Role of the Ser-287-Asn Replacement in the Hydrolysis Spectrum Extension of AmpC β-Lactamases in Escherichia coli

Hedi Mammeri,1,2* Moreno Galleni,3 and Patrice Nordmann2

Service de Bactériologie-Hygiène, Centre hospitalier universitaire d’Amiens, Hôpital Nord, 80000 Amiens, France; Service de Bactériologie-Virologie-Hygiène, Unité INSERM 914 Emerging Resistance to Antibiotics, Hôpital de Bicêtre, Assistance Publique/Hôpitaux de Paris, Faculté de Médecine et Université Paris Sud, 94275 le K.-Bicêtre, France; and Center for Protein Engineering, Université de Liège, Liège B-4000, Belgium

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Two AmpC variants harboring the S287N substitution were obtained by mutagenesis from cephalosporinases representative of the phylogenetic groups A and B2 of Escherichia coli. Their biochemical characterization revealed that the S287N replacement led to an important increase in the catalytic efficiency toward extended-spectrum cephalosporins in the AmpC β-lactamase of group A only.

Escherichia coli is a heterogeneous species which can be divided into four main phylogenetic groups: A, B1, B2, and D (2). Most of the E. coli isolates of the commensal digestive flora belong to group A, whereas E. coli isolates responsible for extraintestinal infections belong mainly to group B2 (2). The chromosome-borne ampC gene of E. coli, which codes for a cephalosporinase, exhibits three distinctive patterns matching the phylogenetic groups A+B1, B2, and D (9). The two ampC allele groups corresponding to the phylogenetic groups A+B1 and B2 are internally highly homogeneous (9).

In wild-type E. coli isolates, the chromosomal AmpC β-lactamase is usually produced at a low level due to a weak promoter (1). Mutations in the promoter region may induce a constitutive overproduction of the enzyme responsible for resistance to early-generation cephalosporins.

Recently, cephalosporinases with broadened substrate activity have been reported among E. coli isolates of the phylogenetic groups A or B1 (6, 9). These extended-spectrum AmpC (ESAC) β-lactamases derived from the parental wild-type enzymes by either insertion, deletion, or amino acid substitutions in the R2 binding site (Fig. 1) (11). The ESAC β-lactamases exhibited increased catalytic efficiencies toward all the extended-spectrum cephalosporins (ESCs) and also slightly against imipenem (5, 10). They conferred resistance to ceftazidime, reduced significantly susceptibilities to cefotaxime and cefepime (11), and also reduced susceptibilities to ertapenem and imipenem in combination with decreased membrane permeability (7). Recent studies identified the S287N replacement as a frequent site of hydrolysis-spectrum extension in the AmpC β-lactamase of E. coli (6, 9).

Herein, a detailed site-directed mutagenesis study was undertaken to elucidate the functional changes induced by this substitution. The ampC genes from E. coli EC2 and E. coli EC6, which are representative of the two main ampC allele groups A+B1 and B2 (9), were cloned into pCR-BluntII-Topo (Invitrogen), as previously described (10), thus giving rise to E. coli TOP10(pAmpC-A) and E. coli TOP10(pAmpC-B2). These recombinant clones harbored the plasmids pAmpC-A and pAmpC-B2, respectively. A comparison of the deduced amino acid sequences showed that the encoded cephalosporinases mainly differed by 12 amino acids at positions 24, 175, 194, 235, 238, 239, 241, 282, 288, 296, 300, and 351.

The mutagenesis experiments were performed using the site-directed mutagenesis kit (Stratagene), the recombinant plasmids pAmpC-A and pAmpC-B2 as templates, and the primer pairs EC2-S287N-1 (5′-CTGACAGCATCATTAACG GCAATGGCAATAAATAATGGCAGCTGGAG-3′) and EC2-S287N-2 (5′-CTGGCAGTGCATTTAATGCGATTCGCG TTAATGATGCTGTCAG-3′) and EC6-S287N-1 (5′-CTGAC ATCATATTTACGGCAATGACAATAAATAATGGCAG CTGGAG-3′) and EC6-S287N-2 (5′-CTGAGCAGTGCATTTAATGCGATTCGCG TTAATGATGCTGTCAG-3′), respectively. It gave rise to the recombinant plasmids pAmpC-A-S287N and pAmpC-B2-S287N, respectively, which were subsequently transformed into E. coli TOP10 (Stratagene). A sequence analysis of the inserts confirmed the presence of the expected mutation which led to the S287N replacement in the mature β-lactamases AmpC-A-S287N and AmpC-B2-S287N, respectively.

Analysis of the MICs (Table 1), which were determined as previously described (10), showed that the wild-type cephalosporinases AmpC-A and AmpC-B2 conferred almost identical levels of resistance to β-lactams, although the MICs for E. coli TOP10(pAmpC-A) were twofold higher than those for E. coli TOP10(pAmpC-B2). AmpC-A and AmpC-B2 conferred high levels of resistance to narrow-spectrum cephalosporins, such as cefalotin and cefoxitin, but spared ESCs, according to CLSI criteria (3).

The resistance level conferred by the AmpC-B2-S287N mutant was only slightly increased compared to that conferred by the parental AmpC-B2. Moreover, the MICs of ESCs for E. coli TOP10(pAmpC-B2-S287N) remained in the susceptibility range. On the other hand, the S287N replacement which occurred in AmpC-A-S287N induced an important increase in the resistance level to ESCs compared to that of the parental AmpC-A. The variant AmpC-A-S287N conferred a high level
of resistance to ceftazidime and significantly reduced susceptibilities to other ESCs, including cefepime. The MICs of imipenem were unchanged for the four recombinant clones.

