High-Level Resistance to Ceftazidime Conferred by a Novel Enzyme, CTX-M-32, Derived from CTX-M-1 through a Single Asp240-Gly Substitution

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The emergence of plasmid-mediated extended-spectrum β-lactamases in members of the family Enterobacteriaceae has become a worldwide problem (3, 4, 6, 7, 11–13, 16).

Most extended-spectrum β-lactamases are derivatives of TEM-1, TEM-2, or SHV-1 enzymes; however, there are an increasing number of reports that describe the worldwide emergence of β-lactamases belonging to other families, such as CTX-M and/or OXA derivatives (8).


Here we report the molecular characterization of a new CTX-M β-lactamase derived from CTX-M-1 through a single Asp240-Gly substitution, CTX-M-32. In addition, we report experimental data showing that substitution of this amino acid is itself sufficient to confer hydrolytic activity against ceftazidime.

Patterns of antibiotic susceptibility shown by the clinical strain Enterobacter cloacae isolated from pleural liquid with high levels of resistance to ceftaxime, ceftazidime, and aztreonam harbors a novel CTX-M gene (blaCTX-M-32) whose amino acid sequence differs from that of CTX-M-1 by a single Asp240-Gly substitution. Moreover, by site-directed mutagenesis we demonstrated that this replacement is a key event in ceftazidime hydrolysis.

Escherichia coli TG1 transformant harboring the pMC-2 plasmid showed higher MICs of the affected antibiotics, probably due to more copies of the bla gene.

Isoelectric focusing was performed using polyacrylamide gels containing Ampholine, within a pH range of 3.5 to 9.5, as previously described (17). The clinical isolate produced one enzyme with a pI of 9.0.

In the present study the E. coli XL1-Blue MRF’Kan strain (Stratagene Europe, Amsterdam, The Netherlands) was used in the conjugation experiments.

The clinical strain JC19325 had one plasmid which harbored a β-lactamase with a pI of 9.0 that was transferred by conjugation into E. coli XL1-Blue MRF’Kan using kanamycin (25 µg/ml) and ceftaxime (2 µg/ml) as selective antibiotics. A few of the transconjugants which grew harbored an identical plasmid of approximately 15 kb, which was named pMC-1.

Plasmid DNA was isolated by the alkaline lysis method (23) from the transconjugant that produced a single β-lactamase with a pI of 9.0. Plasmid DNA was digested with KpnI and ligated to the plasmid vector pBGS18− (25); afterwards, the ligation mixture was introduced into E. coli TG1 cells by transformation with CaCl2, and transformants were detected on Luria-Bertani agar plates with cefotaxime (25 µg/ml) and ceftazidime (25 µg/ml) as selective antibiotics. A few of the transconjugants which grew harbored an identical plasmid of approximately 15 kb, which was named pMC-1.

Patterns of antibiotic susceptibility shown by the clinical strain E. coli JC19325, as well as its transconjugant and transformants, are shown in Table 1. The MICs were determined by E-test and interpreted according to the method of the National Committee for Clinical Laboratory Standards (18). The clinical strain JC19325 showed a high level of resistance to ceftaxime, ceftazidime, and aztreonam (MICs of >256 µg/ml), cefoxitin (MIC of >256 µg/ml), and cefepime (MIC of 64 µg/ml). Moreover, clavulanic acid acted synergistically with amoxicillin, cefotaxime, and ceftazidime (E-test; ABBiodisk, Solna, Sweden), thus indicating the presence of a class A β-lactamase (9). An

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was the inverted repeat right (IRR) sequence of ISEcp1B 80 bp upstream of the ATG start codon of CTX-M-32. No putative promoter sequences were found in the 80-bp sequence that separated the IRR of ISEcp1B from the ATG site of the bla\textsubscript{CTX-M-32} gene; moreover, this IRR provided −35 and −10 promoter sequences, thus probably contributing to the expression of the bla\textsubscript{CTX-M-32} gene. (iii) Third, this IRR was downstream of a tnpA gene that encoded the transposase of IS5. Figure 1 shows the 2,326-bp sequence of the original 4-kbp KpnI fragment.

To purify the CTX-M enzyme, the bla\textsubscript{CTX-M-32} gene was cloned in the pGEX-6P-1 vector, which allowed a fusion protein between glutathione S-transferase (GST) and the CTX-M \( \text{β-lactamase} \) was used at a 1,670 μM concentration. The CTX-M-32 \( \text{β-lactamase} \) was purified to homogeneity following the manufacturer’s directions for the GST gene fusion system (Amersham Pharmacia Biotech, Europe GmbH). The purified protein appeared on sodium dodecyl sulfate-polyacrylamide gel electrophoresis as a band of 28 kDa (≥99% pure) (Fig. 2).

For kinetic experiments, CTX-M-32 \( \text{β-lactamase} \) was used at a 1,800 μM concentration. The CTX-M-32 \( \text{β-lactamase} \) showed a hydrolytic profile similar to that expected for a molecular class A CTX-M enzyme (6), with the \( K_m \) for ampicillin lower than the \( K_m \) for cefalothin, a \( K_m \) for cefotaxime of 0.0001 and 1.5; therefore, a lower catalytic efficiency with respect to cefazidime was detected with CTX-M-1, according to the differences in cefazidime MICs between CTX-M-32 and CTX-M-1 enzymes (Table 1).

