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Reduced Susceptibility to Cefepime among *Escherichia coli* Clinical Isolates Producing Novel Variants of CMY-2 β-Lactamase▽

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Here we describe three *Escherichia coli* clinical isolates with reduced susceptibility to cefepime. Sequencing of the bla_{CMY} genes revealed two novel variants (CMY-33 and -44) with two- to four-amino-acid deletions in the H-10 helix. The deletions were responsible for 12- to 24-fold increases in the MICs of cefepime.

CMY-2 is the most commonly encountered plasmid-mediated class C β-lactamase, often found in *Escherichia coli* and *Salmonella enterica* serovars (10, 13). The spectrum of resistance conferred by CMY-2 is typical of class C enzymes: CMY-2 confers resistance to penicillins, cephalosporins (including cephamycins), and aztreonam. In contrast, gram-negative bacteria harboring CMY-2 are susceptible to cefepime. Cefepime, a cephalosporin with methoxyimino and aminothiazolyl moieties, is a substrate that is stable against hydrolysis by most class C β-lactamases, both chromosomal and plasmid mediated. Here, we report the identification of two variants of CMY-2 β-lactamases, designated CMY-33 and CMY-44, which confer reduced susceptibility to cefepime.

E. coli strains producing these variants were isolated from three unrelated patients who were admitted to the University of Pittsburgh Medical Center (UPMC) Presbyterian Campus between 2006 and 2008. The *E. coli* strains under study were bloodstream and urinary tract isolates. Two of the patients received cefepime for 6 to 10 days within the 2 months preceding the isolation of the organisms. The MICs of the representative β-lactams for these clinical isolates were measured by Etest (AB Biodisk, Solna, Sweden). According to the Etest results, the *E. coli* isolates were all highly resistant to oxyiminocephalosporins, including cefuroxime, ceftazidime, and cefotaxime, and also showed reduced susceptibility to cefepime (MIC range, 6 to 96 μg/ml) (Table 1). Analytical isoelectric focusing revealed β-lactamase activity with pIs of >9.0 for all isolates and 5.4 for isolates YD006 and 34943, suggesting the presence of class C β-lactamases and TEM-1, respectively (data not shown). To define the β-lactamases causing the extended-spectrum cephalosporin resistance, PCR and sequencing were performed to detect the bla_{TEM}-, bla_{SHV}-, and bla_{CTX-M}-type extended-spectrum β-lactamase (ESBL) genes (4). For the amplification and sequencing of the bla_{CMY-2}-type β-lactamase genes, the following primer set was used: CMY-flanking-F (5′-CCGGACACCTTTTTGCTTTT-3′) and CMY-flanking-R (5′-TATCCTGGGCCTCATCGTCAGTTA-3′). The PCR amplification identified bla_{TEM-1} in isolates YD006 and 34943 but no class A ESBL genes in any of the isolates. In contrast, the PCR amplified bla_{CMY} in all three isolates. The conjugal transfer of the bla_{CMY} gene using *E. coli* J53 Azi^r (azide resistant) as the recipient (kind gift from George A. Jacoby) was successful with *E. coli* 34943 but not with *E. coli* 1285 or *E. coli* YD006. However, a plasmid was successfully transformed into *E. coli* DH10B by electroporation for all three isolates, indicating the plasmidic location of these bla_{CMY-2} variants. These plasmids appeared unrelated to each other by restriction endonuclease digestion (Fig. 1). The plasmid from *E. coli* YD006 conferred resistance to tetracycline, chloramphenicol, and sulfisoxazole in addition to cephalosporins; resistance to aminoglycosides was not observed. The plasmids from *E. coli* 1285 and 34943 only conferred resistance to cephalosporins. Sequencing analysis revealed the presence of novel CMY-2 variants, bla_{CMY-33} in isolates 1285 and YD006 and bla_{CMY-44} in isolate 34943, respectively. A two-amino-acid deletion (Leu293 and Ala294) was detected in the translated sequence of bla_{CMY-33} compared with that of the other CMY-2 variant. The translated sequence of bla_{CMY-44} had an additional two-amino-acid deletion of Ala295 and Leu296 compared with that of bla_{CMY-33} (Fig. 2). These deletions were located within or in the proximity of the H-10 helix of the enzymes (alternatively, referred to as the R2 loop) (7).

