Characterization of the New AmpC β-Lactamase FOX-8 Reveals a Single Mutation, Phe313Leu, Located in the R2 Loop That Affects Ceftazidime Hydrolysis

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A novel class C β-lactamase (FOX-8) was isolated from a clinical strain of Escherichia coli. The FOX-8 enzyme possessed a unique substitution (Phe313Leu) compared to FOX-3. Isogenic E. coli strains carrying FOX-8 showed an 8-fold reduction in resistance to ceftazidime relative to FOX-3. In a kinetic analysis, FOX-8 displayed a 33-fold reduction in \( k_{cat}/K_m \) for ceftazidime compared to FOX-3. In the FOX family of β-lactamases, the Phe313 residue located in the R2 loop affects ceftazidime hydrolysis and alters the phenotype of E. coli strains carrying this variant.

AmpC β-lactamases are clinically important cephalosporinases, particularly in Enterobacteriaceae (1, 2). To date, none of the commercially available β-lactamase inhibitors (clavulanic acid, sulbactam, and tazobactam) are effective against high-level class C producers (3). New extended-spectrum class C enzymes that are capable of hydrolyzing cephalosporins with large side chains, and even imipenem, are emerging (4). These enzymes differ from typical AmpC β-lactamases because of amino acid insertions, deletions, and substitutions (1, 2, 4). The three regions involved in these modifications are the omega loop, R2 loop, and helix H-2 (1, 2, 4).

FOX-type enzymes are plasmid-encoded AmpC β-lactamases that are especially active against cefoxitin (5–10). Although at least 10 variants have been described previously (http://www.lahey.org/Studies), structural data are not yet available. An amino acid trimer, Gly306/Asn307/Ser308 deletion in the FOX-4 enzyme has been suggested to be wide enough to accommodate substrates with large R2 groups.

In the present study, we compared microbiological and biochemical data on FOX-3 and FOX-8 enzymes in order to investigate the role of Phe313 (present in the R2 loop of FOX enzymes) in cephalosporin hydrolysis.

Escherichia coli HGURS42015 carrying the \( \text{bla}_{FOX-8} \) gene was isolated from a urine culture from an 80-year-old female patient admitted to the Reina Sofia Hospital (Murcia, Spain) in 2009. By PCR and using oligonucleotides specific for AmpC-type β-lactamases (12), a positive result for \( \text{bla}_{FOX-8} \)-type genes was observed. After DNA sequencing, a new \( \text{bla}_{FOX-8} \) variant, \( \text{bla}_{FOX-8}^{*} \), corresponding to a Phe313Leu replacement compared to the FOX-3 enzyme, was detected.

We used pulsed-field gel electrophoresis (PFGE) with S1 nuclease digestion of whole-genome DNA (S1-PFGE) and PCR-based replicon typing (PBRT) to characterize the plasmids as described previously (13). We used PFGE with I-CeuI digestion of whole-genome DNA, as described by Liu et al. (14), to determine whether the \( \text{bla}_{FOX-8} \) gene was located in the chromosome. We successfully transferred and hybridized the S1-PFGE-I-CeuI gels with the FOX probe (data not shown). Unfortunately, hybridization signals were not conclusive. We next isolated a plasmid of ca. 6 kbp from E. coli HGURS42015. After transformation, this plasmid harboring \( \text{bla}_{FOX-8} \) was obtained. Total DNA of these transformants failed to amplify with \( \text{bla}_{FOX-3} \) gene primers. The repeated conjugation experiments with a kanamycin-resistant host E. coli strain were unsuccessful. Although experiments suggested a chromosomal location for \( \text{bla}_{FOX-8} \), further studies should be performed to confirm this affirmation.

We elucidated the genetic environment of \( \text{bla}_{FOX-8} \) by PCR and sequencing. First, we amplified the \( \text{bla}_{FOX-8} \) Gene by PCR and fully sequenced the gene by using primers FOX3F (5’ATGCACACACGACGCGTC) and FOX3R (5’TCACTCAGCCCTAAGTGA). We then mapped and sequenced the genetic context by using primers for class 1 integrons (15) and inverse primers for \( \text{bla}_{FOX-3} \) and \( \text{bla}_{FOX-8} \) (5’CGCAGCTGTTGTACGC; forward) and FOX3Ri (5’AGTCAGTTGGCCAGTTA; reverse), which introduced the KpnI and EcoRI restriction sites, respectively. We cloned the \( \text{bla}_{FOX-8} \) and \( \text{bla}_{FOX-3} \) genes in the plasmid pBGS18-CTX, under the promoter of the blaCTX-M-14 gene, in E. coli strain TG1 (11). We used PCR to obtain the \( \text{bla}_{FOX-8} \) gene from E. coli HGURS42015 and the \( \text{bla}_{FOX-3} \) gene by PCR with a bacterial strain harboring the \( \text{bla}_{FOX-3} \).
gene (a kind gift of Guillaume Arlet, Hôpitaux Universitaires Est Parisiens). We transformed both constructs into E. coli TG1, and the MICs of selected β-lactam antibiotics were obtained (Table 1).