Three different enzymes, CTX-M-15, -16, -19 and, recently, CTX-M-27 have been reported to be associated with cefazidime hydrolysis (4, 5, 20, 21). The amino acid changes associated with the phenotype of cefazidime hydrolysis were a Pro-to-Ser substitution at position 167 in CTX-M-19 with respect to CTX-M-18 (20) and an Asp-to-Gly substitution at position 240 in CTX-M-16 with respect to CTX-M-9 (4) and in CTX-M-27 with respect to CTX-M-14 (5). In agreement with these previous results, we also report that the Asp240 substitution is a key factor in the evolution of CTX-M \( \text{β-lactamases} \), as it increases their hydrolytic activity toward cefazidime.

Regarding the CTX-M enzymes, to our knowledge only six different enzymes have been published in the group 1 CTX-M

<table>
<thead>
<tr>
<th>Antibiotics</th>
<th>JC19325 (produces CTX-M-32)</th>
<th>XLI(pMC-3') (produces CTX-M-32)</th>
<th>TG1 (produces CTX-M-32)</th>
<th>TG1(pMC-2') (produces CTX-M-32)</th>
<th>TG1(pMC-3') (produces CTX-M-1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amoxicillin</td>
<td>&gt;256</td>
<td>&gt;256</td>
<td>3</td>
<td>&gt;256</td>
<td>&gt;256</td>
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<tr>
<td>Amoxicillin + clavulanate</td>
<td>12</td>
<td>6</td>
<td>2</td>
<td>4</td>
<td>8</td>
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<tr>
<td>Piperacillin</td>
<td>&gt;256</td>
<td>&gt;256</td>
<td>0.38</td>
<td>&gt;256</td>
<td>&gt;256</td>
</tr>
<tr>
<td>Cephalothin</td>
<td>&gt;256</td>
<td>&gt;256</td>
<td>3</td>
<td>&gt;256</td>
<td>&gt;256</td>
</tr>
<tr>
<td>Cefuroxime</td>
<td>&gt;256</td>
<td>&gt;256</td>
<td>1.5</td>
<td>&gt;256</td>
<td>&gt;256</td>
</tr>
<tr>
<td>Cefotaxime</td>
<td>&gt;256</td>
<td>&gt;256</td>
<td>2</td>
<td>&gt;256</td>
<td>&gt;256</td>
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<tr>
<td>Cefotaxime + clavulanate</td>
<td>&gt;1</td>
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<td>0.02</td>
<td>&gt;256</td>
<td>&gt;256</td>
</tr>
<tr>
<td>Cefazidime</td>
<td>128</td>
<td>96</td>
<td>0.06</td>
<td>&gt;256</td>
<td>6</td>
</tr>
<tr>
<td>Cefazidime + clavulanate</td>
<td>&gt;4</td>
<td>0.25</td>
<td>0.06</td>
<td>&gt;256</td>
<td>0.19</td>
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<tr>
<td>Cefepime</td>
<td>64</td>
<td>16</td>
<td>0.02</td>
<td>64</td>
<td>48</td>
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<tr>
<td>Aztreonam</td>
<td>&gt;256</td>
<td>&gt;256</td>
<td>0.03</td>
<td>&gt;256</td>
<td>48</td>
</tr>
<tr>
<td>Imipenem</td>
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<td>0.25</td>
<td>0.12</td>
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<td>Meropenem</td>
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<td>0.03</td>
<td>0.008</td>
<td>0.02</td>
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</tr>
</tbody>
</table>

\( ^* \) Clavulanate was used at 4 μg/ml.
\( ^b \) Transconjugant harboring CTX-M-32.
\( ^c \) Transformant harboring CTX-M-32 \( \text{β-lactamase} \) gene.
\( ^d \) Transformant harboring CTX-M-32mut or CTX-M-1 \( \text{β-lactamase} \) gene.
FIG. 1. Nucleotide sequence of a 2,326-bp DNA fragment of the pMC-2 plasmid. The deduced amino acid sequence is indicated in single-letter code below the nucleotide sequence. Stop codons are indicated by asterisks. The /H11002 and /H11002 promoter sequences of the \bla CTX-M-32 gene and the IRR sequence of IS\Ecp1 are underlined and indicated by bold letters, as is the /H11001 position of the transcriptional start of the \bla CTX-M-32 gene (10). The CTX-M-32 and transposase of IS\5 proteins are indicated by arrows. Bold amino acids are those conserved in class A \beta-lactamases (15). Oligonucleotides used for sequencing are indicated by arrows, and KpnI restriction sites delimiting the 4-kbp insert are also underlined.
FIG. 1—Continued.
enzymes: CTX-M-1, -3, -10, -12, -15, and -32 (3, 16, 19, 20). Among these, only CTX-M-15 and -32 showed more efficient ceftazidime hydrolysis than their parental enzymes, CTX-M-3 and CTX-M-1, respectively. The two former enzymes share the same amino acid substitution, although CTX-M-15 differs from CTX-M-32 in four additional amino acid changes. In terms of evolution, CTX-M-32 is probably an ancestor between CTX-M-1 and CTX-M-15 and constitutes a step forward in the evolution of β-lactamase in broad-spectrum hydrolysis of antibiotics such as ceftazidime.

In summary, we report the genetic and biochemical characterization of a new CTX-M enzyme, CTX-M-32. This is the fourth report of a CTX-M β-lactamase isolation in Spain, as CTX-M-9, CTX-M-10, and CTX-M-14 have previously been isolated in this country (7, 19, 22, 24).

Nucleotide sequence accession number. The GenBank accession number for the CTX-M-32 β-lactamase is AJ557142.

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REFERENCES