A kinetic analysis was performed for the variants and their parental β-lactamases as previously described (10). AmpC enzymes were purified to near homogeneity (≥95%) as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis. The catalytic efficiencies (kcat/Km) of the

FIG. 1. Ribbon representation of the crystallographic structure of the E. coli K-12 β-lactamase bound to ceftazidime (14). The atomic coordinates are available on the Internet (www.ncbi.nlm.nih.gov/entrez) with the accession number IIEL. The representation has been determined using the software Swiss-Pdb Viewer available on the Internet (www.expasy.org/spdbv/) (4, 12, 13). The antibiotic is represented inside the active site. The ribbon representation has been used for the following secondary structures involved in the substrate recognition: helix H-11, helix H-9, helix H-10, the R2 loop, β strand B-3, and the Ω loop. The R1 binding site, which accommodates the oxyimino substituent (R1) at position C-7 of the β-lactam nucleus in ESCs, is surrounded by the Ω loop, whereas the R2 binding site represents the opposite region interacting with the right part of the β-lactam ring including the R2 side chain at C-3 and the carboxylate group at C-4.

### TABLE 1. MICs of β-lactams for the recombinant clones E. coli TOP10(pAmpC-A), E. coli TOP10(pAmpC-A-S287N), E. coli TOP10(AmpC-B2), E. coli TOP10(pAmpC-B2-S287N), and the recipient strain E. coli TOP10

<table>
<thead>
<tr>
<th>β-Lactam**</th>
<th>E. coli TOP10 (pAmpC-A)*</th>
<th>E. coli TOP10 (pAmpC-A-S287N)*</th>
<th>E. coli TOP10 (pAmpC-B2)*</th>
<th>E. coli TOP10 (pAmpC-B2-S287N)*</th>
<th>E. coli TOP10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amoxicillin</td>
<td>&gt;512</td>
<td>&gt;512</td>
<td>&gt;512</td>
<td>&gt;512</td>
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<tr>
<td>Amoxicillin-CLA</td>
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<td>512</td>
<td>256</td>
<td>512</td>
<td>2</td>
</tr>
<tr>
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<td>2</td>
<td>2</td>
<td>64</td>
<td>1</td>
</tr>
<tr>
<td>Ticarcillin-CLA</td>
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<td>256</td>
<td>2</td>
<td>64</td>
<td>1</td>
</tr>
<tr>
<td>Pipercillin</td>
<td>4</td>
<td>8</td>
<td>2</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>PIPERACILLIN-TZB</td>
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<td>8</td>
<td>4</td>
<td>4</td>
<td>1</td>
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<tr>
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<td>&gt;512</td>
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<td>32</td>
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<td>128</td>
<td>2</td>
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<tr>
<td>Ceftazidime</td>
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<td>1</td>
<td>0.06</td>
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<td>0.06</td>
<td>0.125</td>
<td>0.06</td>
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<tr>
<td>Cefepime</td>
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<td>16</td>
<td>0.06</td>
<td>0.06</td>
<td>0.06</td>
</tr>
<tr>
<td>Imipenem</td>
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<td>0.06</td>
<td>0.06</td>
<td>0.06</td>
<td>0.06</td>
</tr>
</tbody>
</table>

*CL, clavulanic acid at 2 mg/liter; TZB, tazobactam at 4 mg/liter.

**E. coli TOP10(pAmpC-A) and E. coli TOP10(pAmpC-B2) produced the wild-type AmpC-A and AmpC-B2, whereas E. coli TOP10(pAmpC-A-S287N) and E. coli TOP10(pAmpC-B2-S287N) produced variant cephalosporins harboring the S287N replacement.
characterization of AmpC harboring S287N replacement

purified wild-type β-lactamase AmpC-A against cephalosporins and imipenem mirrored those of the purified wild-type AmpC-B2, although they were 2- to 10-fold higher, which was mainly due to lower $K_m$ values (Table 2).

The S287N substitution, which occurred in AmpC-A-S287N and AmpC-B2-S287N, led to important increases in affinities for all the cephalosporins and for imipenem, whereas the hydrolysis rates ($k_{cat}$) were decreased. The resulting catalytic efficiencies were weakly decreased for the narrow-spectrum cephalosporins, whereas they were increased against ESCs, including cefepime, and imipenem. Moreover, the catalytic efficiencies exhibited by AmpC-A-S287N against ESCs and imipenem were higher than those exhibited by AmpC-B2-S287N. The hydrolysis of aztreonam was not detected for both wild-type and variant cephalosporinases.

This study highlighted the contribution of the S287N replacement in the hydrolysis-spectrum extension of class C β-lactamases. It revealed that the natural polymorphism of AmpC in E. coli may constitute a genetic background facilitating the emergence of resistant mutants among E. coli clinical isolates of group A and its closely related phylogenetic group, B1. It should explain, almost in part, the high prevalence of strains belonging to these phylogenetic groups among ESAC-producing E. coli clinical isolates (6, 9, 10).

Interestingly, the wild-type AmpC β-lactamase produced by Serratia marcescens and the plasmid-borne CMY-1 β-lactamase and its derivatives, such as CMY-10, have an asparagine residue at position 287. These enzymes conferred higher levels of resistance to ESCs than the wild-type AmpC β-lactamases of E. coli (5, 8). Therefore, Asn-287 may contribute in part to the natural expanded spectrum of these cephalosporinases, although other structural discrepancies, such as a shortened R2 loop for the CMY-1-like β-lactamases (5), may also be involved.

Nucleotide sequence accession numbers. The nucleotide sequences of the $bla_{AmpC}$ genes of E. coli EC2 and E. coli EC6 have been deposited in the EMBL nucleotide sequence database under accession numbers EU497239 and DQ092425, respectively.

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**REFERENCES**


7. Mammeri, H., P. Normann, A. Berkani, and F. Eb. Contribution of extend-