In class C β-lactamases, certain amino acid deletions in the H-10 helix are implicated in the broadening of the substrate spectrum to include cefepime (2, 5, 8). To establish the role of these deletions in the reduced susceptibility to cefepime, isogenic *E. coli* strains producing CMY-2, CMY-33, or CMY-44 were constructed. In brief, the three bla_{CMY} genes were amplified by PCR and directionally cloned to vector pBCSK− as described previously (3). *E. coli* DH10B was then transformed

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TABLE 1. Susceptibility of *E. coli* strains to representative β-lactams

β-Lactam	MIC (μg/ml) for *E. coli* strain:						
	1285 (CMY-33)	YD006 (CMY-33)	34943 (CMY-44)	DH10B (pCMY-33)[a]	DH10B (pCMY-44)[a]	DH10B (pCMY-2)[a]	DH10B (pBCSK−)
Ampicillin	>256	128	>256	>256	>256	>256	3
Piperacillin	>256	12	>256	64	32	128	2
Piperacillin-tazobactam	128	2	96	6	16	4	1
Cefuroxime	>256	96	>256	128	64	>256	6
Ceftazidime	>256	>256	>256	>256	>256	>256	0.5
Cefotaxime	>256	8	32	12	8	16	0.064
Cefoxitin	>256	24	>256	24	32	>256	6
Cefepime	96	6	32	6	3	0.25	0.032
Aztreonam	64	6	24	16	4	8	0.064
Ertapenem	1	0.064	4	0.032	0.064	0.094	0.012

[a] Isogenic strains of *E. coli* DH10B with pBCSK− vector containing bla_{CMY-33}, bla_{CMY-44}, and bla_{CMY-2}, respectively.

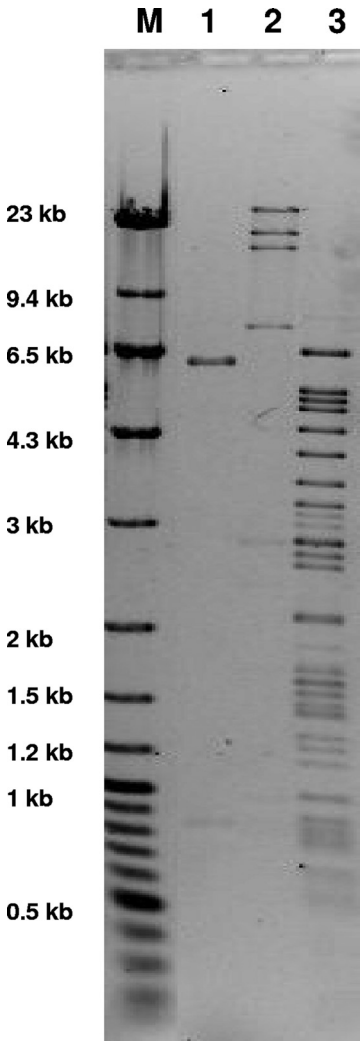

FIG. 1. Plasmid restriction analysis of the bla_{CMY}-bearing plasmids in *E. coli* DH10B transformants corresponding to the clinical isolates. Lane M, molecular weight markers (λ DNA-HindIII digest [New England Biolabs, Ipswich, MA] plus GeneRuler 100-bp Plus DNA ladder [Fermentas, Glen Burnie, MD]); lane 1, 1285; lane 2, YD006; lane 3, 34943. The plasmids were extracted by the standard alkaline lysis method, digested with PstI (New England Biolabs), and electrophoresed in a 0.7% agarose gel.

with these recombinant plasmids. The MICs against representative β-lactams are presented in Table 1.

