Emergence of Ertapenem Resistance in *Escherichia coli*

Clinical Isolate producing Extended-Spectrum AmpC (ESAC) \(\beta\)-Lactamase

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Running title: Ertapenem resistance in ESAC-producing *E. coli* isolate

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The *Escherichia coli* isolate MEV responsible for bloodstream infection was resistant to penicillins, cephalosporins, and ertapenem. Molecular and biochemical characterization revealed the production of a novel chromosome-borne extended-spectrum AmpC β-lactamase with Ser-282 duplication and increased carbapenemase activity. This study demonstrates for the first time that chromosome-borne ESAC β-lactamases can contribute to the emergence of ertapenem resistance in *E. coli* clinical isolates.
*Escherichia coli* produces a chromosomal AmpC β-lactamase at a very low level because of a weak promoter (4). Spontaneous mutations affecting the promoter region can induce overproduction of this enzyme conferring resistance to narrow-spectrum cephalosporins (20). Moreover, structural alterations in the R2 binding site that accommodates the R2 lateral side chain of β-lactams can broaden the hydrolysis spectrum of AmpC β-lactamases toward extended-spectrum cephalosporins (ESCs) including cefepime (14,28). Extended-spectrum AmpC (ESAC) β-lactamases, which constitute the novel group 1e in the updated functional classification of Bush and Jacoby (3), also exhibited increased catalytic efficiency against carbapenems as compared to parental enzymes (28). However, at the present time, the phenotypic expression of this weak carbapenemase activity was only detected in porin-deficient *E. coli* recombinant clones (24).

We described here for the first time the emergence of ertapenem resistance in ESAC-producing *E. coli* clinical isolate. *E. coli* MEV was recovered in two blood cultures from a 50-year-old patient suffering from myeloma at the Morvan hospital (Brest, France) in April 2008. The patient had been hospitalized multiple times in the previous six months and had received multiple courses of antibacterials. On admission, the patient was empirically treated by vancomycin, ceftazidime and ciprofloxacin. Once the bacteriological documentation became available, the treatment was changed to imipenem and vancomycin during 13 days combined with amikacin.
during the first 5 days. The course of the infection was favorable.

Susceptibility testing, which was performed as previously described (26), showed that *E. coli* MEV was resistant to penicillins, cephalosporins, and ertapenem, but remained susceptible to imipenem according to the revised CLSI criteria (7) (Table 1). The resistance was not antagonized by clavulanic acid whereas the susceptibility to ESCs and ertapenem was partially restored by cloxacillin and phenylalanine arginine–β-naphthylamide (PAβN) (Sigma-Aldrich), in agreement with AmpC β-lactamase production and efflux overexpression, respectively (3,5). Furthermore, *E. coli* MEV was resistant to fluoroquinolones, gentamicin and tobramycin.

The PCR screening revealed that *E. coli* MEV did not harbor plasmid-mediated *ampC* genes (29). Moreover, isoelectric focusing analysis of the culture extract of *E. coli* MEV, which was performed as previously described (23), gave one β-lactamase activity with pl value of 9.0. The entire chromosome-borne *ampC* gene was amplified and analysed as described previously (20). As compared to the wild-type promoter of *E. coli* K12, DNA sequence analysis of the *bla*<sub>AmpC-MEV</sub> gene promoter showed a C→T transition at position -42 that made a perfect TTGACA box upstream of native -35 sequence. Moreover, the T→A transversion at position -18 resulted in a new -10 box separated by 17-bp from the new -42 box giving rise to a strong promoter (4). The analysis of the coding sequence of the *bla*<sub>AmpC-MEV</sub> gene revealed a 3-bp insertion as compared to the *bla*<sub>AmpC-A</sub> gene that coded for the wild-type cephalosporinase of *E. coli* strains.
belonging to the phylogenetic group A (6,21,25). This insertion led to a duplication of the serine residue located at position 282 in the H-9 helix (Fig. 1) (9,20,28). This structural alteration was reported here for the first time.

The coding regions of the *bla*<sub>AmpC-MEV</sub> and *bla*<sub>AmpC-A</sub> genes were cloned into pCR-BluntII-Topo (Invitrogen, Cergy-Pontoise, France) and the recombinant plasmids were subsequently transformed into *E. coli* TOP10 strain, giving rise to *E. coli* TOP10 (pMEV) and *E. coli* TOP10 (pAmpC-A) recombinant clones, respectively (26). In all of the recombinant plasmids, the orientation of the clone insert was the same, with the *ampC* gene under the transcriptional control of the *lacZ* promoter flanking the cloning site.

