Novel Plasmid-Encoded Class C β-Lactamase (MOX-2) in *Klebsiella pneumoniae* from Greece

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*Klebsiella pneumoniae* KOL, a clinical strain resistant to various β-lactams, was isolated from the stools of a patient from Greece. This strain harbored a new pl 9.1 plasmid-mediated AmpC β-lactamase with unusually high levels of hydrolytic activity for cefoxitin and cefotetan that we named MOX-2. Sequencing of **bla**MOX-2 revealed 93.2, 92.9, 92.7, and 73.1% identities with the deduced amino acid sequences of CMY-8, MOX-1, CMY-1, and the AmpC β-lactamase of *Aeromonas sobria*, respectively.

Plasmid-encoded AmpC-type β-lactamases have been described in clinical strains of various Enterobacteria. For some of them, the amino acid and nucleotide sequences of the corresponding genes are very similar to those of the chromosome-encoded AmpC β-lactamases of *Enterobacter cloacae* (ACT-1 and MIR-1), *Citrobacter freundii* (CMY-2, CMY-4, CMY-5, and LAT-1), *Morganella morganii* (DHA-1 and DHA-2), and *Haemophilus alvei* (ACC-1) (15). The phylogeny of MOX-1, CMY-1, and FOX-type enzymes (5, 7, 12) is unclear as they show lower sequence similarities (≤77%) to those of the chromosomally encoded AmpC β-lactamases of *Aeromonas sobria*, *Pseudomonas aeruginosa*, and *Serratia marcescens* (15, 18).

A strain of *Klebsiella pneumoniae* (KOL) resistant to various β-lactam antibiotics, including cephalexin, was isolated in 1997 at Lariboisière Hospital, Paris, France, from the stools of a patient transferred from an intensive care unit in Athens, Greece, for treatment of a carotid cavernous fistula after a road accident (S. Boyer, L. Raskine, B. Hanau, A. Philippon, 3rd Service de Neuroradiologie, CHU Lariboisière, Paris, France, May 15, 2021 by guest http://aac.asm.org/ Downloaded from 1997). He had previously received ampicillin-sulbactam and pefloxacin for a patient from Greece. This strain harbored a new pI 9.1 plasmid-mediated AmpC β-lactamase with unusually high levels of hydrolytic activity for cefoxitin and cefotetan that we named MOX-2. Sequencing of **bla**MOX-2 revealed 93.2, 92.9, 92.7, and 73.1% identities with the deduced amino acid sequences of CMY-8, MOX-1, CMY-1, and the AmpC β-lactamase of *Aeromonas sobria*, respectively.

**Klebsiella pneumoniae** KOL was highly resistant to *β*-lactams, was isolated from the stools of a patient from Greece. This strain harbored a new pl 9.1 plasmid-mediated AmpC β-lactamase with unusually high levels of hydrolytic activity for cefoxitin and cefotetan that we named MOX-2. Sequencing of **bla**MOX-2 revealed 93.2, 92.9, 92.7, and 73.1% identities with the deduced amino acid sequences of CMY-8, MOX-1, CMY-1, and the AmpC β-lactamase of *Aeromonas sobria*, respectively.

Plasmid DNA was extracted from the transconjugant according to the method of Kado and Liu for large plasmids (8). Its analysis revealed one plasmid of about 130 kb (data not shown).

Analytical isoelectric focusing was performed on a polyacrylamide gel with sonicated crude cell extracts as described previously (11). Three bands of β-lactamase activity with pls of 9.1, 5.4, and 8.2 were detected in *K. pneumoniae* KOL and its transconjugant. A fourth band (pl 7.6) was present only in *K. pneumoniae* KOL (data not shown).

The β-lactamase with a pl of 9.1 from the transconjugant was characterized after purification as previously described (4). The kinetic constants, *k*_cat and *K*_m, for substrates were determined by computerized microacidimetric assay at pH 7.0 and 37°C in 0.1 M NaCl as described by Labia et al. (9). One unit of β-lactamase activity was defined as the amount of enzyme required to hydrolyze 1 μmol of benzylpenicillin per min at pH 7.0 and 37°C.

