperacillin-tazobactam, ceftazidime-clavulanate, and cefotaxime-clavulanate, the inhibitors were tested at a fixed concentration of 4 μg/ml.
[b]The corresponding DH5α transformant of *E. coli* HS37, with plasmid pHS37 harboring *bla*$_{\text{CTX-M-190}}$.
[c]NA, breakpoint criterion was not available in the CLSI interpretive standards.

**TABLE 3** Kinetic parameters of CTX-M-190 and CTX-M-55[a]

| Substrate | CTX-M-190 | | | CTX-M-55 | | |
|---|---|---|---|---|---|---|
| | $K_m$ (μM) | $k_{cat}$ ($s^{-1}$) | $k_{cat}/K_m$ ($\mu M^{-1}$ $s^{-1}$) | $K_m$ (μM) | $k_{cat}$ ($s^{-1}$) | $k_{cat}/K_m$ ($\mu M^{-1}$ $s^{-1}$) |
| Ampicillin | 46.9 | 101.7 | 2.2 | 114.9 | 422.6 | 3.7 |
| Nitrocefin | 26.6 | 21.9 | 0.82 | 25.5 | 9.2 | 0.36 |
| Piperacillin | 44.3 | 58.4 | 1.3 | 21.9 | 46.4 | 2.1 |
| Cefotaxime | 11.3 | 164.3 | 14.5 | 16.6 | 125.8 | 7.6 |
| Ceftazidime | 378.7 | 5.5 | 0.015 | 758.7 | 6.9 | 0.009 |
| Cefepime | 168.4 | 16.4 | 0.097 | 594.1 | 7.3 | 0.012 |

[a]Data are the averages of the results obtained from three independent experiments.

cephalosporins (cefotaxime, ceftazidime, and cefepime), except that the CTX-M-55-producing DH5α clone was intermediate to cefepime, while all strains were susceptible to cefoxitin, imipenem, and ertapenem.

Interestingly, tazobactam and sulbactam did not restore the susceptibility of *E. coli* HS37, the corresponding pHS37 transformant, and the CTX-M-190-producing DH5α clone to penicillins, and their MICs of piperacillin-tazobactam and ampicillin-sulbactam were 8- to 32-fold higher than those of the CTX-M-55- and CTX-M-15-producing clones (Table 2). In contrast, clavulanate did significantly reduce the MICs of amoxicillin, ceftazidime, and cefotaxime in *E. coli* HS37 and all of the DH5α clones.

**Fifty-percent inhibitory concentration ($IC_{50}$) determination and β-lactamase kinetic analysis.** Recombinant pET28a(+) (Novagen, Darmstadt, Germany) expression derivatives were constructed for CTX-M-190, CTX-M-55, and CTX-M-15 β-lactamases. The above-mentioned BamHI-EcoRI-digested PCR products were ligated into a BamHI-EcoRI-digested pET28a(+) expression vector, which was then introduced by electroporation into *E. coli* BL21(DE3) (Novagen, Darmstadt, Germany). The expression of the three kinds of CTX-M β-lactamases was induced by 0.75 mM IPTG (isopropyl-β-D-thiogalactopyranoside) as previously described (9), and proteins were purified by using nickel magnetic beads for His tag protein purification (Biotool, Houston, TX, USA), following the manufacturer's instructions. Purity was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis.

The concentration of each β-lactamase inhibitor required to attain 50% enzyme inhibition ($IC_{50}$) was determined after incubation of the purified CTX-M enzymes and inhibitors for 10 min at 30°C. The reporter substrate was ampicillin, which was used at a concentration of 100 μM (10). The $IC_{50}$s of tazobactam and sulbactam for CTX-M-190 were 77- and 55-fold higher than those of CTX-M-55 (Table 1). In contrast, CTX-M-190 shared a similar $IC_{50}$ for clavulanate with CTX-M-55, and the $IC_{50}$ of clavulanate for CTX-M-190 was sevenfold lower than that of CTX-M-15.