The AmpC-MEV and AmpC-A β-lactamases were extracted and purified, as described previously (26), from the *E. coli* TOP10 recombinant strains yielding two extracts containing 0.38 mg/ml and 1.52 mg/ml of proteins, respectively. The homogeneity of the purified extracts was assessed by SDS–PAGE analysis (> 99%) (15). The specific activities, determined with 100 µM of cephalothin as substrate, were 60 µmol/min×mg of protein and 490 µmol/min×mg of protein, respectively. The kinetic parameters, which were determined as previously described (26), are presented Table 2. The *k<sub>cat</sub>* and *K<sub>m</sub>* values of AmpC-MEV β-lactamase were respectively increased and decreased with respect to those of AmpC-A for all ESCs and imipenem, accounting for enhanced catalytic efficiencies (*k<sub>cat</sub>/K<sub>m</sub>*).

Surprisingly, the hydrolysis of ertapenem by AmpC-MEV and
AmpC-A extracts was not detectable. Such discrepancy between phenotypical and biochemical results were already reported (22), and might be attributable to the low but non-zero deacylation rate of AmpC β-lactamases for this compound (16).

MICs for *E. coli* TOP10 (pMEV) and *E. coli* TOP10 (pAmpC-A) recombinant strains are presented in Table 1. AmpC MEV β-lactamase conferred higher level of resistance to all oxyiminocephalosporins as compared to AmpC-A enzyme, thus confirming that Ser-282 insertion extended the hydrolysis spectrum. In the opposite, *E. coli* TOP10 (pMEV) strain remained susceptible to ertapenem, although the MIC was slightly increased as compared to that of wild-type *E. coli* TOP10 strain (five-fold increase). This result suggested that *E. coli* MEV isolate expressed an additional mechanism of resistance affecting the susceptibility to ertapenem.

The AcrAB efflux transporter and the marA gene, which encodes a transcriptional activator of the acrRAB operon, was determined by quantitative RT-PCR as previously described (11,13). It showed that the expression of the AcrAB transporter was significantly increased in *E. coli* MEV (12-fold increase) whereas the expression of the MarA transcriptional activator was decreased (11-fold decrease) (Fig. 2).

OMP profile of the *E. coli* clinical isolate MEV was analyzed with sodium dodecyl sulfate containing-polyacrylamide gel electrophoresis (SDS-PAGE), as described previously (30), and *E. coli* control strains expressing OmpC and/or OmpF porins (27). Comparison of the OMP
profiles showed weak expression of OmpC and lack of OmpF protein in *E. coli* MEV (Fig. 3), which might explain the additional resistance to ertapenem in *E. coli* clinical isolate MEV (10,30).

Amplification of the *acrR* gene, which encodes a transcriptional repressor of the *acrA* gene (19), was performed using the primers AcrR-F 5'-GCTGCGTTTATATTATCGTCGTGC-3' and AcrR-R 5'-GTCAAACCAGCAAGATATCAGACG -3', and a standard PCR protocol (denaturation for 10 min at 94°C; 35 cycles of 1 min at 94°C, 1 min at 55°C, and 1 min at 72°C; and a final extension step of 10 min at 72°C). It yielded a 815-bp amplicon for *E. coli* TOP10 strain whereas it was negative for *E. coli* MEV, suggesting that the AcrAB efflux overexpression in *E. coli* MEV was related to the deletion of the *acrR* gene. Whole-cell DNA of *E. coli* MEV was extracted and transferred onto a nylon membrane (31). Hybridization of the membrane with a fluorescein-labeled probe that was made of the PCR product of the *acrR* gene of *E. coli* TOP10 strain (23) failed, thus confirming the deletion of the *acrR* gene in *E. coli* MEV. Nevertheless, further studies, such as homologous recombination, are needed to confirm whether AcrAB efflux transporter overexpression contribute to ertapenem resistance in *E. coli*.

Carbapenem resistance is an emerging phenomenon among *E. coli* clinical isolates. To date, it was related to transmissible β-lactamases with strong carbapenemase activity, such as metallo-β-lactamases, KPC-type, and OXA-48-type, or transmissible β-lactamases with weak carbapenemase activity, such as CTX-M-type or some plasmid-mediated AmpC β-
lactamases, in combination with lack of outer membrane permeability (2,12,17,18,22,30,32). This study demonstrates for the first time that chromosome-borne ESAC β-lactamases can also contribute to the emergence of ertapenem resistance in *E. coli* clinical isolates.