The *E. coli* DH10B strains producing CMY-33 and CMY-44 demonstrated reduced susceptibility to cefepime compared with that producing CMY-2 by 12- to 24-fold, whereas resistance to ceftazidime and cefotaxime was maintained (Table 1). On the other hand, the MICs of cefuroxime and cefoxitin, which are usually excellent substrates of class C β-lactamases including CMY-2, were lowered for isolates producing CMY-33 and -44. These results suggested that the amino acid deletions observed in CMY-33 and -44 may be responsible for the reduced cefepime susceptibility in these clinical isolates. Given the variable degree of resistance to cefepime among the clinical isolates, however, it is plausible that other mechanisms augmenting cefepime resistance are present, especially in isolate 1285.

Certain amino acid changes (insertions, deletions, or substitutions) in the H-10 helix are believed to result in structural alterations that enable better accommodation of the R2 side chains of cephalosporins, including cefepime, into the active site of class C β-lactamases and thus enhance the hydrolysis of these substrates (7). Examples of insertions and substitutions in class C enzymes that lead to a clinically significant reduction of cefepime susceptibility include chromosomal AmpC of *E. coli* BER (9) and *Enterobacter aerogenes* Ear2 (1) and plasmid-mediated CMY-19 in *Klebsiella pneumoniae* (12). Examples of deletions leading to this property include chromosomal AmpC of *Enterobacter cloacae* CHE (2), *Serratia marcescens* HD (8), and *E. coli* HKY28 (5). All of these variant enzymes were reported as sporadic events, and the understanding is that cefepime maintains excellent activity against organisms producing class C β-lactamases (11). However, our finding of three independent clinical cases producing two CMY-2 variants with this property during a relatively brief period of time suggests that bla_{CMY-2}, the most common plasmid-mediated class C β-lactamase gene in *E. coli*, has the potential to develop variants with reduced susceptibility to cefepime, likely under the selective pressure from excessive use. Clinical microbiologists should be aware that reduced susceptibility to cefepime may occur in variants of CMY-2, which are not easily identified in the clinical laboratory and thus may be overlooked. The continuing discovery of plas-

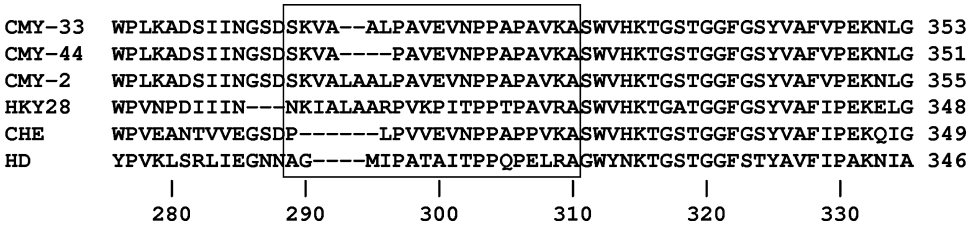

FIG. 2. A sequence alignment of amino acid residues in the H-10 helix region of class C β-lactamases. Partial amino acid sequences of CMY-33 (GenBank accession no. EU496816), CMY-43 (GenBank accession no. FJ437066), and CMY-2 (GenBank accession no. X91840) are aligned along with AmpC β-lactamases of *Escherichia coli* HKY28 (AB108683), *Enterobacter cloacae* CHE (AJ278994), and *Serratia marcescens* HD (AY336102). Amino acids within the R2 loop are represented by a square box. The numbering scheme of the amino acid residues of P99 is adopted (6).

mid-mediated AmpCs has significant implications for infection control measures.

Nucleotide sequence accession numbers. The sequences determined in this study appear in the GenBank/EMBL/DDBJ database under accession numbers EU496816 and FJ437066.

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