The wild-type CTX-M-190 and CTX-M-55 β-lactamases, each with a molecular mass of 32.7-kDa by mass spectrometry analysis, were obtained after thrombin digestion for His tag removal. The hydrolysis of β-lactam compounds by CTX-M-190 and CTX-M-55 was monitored by absorbance variation at the appropriate wavelengths in phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 1.8 mM $KH_2PO_4$, 10 mM $Na_2HPO_4$) at 30°C using a UV-2700 spectrophotometer (Shimadzu, Kyoto, Japan), and steady-state kinetic parameters ($K_m$, $k_{cat}$, and $k_{cat}/K_m$) were determined as previously described (9, 10). As shown by the results in Table 3, the highest catalytic efficiency of CTX-M-190 was observed toward ampicillin and cefotaxime, with $k_{cat}/K_m$ ratios of 2.2 and 14.5 $\mu M^{-1}$ $s^{-1}$, respectively. For cefepime, the $k_{cat}/K_m$ value of CTX-M-190 was eightfold higher than that of CTX-M-55, indicating that CTX-M-190 might have an elevated hydrolytic activity toward cefepime.

**Genetic environments of *bla*$_{\text{CTX-M-190}}$.** The plasmid profiles of *E. coli* HS37 and the transformants were determined by S1 nuclease pulsed-field gel electrophoresis (PFGE), and plasmid pHS37 was characterized by PCR-based replicon typing (11). *E. coli* HS37 only had a single 70-kb plasmid, which was characterized as an IncI1 type. PCR

screening revealed that only $bla_{TEM-1}$ and $bla_{CTX-M-190}$ were found on pHS37, as well as *E. coli* HS37. pHS37 is a self-transferable plasmid, and the frequency of conjugation was approximately $10^{-3}$ recombinants per donor cell (*E. coli* strain J53). pHS37 was extracted from the DH5$\alpha$ transformant with the Qiagen plasmid midikit (Qiagen, Hilden, Germany), and the genetic environment around $bla_{CTX-M-190}$ was determined through primer-walking sequencing. Just like other CTX-M-1 group $\beta$-lactamase genes, $bla_{CTX-M-190}$ was located within the classical genetic platform, IS*Ecp1*-$bla_{CTX-M}$-*orf477* (12). IS*Ecp1* is composed of an open reading frame (*orf*) encoding a transposase with 420 amino acids and two imperfect and inverted repeats, while *orf477* encodes a protein of 110 amino acids with unknown function (12).

Here, we report a novel CTX-M $\beta$-lactamase, CTX-M-190, which displayed resistance to inhibition by tazobactam and sulbactam and retained hydrolytic activity against expanded-spectrum cephalosporins. As irreversible "suicide inhibitors," clavulanate, sulbactam, and tazobactam exert their inhibitive effects by following similar reaction pathways, beginning with the formation of an acyl enzyme species and then proceeding to the formation of transient imine or enamine intermediates (13). Depending on the properties of the $\beta$-lactamase and inhibitor, the reaction will ultimately proceed to deacylation or irreversible inactivation (13). Ser133 is a conserved amino acid among all class A $\beta$-lactamases, and it also serves as a second nucleophile which attacks the transient intermediates and becomes covalently cross-linked to Ser70 in the terminal inactivation of the mechanism-based inhibitors (14). The specific substitution in CTX-M-190, Ser133Thr, may bring about structural changes that discourage the formation of the acyl enzyme complex or reduce the stability of the intermediate species with sulbactam or tazobactam and, thus, lead to deacylation and regeneration of the active CTX-M-190 $\beta$-lactamase.

Unlike the previously reported inhibitor-resistant TEM and SHV enzymes, which are typically most resistant to inhibition by clavulanate and sulbactam (13), CTX-M-190 was resistant to inhibition by tazobactam, showing a different and distinctive pattern. To the best of our knowledge, this is the first report of a CTX-M $\beta$-lactamase with resistance to inhibition by tazobactam and sulbactam in a natural clinical isolate. CTX-M-190 may foreshadow the emergence of a new class of versatile $\beta$-lactamases with even broader hydrolytic and resistance profiles.

Previous studies demonstrated that $\beta$-lactamases from all CTX-M groups were able to acquire mutations reducing BLBLI susceptibility upon exposure to BLBLIs (15). An interesting question is why natural inhibitor-resistant CTX-M variants have not previously been described in clinical isolates. It has been suspected that the phenotypic patterns shown by the inhibitor-resistant CTX-M variants might be obscured by the more common inhibitor-resistant TEM enzymes or the coproduction of other $\beta$-lactamases, as their lack of detection might be more a methodological issue than a real absence in clinical strains (16). The emergence of CTX-M-190 highlights the ongoing and complex evolution of CTX-M $\beta$-lactamases.

**Accession number(s).** The nucleotide sequence of the $bla_{CTX-M-190}$ gene is available in GenBank under accession number KX664469.

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