Our MICs revealed that in an isogenic E. coli background, FOX-8 was 8- to 16-fold more susceptible to cefoxitin and cefazidime than FOX-3 was (Table 1). There was also a 4-fold reduction in the MIC of aztreonam for FOX-8 relative to that for FOX-3. However, for FOX-8, there was a notable increase in the MICs of antibiotics such as ampicillin, piperacillin, and cefotaxime, for which the MICs for FOX-3 were 8- to 16-fold more susceptible to cefoxitin and ceftazidime than FOX-3 was (Table 1). There was also a 4-fold reduction in the MIC of aztreonam for FOX-8 relative to that for FOX-3 for all the tested substrates (Table 2).

The purified proteins (≥99% pure) appeared as a single band on sodium dodecyl sulfate-polyacrylamide gels (data not shown).

In order to monitor hydrolysis of antibiotics by FOX-3 and FOX-8-β-lactamases, we recorded the variation in absorbance resulting from the opening of the β-lactam ring, under the following conditions. We determined the kinetic parameters for nitrocefin and cephalothin from the initial rates by Hanes-Woolf linearization of the Henri-Michaelis-Menten equation. For the other antibiotics, we measured the $K_m$ value as the $K_p$ in a competition experiment, with nitrocefin as the reporter substrate. We then obtained the $k_{cat}/K_m$ values by monitoring hydrolysis of the antibiotic at a concentration $>> 10$ times the $K_m$. In the case of cephalothin, for which the $K_m$ was very high, only the $k_{cat}/K_m$ ratio could be determined under first-order conditions ([S] $\ll K_m$) (11).

The kinetic analyses revealed a decrease in the $k_{cat}/K_m$ ratio for FOX-8 relative to that for FOX-3 for all the tested substrates (Table 2). This effect was significant only for cefoxitin and cefazidime, for which the $k_{cat}/K_m$ values for FOX-8 were, respectively, 26 and 105 times lower than the FOX-3 values. For FOX-8, the $K_m$ was also lower with all the antibiotics tested, except nitrocefin, for which the $K_m$ remained unchanged. The decrease was relatively modest (less than 10-fold modification), except for with cefoxitin.

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>$k_{cat}$ ($s^{-1}$)</th>
<th>$K_m$ ($\mu M$)</th>
<th>$k_{cat}/K_m$ ($\mu M^{-1} s^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>FOX-3</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ampicillin</td>
<td>3.9 $\times$ 10^3 ± 123</td>
<td>99.8 ± 6.38</td>
<td>0.007</td>
</tr>
<tr>
<td>Cephalothin</td>
<td>1.634 ± 114</td>
<td>163 ± 26.7</td>
<td>10</td>
</tr>
<tr>
<td>Cefotaxime</td>
<td>1.742 ± 0.258</td>
<td>0.066 ± 0.007</td>
<td>10</td>
</tr>
<tr>
<td>Cefazidime</td>
<td>8.1 $\times$ 10^{-2} $\times$ 3.7 $\times$ 10^{-2}</td>
<td>76.3 $\times$ 10^{-2} ± 0.0138</td>
<td>10.61</td>
</tr>
<tr>
<td>Cefepime</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Imipenem</td>
<td>0.015 ± 0.0011</td>
<td>0.0125 ± 0.0014</td>
<td>0.00332 ± 0.00026</td>
</tr>
</tbody>
</table>

$^a$ Data are means ± standard deviations (where applicable). ND, not determined. $K_m$ values are very high, and only the $k_{cat}/K_m$ ratio was determined on the basis of the first-order reaction time for hydrolysis.
hypothesize that replacement of a phenylalanine by a less bulky amino acid from the conserved motif displayed as yellow spheres and the R2 loop in green. Leu293, which is equivalent to Leu313 of FOX-8 and Phe313 of FOX-3, is shown as orange structures. (B) Close-up view of the active site, with residues from the conserved motif represented as gray structures.

FIG 1 (A) Representation of the CMY-10 class C β-lactamase, with amino acids from the conserved motif displayed as yellow spheres and the R2 loop in green. Leu293, which is equivalent to Leu313 of FOX-8 and Phe313 of FOX-3, is shown as orange structures. (B) Close-up view of the active site, with residues from the conserved motif represented as gray structures.

