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In Vivo Acquisition of High-Level Resistance to Imipenem in *Escherichia coli*

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Four clonally related *Escherichia coli* strains were isolated successively from bile duct of a girl suffering from sclerosing cholangitis. One of them, selected after an imipenem-containing regimen, was resistant to carbapenems and to broad-spectrum cephalosporins due to a plasmid-mediated cephalosporinase, CMY-2, and the lack of outer membrane proteins OmpF and OmpC.

Resistance to carbapenems, while rare in *Enterobacteriaceae*, can be mediated by several mechanisms (4, 13). Production of β-lactamases capable of hydrolyzing carbapenems has been reported in *Enterobacteriaceae*: mostly in *Enterobacter* spp. and *Serratia* spp. (16). Carbapenem resistance may result also from production of large quantities of chromosomal and plasmid-mediated cephalosporinases combined with decreased drug permeability through the outer membrane. Involvement of chromosomal cephalosporinase is known for *Enterobacter* spp., *Proteus rettgeri*, and *Citrobacter freundii*, whereas plasmid-mediated cephalosporinases are involved in low-level resistance to carbapenems for rare isolates of *Proteus mirabilis*, *Klebsiella pneumoniae*, and *Salmonella enterica* serotype Wien (1, 3, 6, 8, 18, 21). In *Escherichia coli*, reports of low-level resistance to imipenem are exceptional, resulting from AmpC hyperproduction and loss of porins (14, 16, 17, 22). We report here the clinical and microbiological features associated with a carbapenem-resistant *E. coli* isolate that had been selected in vivo by an imipenem-containing regimen. A detailed molecular analysis of the antibiotic resistance mechanisms is provided.

Patient and strains. *E. coli* isolates 1 to 4 were isolated from bile duct of a 10-year-old girl hospitalized several times at the Hôpital Bicêtre (Le Kremlin-Bicêtre, France), because she had sickle cell disease, autoimmune hepatitis, and sclerosing cholangitis. *E. coli* isolates 1 and 3 had been isolated in November 2001, whereas isolates 2 and 4 had been isolated in February 2003. These isolates were identified with the API20E gallery (BioMérieux, La-Balme-les-Grottes, France), and identification was confirmed by sequencing of 16S ribosomal DNA sequencing as described previously (2). *E. coli* isolates 2 and 3 were also obtained from blood cultures in February 2003 and September 2002, respectively. Several treatments with antibiotics were given from November 2001 to February 2003, including the β-lactams amoxicillin, cefotaxime, and imipenem. *E. coli* isolate 4 was recovered in the immediate follow-up of an imipenem-containing regimen (750 mg two times daily for 8 days). Then the patient was treated with ciprofloxacin for 2 weeks and underwent surgery of the bile duct.

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Susceptibility testing. Disk diffusion susceptibility testing with antibiotic-containing disks (Sanofi Diagnostics Pasteur, Marnes-La-Coquette, France) (http://www/sfm.asso.fr) was performed with and without cloxacillin (150 μg/ml), a β-lactam molecule that inhibits cephalosporinase activity (5). MICs were determined by an agar dilution technique as reported previously (19). *E. coli* isolate 1 was susceptible to β-lactams, whereas *E. coli* isolate 2 was resistant to cephalosporins and aztreonam (Table 1). The resistance to β-lactams of *E. coli* isolate 3 was similar to that of *E. coli* isolate 2 (although expressed at a much lower level), whereas *E. coli* isolate 4 was resistant to all β-lactams, including imipenem at a high level (with the exception of cefepime) (Table 1). Antimicrobial susceptibility testing on cloxacillin-containing plates indicated that the resistance levels of *E. coli* isolates 2, 3, and 4 to cephalosporins were decreased after cloxacillin addition (data not shown), suggesting the strong expression of a cephalosporinase.

Molecular investigation and biochemical analysis. The pulsed-field gel electrophoresis technique with restriction enzyme XbaI was used for genotyping *E. coli* clinical isolates (20) and showed indistinguishable patterns between *E. coli* isolates 1 and 2 and between isolates 3 and 4, suggesting two clonal origins (data not shown). Conjugation experiments using a rifampin-resistant *E. coli* strain, DH10B, and selection on Mueller-Hinton agar plates containing 100 μg of rifampin per ml and 100 μg of ampicillin per ml (19) gave transconjugants, using *E. coli* isolates 2, 3, and 4 as donors. An identical 60-kb conjugative plasmid (pLN) was extracted from these transconjugants (19) that conferred resistance to penicillins and expanded-spectrum cephalosporins (Table 1). Standard PCR conditions were used to amplify several β-lactamase genes encoding plasmid-mediated cephalosporinases, including $bla_{\text{CMY-1/2}}$, $bla_{\text{FOX-1}}$, $bla_{\text{MOX-1}}$ (18), and several clavulanic acid-susceptible narrow-spectrum and extended-spectrum β-lactamases (19). PCR amplification and sequencing identified the narrow-spectrum penicillinase gene $bla_{\text{TEM-1}}$ and the plasmid-mediated cephalosporinase $bla_{\text{CMY-2}}$ gene located on the 60-kb plasmid in *E. coli* isolates 2, 3, and 4 and their transconjugants, whereas no acquired β-lactamase gene was detected in *E. coli* isolate 1. Identification of the $bla_{\text{CMY-2}}$ gene in *E. coli* isolates 2, 3, and 4 was consistent with their cephalosporin resistance profile.