On the basis of the *k*_cat values, MOX-2 hydrolyzed cefazolin 156 times faster than benzylpenicillin. Cefoxitin and cefotetan were hydrolyzed 7 and 3 times faster, respectively, than benzylpenicillin. These latter rates of hydrolysis were unusually high. The *k*_cat values were about 100 times higher than the values generally reported for class C β-lactamases (13), but *k*_cat/*K*_m values were close to normal values, suggesting ready deacylation for these antibiotics. In contrast, the *k*_cat of moxalactam remained low (*k*_cat = 0.04 s⁻¹), which is probably related to the carbonyl of the 7-α side chain of this molecule. In terms of *K*_m, MOX-2 had a higher affinity for cefoxitin than for cephalothin or benzylpenicillin. The *K*_m values of MOX-2 for cefotaxime, ceftazidime, and aztreonam were lower than 2262–2265.2002


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Recombinant plasmids were introduced into *E. coli* the CaCl₂ transformation method (16). Transformants with 

fragments were ligated to the vector pBK-CMV (Stratagene, La Jolla, Calif.) and digested with 

substrates.

**TABLE 1. MICs of β-lactams for *K. pneumoniae* KOL; its transconjugant; the clones producing TEM-1, SHV-5, and MOX-2; *E. coli* 53-2; and *E. coli* JM101**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Plasmid</th>
<th>bla</th>
<th>AMX</th>
<th>FOX</th>
<th>CTT</th>
<th>CAZ</th>
<th>CAZ-CLA</th>
<th>CTX</th>
<th>MOX</th>
<th>FEP</th>
<th>IMP</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>K. pneumoniae</em> KOL</td>
<td>pKOL</td>
<td>MOX-2 SHV-5</td>
<td>1,024</td>
<td>1,024</td>
<td>64</td>
<td>256</td>
<td>8</td>
<td>8</td>
<td>4</td>
<td>4</td>
<td>0.125</td>
</tr>
<tr>
<td></td>
<td>pKOL</td>
<td>SHV-5 TEM-1</td>
<td>512</td>
<td>128</td>
<td>4</td>
<td>1</td>
<td>0.125</td>
<td>1</td>
<td>0.25</td>
<td>0.25</td>
<td>0.06</td>
</tr>
<tr>
<td><em>E. coli</em> 53-2 TcKOL</td>
<td>pKOL</td>
<td>MOX-2 SHV-5</td>
<td>4</td>
<td>4</td>
<td>0.125</td>
<td>0.25</td>
<td>0.25</td>
<td>0.06</td>
<td>0.25</td>
<td>0.25</td>
<td>0.03</td>
</tr>
<tr>
<td></td>
<td>pKOL</td>
<td>SHV-5 TEM-1</td>
<td>&gt;1,024</td>
<td>4</td>
<td>0.125</td>
<td>0.5</td>
<td>0.5</td>
<td>0.06</td>
<td>0.125</td>
<td>0.5</td>
<td>0.125</td>
</tr>
</tbody>
</table>

*Abbreviations: bla, β-lactamase; AMX, amoxicillin; FOX, cefoxitin; CTT, cefotetan; CAZ, ceftazidime; CTX, cefotaxime; MOX, moxalactam; FEP, cefepime; IMP, imipenem; CLA, clavulanic acid (2 µg/ml).*

those usually observed, about 1/10 of those reported for chromosome-encoded enzymes (Table 2).

The BLASTN program (1) at the National Center for Biotechnology Information was used for database searches. The ClustalW program (www.infobiogen.fr) was used to align multiple-protein sequences. Open reading frames (ORFs) were identified with the ORF Finder program (www.pasteur.fr).

DNA sequencing of the PCR products of the clones harboring pLRB02 and pLRB03 revealed that these clones produced an SHV-5-type β-lactamase (pI 8.2) and a TEM-1 β-lactamase (pI 5.4), respectively.

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**TABLE 2. Kinetic parameters of purified MOX-2 β-lactamase**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$k_{cat}$ (s⁻¹)</th>
<th>$K_m$ (µM)</th>
<th>$k_{cat}/K_m$ (s⁻¹ µM⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benzylpenicillin</td>
<td>5</td>
<td>9.7</td>
<td>0.51</td>
</tr>
<tr>
<td>Cephalothin</td>
<td>250</td>
<td>78</td>
<td>3.20</td>
</tr>
<tr>
<td>Cephaloridine</td>
<td>180</td>
<td>890</td>
<td>0.20</td>
</tr>
<tr>
<td>Cefazolin</td>
<td>800</td>
<td>712</td>
<td>1.12</td>
</tr>
<tr>
<td>Cefotaxime</td>
<td>0.054</td>
<td>0.9</td>
<td>0.01</td>
</tr>
<tr>
<td>Cefazidime</td>
<td>0.005</td>
<td>0.01</td>
<td>0.005</td>
</tr>
<tr>
<td>Cefepime</td>
<td>1</td>
<td>20</td>
<td>0.05</td>
</tr>
<tr>
<td>Aztreonam</td>
<td>&lt;0.01</td>
<td></td>
<td>0.01</td>
</tr>
<tr>
<td>Cefoxitin</td>
<td>35</td>
<td>300</td>
<td>0.12</td>
</tr>
<tr>
<td>Cefotetan</td>
<td>15</td>
<td>42</td>
<td>0.35</td>
</tr>
<tr>
<td>Moxalactam</td>
<td>0.04</td>
<td>0.30</td>
<td></td>
</tr>
</tbody>
</table>

*$k_{cat}$ values were not calculated for these substrates.

