Selection during Cefepime Treatment of a New Cephalosporinase Variant with Extended-Spectrum Resistance to Cefepime in an Enterobacter aerogenes Clinical Isolate

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Enterobacter aerogenes resistant to cefepime (MIC, 32 μg/ml) was isolated from a patient treated with cefepime for an infection caused by a strain of E. aerogenes overproducing its AmpC β-lactamase (MIC of cefepime, 0.5 μg/ml). The AmpC β-lactamase of the resistant strain had an L-293-P amino acid substitution and a high $k_{cat}/K_m$ ratio for cefepime. Both of these modifications were necessary for resistance to cefepime.

Enterobacter aerogenes produces an inducible chromosome-encoded AmpC cephalosporinase (12). Some isolates display high-level resistance to β-lactams owing to overproduction of the AmpC cephalosporinase or production of an extended-spectrum β-lactamase (2, 4, 6). Constitutive overproduction of the AmpC cephalosporinase confers resistance to broad-spectrum cephalosporins except cefpirome and cefepime. These antibiotics rapidly penetrate gram-negative bacteria and have a high affinity for essential penicillin-binding proteins (7, 9).

The two isolates gave strictly identical XbaI pulsed-field gel electrophoresis patterns, in accordance with the criteria of Tenover et al. (14) (data not shown). Transformation of the Ear1 and Ear2 strains with pNH5, containing the ampD gene (8), resulted in a decrease in the MICs of all of the β-lactams tested; similar decreases were observed for ceftazidime and cefotaxime, although the MIC of cefepime was eight times higher (0.5 versus 0.06 μg/ml) for Ear2 than for Ear1 (Table 1). This result suggested that resistance to cefepime was associated with the ampC gene. Furthermore, the level of resistance to cefepime in Ear2/pNH5 was lower than that in Ear2 by a factor of 64, suggesting that overproduction of the cephalosporinase was involved in resistance to cefepime.

We used the published sequence of the E. aerogenes 97B ampC gene (12) to design primers E1 (5′-TGGCGTCGTCATAACATTATCCG-3′) and E2 (5′-AACCCCGTAGCCCAATGTAAC-3′) for amplification and sequencing of a 1,291-bp fragment (ampC gene with its promoter). The PCR products were cloned into the pCR-Blunt vector (Invitrogen, Cergy-Pontoise, France). Recombinant plasmids p-EarCOL, p-Ear1, and p-Ear2 were used to transform E. coli DH5-α. The MIC of cephalosporinase, isolated from another patient, was studied for comparison. Antibiotic susceptibility patterns were determined by the disk diffusion method with Mueller-Hinton agar (Bio-Rad, Marnes-la-Coquette, France), and MICs were determined by means of the E-Test (AB BioDisk, Solna, Sweden).

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sequences of the intercistronic ampC-ampR regions of the three isolates were 100% identical. The deduced amino acid sequences of the Ear1 and Ear2 cephalosporinases differed from those of EarCOL and *E. aerogenes* 97B (accession no. AF211348) by 11 identical substitutions. A twelfth substitution corresponding to the replacement of leucine-293 with a proline was identified only in the AmpC cephalosporinases of the Ear2 strain and the p-Ear2 transformant (Fig. 1). Thus, the observed level of resistance to cefepime in the p-Ear2 transformant resulted from this single base pair change. This substitution has been described by Vakulenko et al. in the in vitro AmpC variant, which displayed a level of resistance to cefepime similar to that observed in the p-Ear2 transformant (15). The cephalosporinases from Ear1 and Ear2 were purified and characterized as previously described (1). Both Ear1 and Ear2 produced large amounts of the cephalosporinases, 960 and 145 mU/mg of total protein, respectively, whereas ≤10 mU/mg of total protein is produced for inducible enzymes. However, Ear1 produced 6.6 times more enzyme than Ear2, consistent with the observations of Vakulenko et al. for the in vitro AmpC variant (15).

On the basis of $k_{cat}$ values, the two β-lactamases were very similar and the data presented here are similar to what would be expected for class C enzymes. The most striking differences

<table>
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<tr>
<th>Strain</th>
<th>AMX</th>
<th>CF</th>
<th>CTX</th>
<th>CAZ</th>
<th>FEP</th>
<th>CPO</th>
<th>IPM</th>
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<tbody>
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<td>0.064</td>
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<tr>
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<tr>
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<tr>
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<th>CPO</th>
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* AMX, amoxicillin; CF, cefalothin; CTX, cefotaxime; CAZ, ceftazidime; FEP, cefepime; CPO, cefpirome; IPM, imipenem; ATM, aztreonam.

FIG. 1. Multiple amino acid sequence alignment of the AmpC β-lactamases of the following strains: 1, *E. aerogenes* 97B (accession no. AF211348); 2, *E. aerogenes* EarCOL; 3, *E. aerogenes* Ear1; 4, *E. aerogenes* Ear2. Dashes indicate identical amino acids. Conserved residues of class C β-lactamases are underlined. The amino acid substitution in the Ear2 sequence is shown in boldface. Amino acids are numbered in accordance with the conventional numbering of *E. cloacae* P99, with the active-site serine at position 64.
concerned the $K_m$ and $K$ values for cephalosporins, which were generally much lower for the Ear2 enzyme. The lowest values were obtained with cefalothin and cefepime. In terms of the $k_{cat}$/2$K_m$ ratio, cefepime was hydrolyzed 15 times faster with the Ear2 cephalosporinase than with the Ear1 enzyme whereas the difference was much smaller for cepfime (Table 2). The increase in this ratio resulted principally from the decrease in $K_m$. These results are consistent with those reported by Vakulenko et al. (15). However, the Ear2 isolate was more resistant to cefepime owing to a combination of overproduction of the cephalosporinase and the ampC gene mutation. This mutation seems to have caused changes in the structure of the $\beta$-lactama
tase, leading to changes in the catalytic properties of the enzyme, resulting in an extension of the substrate spectrum to include cefepime. As suggested by Vakulenko et al., the structural change in the enzyme would result in more space being available to accommodate $\beta$-lactams, in particular cefepime and ceftazidime, increasing affinity and the hydrolysis rate (15). The amino acid leucine-293 is part of the S(N)KVALA sequence conserved in the AmpC $\beta$-lactamases of E. cloacae and E. aerogenes. This sequence, located close to the end of the H-10 helix in the three-dimensional structure of the cephalosporinase, is absent from the cephalosporinase of clinical isolate CHE (1). Moreover, Morosini et al. found, in the AmpC variant, a V-298-E substitution close to L-293 (10). The identified substitutions and deletion are located in the same region, suggesting that this region is a potential hot spot for determining resistance to cefepime. In conclusion, we report here the obtainment of a clinical isolate of Enterobacter sp. highly resistant to cefepime. As for the CHE clinical isolate, the high level of resistance to cefepime was associated with a combination of amino acid substitution in the AmpC cephalosporinase and overproduction of the $\beta$-lactamase. The strain was probably selected by the cefepime treatment used. Cefepime is a useful antibiotic for the treatment of infections caused by derepressed AmpC-producing enterobacteria (13). Our observations suggest that increases in the use of this $\beta$-lactam may result in the selection of strains producing extended-spectrum AmpC $\beta$-lactamases.

**Nucleotide sequence accession numbers.** The EMBL accession numbers for the nucleotide sequences reported in this paper are AJ544161, AJ544162, and AJ544163.

**REFERENCES**