**Nucleotide sequence accession number.** The nucleotide sequence of the *bla*<sub>AMPC-MEV</sub> gene of *E. coli* MEV has been deposited in EMBL nucleotide sequence database under accession number HQ419012.

**ACKNOWLEDGMENTS**

We thank Stephanie Trudel for technical assistance in sequencing experiment.

**REFERENCES**


TABLE 1. MICs of β-lactams for *E. coli* MEV, the *E. coli* TOP10 (pMEV) and *E. coli* TOP10 (pAmpC-A) recombinant clones, and the *E. coli* TOP10 recipient strain.

<table>
<thead>
<tr>
<th>β-Lactams</th>
<th><em>E. coli</em> MEV&lt;sup&gt;a&lt;/sup&gt;</th>
<th><em>E. coli</em> MEV&lt;sup&gt;a&lt;/sup&gt;</th>
<th><em>E. coli</em> MEV&lt;sup&gt;a&lt;/sup&gt;</th>
<th><em>E. coli</em> TOP10</th>
<th><em>E. coli</em> TOP10</th>
<th><em>E. coli</em> TOP10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amoxicillin</td>
<td>&gt;512</td>
<td>16</td>
<td>&gt;512</td>
<td>&gt;512</td>
<td>512</td>
<td>2</td>
</tr>
<tr>
<td>Amoxicillin-CLA&lt;sup&gt;b&lt;/sup&gt;</td>
<td>512</td>
<td>8</td>
<td>512</td>
<td>512</td>
<td>512</td>
<td>2</td>
</tr>
<tr>
<td>Ticarcillin</td>
<td>256</td>
<td>4</td>
<td>128</td>
<td>256</td>
<td>8</td>
<td>1</td>
</tr>
<tr>
<td>Ticarcillin-CLA&lt;sup&gt;b&lt;/sup&gt;</td>
<td>128</td>
<td>4</td>
<td>64</td>
<td>128</td>
<td>8</td>
<td>1</td>
</tr>
<tr>
<td>Piperacillin</td>
<td>256</td>
<td>4</td>
<td>128</td>
<td>256</td>
<td>8</td>
<td>1</td>
</tr>
<tr>
<td>Piperacillin-TZB&lt;sup&gt;b&lt;/sup&gt;</td>
<td>128</td>
<td>4</td>
<td>64</td>
<td>64</td>
<td>8</td>
<td>1</td>
</tr>
<tr>
<td>Cefotaxime</td>
<td>64</td>
<td>0.5</td>
<td>16</td>
<td>32</td>
<td>1</td>
<td>0.06</td>
</tr>
<tr>
<td>Ceftazidime</td>
<td>256</td>
<td>1</td>
<td>128</td>
<td>256</td>
<td>2</td>
<td>0.06</td>
</tr>
<tr>
<td>Cefepime</td>
<td>16</td>
<td>0.5</td>
<td>2</td>
<td>2</td>
<td>0.06</td>
<td>0.06</td>
</tr>
<tr>
<td>Imipenem</td>
<td>0.5</td>
<td>0.25</td>
<td>0.5</td>
<td>0.25</td>
<td>0.06</td>
<td>0.06</td>
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<tr>
<td>Antibiotic</td>
<td>MIC (µg/ml)</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Ertapenem</td>
<td>1, 0.06, 0.5, 0.125, 0.016, 0.008</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Meropenem</td>
<td>0.25, 0.125, 0.25, 0.032, 0.032, 0.032</td>
<td></td>
<td></td>
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</tbody>
</table>

- E. coli MEV and E. coli TOP10 (pMEV) produced the extended-spectrum AmpC MEV β-lactamase, E. coli TOP10 (pAmpC-A) produced the narrow-spectrum AmpC-A β-lactamase, whereas E. coli TOP10 recipient strain did not produce β-lactamase.

- CLA, clavulanic acid at 2 µg/ml; TZB, tazobactam at 4 µg/ml.

- CLOXA, MICs obtained using 250 µg/ml cloxacillin-containing Mueller Hinton agar plates.

- phenylalanine arginine–β-naphthylamide (PAβN) at 26.3 µg/ml (5).
TABLE 2. Kinetic parameters of AmpC MEV β-lactamase and the wild-type AmpC-A enzyme.