The 6,170-bp DNA insert of pLRB01 was sequenced, and four ORFs were identified (Fig. 1). An ORF of 1,149 bp (nucleotides [nt] 4620 to 5768) encoded a putative protein of 382 amino acids, with an estimated molecular mass of 38.5 kDa, preceded by putative Shine-Dalgarno and promoter consensus sequences [TTGGCG(N)₁₆TACTTG]. The product of this ORF was most similar to the plasmid-encoded β-lactamases CMY-8 (19) (93.2% sequence identity), MOX-1 (7) (92.9% sequence identity), and CMY-1 (5) (92.7% sequence identity), followed by the chromosone-encoded AmpC β-lactamase of *A. sobria* (73.1% sequence identity). It showed a lower level of similarity to the AmpC β-lactamases of *E. cloacae*, *C. freundii*, *M. morganii*, *H. alvei*, *E. coli* (43 to 47% sequence identity), and *P. aeruginosa* (54% sequence identity). We therefore identified this ORF as *bla*MOX-2. There were several conserved serine β-lactamase motifs: the SXSK motif from the active site, the typical class C motif YXN, and the KTG domain. Alignment with the amino acid sequences of MOX-1, CMY-1, and CMY-8 showed two nonconservative substitutions in the region close to the active site: Ser-74→Arg and Thr-92→Pro.
usually, the residue following element one, SXXK (residues 88 and 91) in class C \( \beta \)-lactamases, is a threonine. Element one is located at the beginning of the highly conserved helix H2. Thus, the presence of a proline in this position distorts this helix and, consequently, the active site. Analysis of the nucleotide sequence upstream from the start codon showed that no \( \textit{ampR} \) gene was present.

The other ORFs were on the opposite DNA strand. ORF2 is located 212 bp upstream from \( \textit{bla}_{\text{MOX-2}} \) and encodes a putative 375-amino-acid protein 40% identical to the transposase of the \( \text{IS}_{1358} \) from \( \textit{Vibrio anguillarum} \) (10) (accession no. VAU93590). ORF3 is located 826 nt upstream from ORF2 and encodes a putative protein of 275 amino acids displaying no significant similarity to any protein in the database. A 612-bp ORF (nt 1 to 612) lacking 5’ sequences was identified 1,010 bp upstream from ORF3. It encodes a 204-amino-acid product 100% identical to the putative transposase of \( \text{IS}_{186} \) of \( E. \text{coli} \) K12 (accession no. X03123). A 22-bp inverted repeat was identified 32 nt downstream (nt 645 to 666) from this ORF.

We analyzed the regions flanking \( \textit{bla}_{\text{MOX-2}} \) and did not find the characteristic structures of an integron or sequences similar to those flanking \( \textit{bla}_{\text{CMY-8}} \) (19), \( \textit{bla}_{\text{MOX-1}} \) (7), and \( \textit{bla}_{\text{CMY-1}} \) (5). The means by which the \( \textit{bla}_{\text{MOX-2}} \) gene was inserted were not clear.

**FIG. 1.** Genetic organization of the genes identified on the 6,170-bp DNA insert of \( p\text{LRB01} \). The directions of gene transcription are indicated by arrows. IR, inverted repeat.

**FIG. 2.** Multiple-sequence alignment of the deduced amino acid sequence of the MOX-2 \( \beta \)-lactamase with those of the MOX-1, CMY-1, and CMY-8 \( \beta \)-lactamases. Dashes indicate identical amino acids.
We did not determine the exact phylogenetic origin of \( \text{b} \text{la} \text{MOX-2} \). Its G+C content of 63.4% and its observed 73.1% identity with the \( A. \text{sobria} \) cephalosporinase suggest that the parental strain may have been a bacterium of the \( \text{Aeromonas} \) genus (G+C content of 57 to 63%).

**Nucleotide sequence accession number.** The EMBL accession number for the nucleotide sequence reported in this paper is AJ276453.

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