(23-fold decrease). The effects on the $k_{cat}$ and $K_m$ of cefoxitin compensated each other and led to similar catalytic efficiencies ($k_{cat}/K_m$). Ceftazidime was therefore the only substrate for which FOX-8 had a significantly lower catalytic efficiency (33-fold) than FOX-3. The catalytic efficiencies of FOX-3 and FOX-8 were found to be very similar for the other substrates. These data are consistent with the MIC data, except for the case with cefoxitin. In the latter case, the apparently higher affinity did not seem to compensate the reduced activity in vivo. Finally, the substitution did not affect the low-level inhibition properties of classical inhibitors.

To rule out an effect of enzyme stability on kinetic parameters, we also performed temperature inactivation studies with pure samples and confirmed that the FOX-3 and FOX-8 proteins displayed similar stabilities (68% ± 3% and 62% ± 6% of residual activity with nitrocefin, after incubation for 40 min at 50°C, with FOX-3 and FOX-8, respectively).

FOX-8 is the second FOX enzyme described in Spain (FOX-4 was the first). Five FOX β-lactamases have been published to date. FOX-8 has a single amino acid difference with respect to FOX-3, with a leucine instead of a phenylalanine at position 313. FOX-8 is the only FOX enzyme with a leucine at this position in the R2 loop. However, this substitution is not unique in the class C β-lactamases. The closely related CMY-10 (76% amino acid sequence identity) is also characterized by a leucine at the same position, as in most of the AmpC enzymes (17).

No tridimensional structure is available for the FOX enzymes, and the most relevant structural data available are the data for CMY-10 (17). The CMY-10 structure was therefore used to locate the Phe313Leu mutation and to analyze its potential effect on the catalytic mechanism. In previous work, CMY-10 was employed to model FOX-4 (11); we anticipate that a significant difference is not expected in the overall structure of the enzyme and more specifically in the R2 loop (Fig. 1A). The same is probably also true for FOX-3 and FOX-8, because they do not have any other substitutions in this area, apart from Phe313Leu.

The Phe313Leu mutation lies in close proximity to Tyr151, which is part of the second conserved motif of class C β-lactamases and is involved in the catalytic mechanism (Fig. 1B). This position also defines one edge of the active site. In this context, we hypothesize that replacement of a phenylalanine by a less bulky amino acid could lead to a slightly wider and more accessible active site. This is consistent with the general decrease in $K_m$ observed for most substrates. The greater effect observed for cefoxitin may be due to the specific constraints of this substrate resulting from the presence of a methoxy group in position 7α on the β-lactam ring. However, it is also possible that the reduced activity may be due to fewer constraints on the second motif tyrosine, leading to a less optimal positioning of this critical residue. However, the difference in the magnitudes of the effects observed for the various substrates cannot be explained with the available data. A potential reason for the very high impact on ceftazidime hydrolysis could be the combination of two large side chains at the position 3 and 7 characterizing this antibiotic.

The kinetic parameters of FOX-3 can also be compared with those previously reported for FOX-4 (11). Eight antibiotics have been tested for FOX-3 and FOX-4, and the kinetic parameters of three of these (nitrocefin, imipenem, and cefotaxime) display significant differences. For imipenem and cefotaxime, the $k_{cat}$ increased by 32 and 17 times, respectively, for FOX-4 relative to FOX-3, while the $k_{cat}$ for nitrocefin increased by 29 times. These differences lead to significant modifications in the catalytic efficiencies for nitrocefin and imipenem (30-fold decrease and 72-fold increase, respectively) but not for cefotaxime. In the latter case, the increase in $k_{cat}$ was partly reversed by a slight increase in $K_m$. We attempted to correlate the difference in specificity between FOX-3 and FOX-4 with the 10 amino acids not conserved in the two enzymes. However, judging by the position in the structure of CMY-10, none of these seems to be capable of inducing any significant change potentially responsible for the observed effect.

The fact that other class C β-lactamases possess a leucine in the same position and significantly hydrolyze ceftazidime may reduce the effect of Phe313Leu on the FOX group. In Aeromonas caviae, the CAV-1 enzyme (a putative precursor of FOX enzymes), which possesses a phenylalanine residue, displays good activity against ceftazidime but not cefoxitin. Other mutations must be established in the Phe313 position to clarify these questions.

In summary, a new FOX-8 β-lactamase carried in a class I integron has been described in Spain. The microbiological and biochemical study of this enzyme in comparison with FOX-3 highlights the Phe313Leu mutation, located in the α10 helix of R2 loop, as being involved in ceftazidime hydrolysis.

Nucleotide accession number. The nucleotide sequence for the blaFOX-8 gene has been deposited in the GenBank database under accession number HM565917.

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