<table>
<thead>
<tr>
<th>β-Lactams</th>
<th>AmpC-MEV</th>
<th>AmpC-A</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$k_{cat}$ (s$^{-1}$)</td>
<td>$K_m$ (μM)</td>
</tr>
<tr>
<td>Cephalothin</td>
<td>135 ±15</td>
<td>18 ±2</td>
</tr>
<tr>
<td>Cefaloridin</td>
<td>160 ±12</td>
<td>38 ±4</td>
</tr>
<tr>
<td>Cefoxitin$^a$</td>
<td>0.056 ±0.01</td>
<td>0.08 ±0.01</td>
</tr>
<tr>
<td>Cefotaxime$^a$</td>
<td>0.25 ±0.005</td>
<td>0.05 ±0.01</td>
</tr>
<tr>
<td>Ceftazidime$^a$</td>
<td>0.78 ±0.03</td>
<td>0.4 ±0.08</td>
</tr>
<tr>
<td>Cefepime</td>
<td>1.5 ±4</td>
<td>60 ±0.025</td>
</tr>
<tr>
<td>Imipenem$^a$</td>
<td>0.01 ±0.005</td>
<td>0.3 ±0.08</td>
</tr>
</tbody>
</table>

$^a$ For those compounds with a $K_m$ values less than 5 μM, $K_i$ were determined instead of $K_m$, using cefaloridin as substrate. The values are at least the mean of three independent experiments.

$^b$, ND, not determinable.
± represents the standard deviation.
FIG. 1. Alignement of the amino acid sequences of wild-type AmpC and ESAC β-lactamases of *E. coli* clinical isolates. AmpC-A is a narrow-spectrum enzyme from *E. coli* EC2 (21,25), whereas the other β-lactamases are variants exhibiting extended-hydrolysis spectrum. AmpC MEV, AmpC BER, and AmpC ECB33 enzymes were produced by *E. coli* MEV (in this study), *E. coli* BER (26), and *E. coli* ECB33 isolates (20), respectively; AmpC EC14, AmpC EC15, AmpC EC16, and AmpC EC18 β-lactamases were produced by *E. coli* EC14, *E. coli* EC15, *E. coli* EC16, *E. coli* EC18 isolates, respectively (25); AmpC<sup>D</sup> was produced by *E. coli* HKY28 isolate (9); AmpC<sub>7014517</sub>, AmpC<sub>8009162</sub>, and AmpC<sub>EC80</sub> β-lactamases, were produced by *E. coli* 7014517, *E. coli* 8009162, and *E. coli* EC80 isolates, respectively (1,8). The amino acids in the R2 loop are shaded in light grey whereas those in the H-9 helix are shaded in dark grey.

FIG. 2. RT-PCR analysis of *marA* and *acrA* expression. Total bacterial RNA was isolated from mid-log phase cultures of *E. coli* MEV isolate and wild type *E. coli* TOP10 strain. The normalized expression ratios of *acrA* and *marA* genes are shown in panels A and B respectively. The error bars represent the standard deviation for the mean of triplicate samples.

FIG. 3. OMP profiles of *E. coli* strains. OMPs were profiled by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Lanes 1, *E. coli* TOP10 is a wild-type strain; Lane 2, *E. coli* isolate JF703 expressing
OmpC alone (27) ; Lane 3, *E. coli* clinical isolate MEV. Horizontal arrows on the left indicate positions of the OMPs OmpC and OmpF.
<table>
<thead>
<tr>
<th></th>
<th>AmpC-A</th>
<th>AmpC-MEV</th>
<th>AmpC-ECB33</th>
<th>AmpC-BER</th>
<th>AmpC-EC14</th>
<th>AmpC-EC15</th>
<th>AmpC-EC16</th>
<th>AmpC-EC18</th>
<th>AmpC-D</th>
<th>AmpC-7014517</th>
<th>AmpC-8009162</th>
<th>AmpC-EC80</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EMLDPVNI-S-I-INGSGNIAL--AA---HPYRAITPPPAVRA34VH</td>
<td>..........-..........-S..........--..---...................</td>
<td>..........-..........-J..........--..---...................</td>
<td>..........-..........--..---..AA..........--..---...................</td>
<td>..........-..........--..---..L..........--..---...................</td>
<td>..........-..........--..---..P ..........--..---...................</td>
<td>..........-..........--..---..C..........--..---...................</td>
<td>..........-..........--..---..N..........--..---...................</td>
<td>..........-..........--..---..LAA ..........--..---...................</td>
<td>..........-..........--..---..V ..........--..---...................</td>
<td>..........-..........--..---..P ..........--..---...................</td>
<td></td>
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</table>
A. Relative *acrA* mRNA transcription

B. Relative *marA* mRNA transcription