TABLE 1. MICs of β-lactams for E. coli clinical isolates 1 to 4, transconjugant E. coli DH10B(pLN), and reference strain E. coli DH10B

β-Lactam(s)	MIC (μg/ml) for *E. coli* isolate or strain:					
	1	2	3	4	DH10B(pLN)	DH10B
Amoxicillin	4	>512	>512	>512	>512	2
Ticarcillin	2	>512	256	512	>512	2
Ticarcillin + CLA	1	>512	256	512	256	1
Piperacillin	1	>512	256	128	>512	1
Piperacillin + TZB	1	128	8	128	256	1
Cephalothin	8	>512	>512	>512	>512	4
Ceftazidime	0.06	>512	256	>512	512	0.06
Cefotaxime	0.12	128	16	>512	256	0.12
Cefepime	<0.06	4	0.12	16	1	<0.06
Cefpirome	0.06	8	1	128	4	0.06
Moxalactam	0.12	64	4	>512	8	0.25
Aztreonam	0.06	512	16	512	512	0.12
Imipenem	0.06	0.25	0.25	>32	0.25	0.12
Meropenem	0.06	0.12	0.03	8	0.12	0.06

[a] CLA, clavulanic acid at a fixed concentration of 2 μg/ml; TZB, tazobactam at a fixed concentration of 4 μg/ml.

The outer membrane protein (OMP) profiles of the *E. coli* isolates were analyzed to explain imipenem resistance of *E. coli* isolate 4. OMP studies were performed with sodium dodecyl sulfate-containing polyacrylamide gel electrophoresis as described previously (15, 20) and *E. coli* control strains expressing either OmpC or OmpF (12). The OMP profiles of *E. coli* isolates 1 and 2 were identical, expressing two major porins, OmpF and OmpC, which comigrated, but missing porin OmpA (Fig. 1). Comparison of the OMP profiles of *E. coli* isolates 3 and 4 showed expression of OmpA in both strains, lack of OmpC in both strains, and lack of OmpF protein in *E. coli* isolate 4, whereas it was detected in *E. coli* isolate 3 (Fig. 1). Using whole-cell DNA of *E. coli* isolates 3 and 4 as a template and primers EcOmpFA (5′-CAGGTACTGCAAACGCTGC-3′) and EcOmpFB (5′-GTCAACATAGGTGGACAT G-3′) annealing at the ends of the OmpF gene of *E. coli* (15, 20), a 953-bp internal fragment of the OmpF gene was obtained (data not shown). Sequencing identified a wild-type OmpF gene for *E. coli* isolate 3, whereas that of *E. coli* isolate 4 had a 2-bp deletion located in the middle of the gene. This deletion introduced a premature stop codon in the protein leading to a truncated 187-amino-acid OmpF protein (normal size, 362 amino acids). Using primers EcOmpCA (5′-GTTAAAGTAC TGTCCCTCCTG-3′) and EcOmpCB (5′-GAACTGGTAAA CCAGACCCAG-3′), a 1,086-bp internal fragment of the OmpC gene of *E. coli* was amplified by using whole-cell DNA of *E. coli* isolates 1 and 2 as templates, whereas no amplification was obtained for *E. coli* isolates 3 and 4 (data not shown). Lack of an entire OmpF protein in *E. coli* isolate 4 in addition to lack of OmpC might explain additional resistance to imipenem.

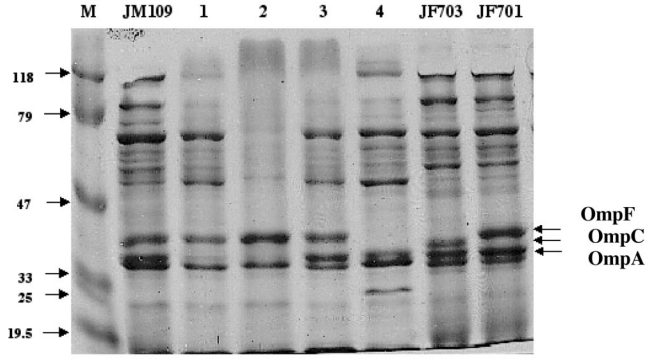

FIG. 1. OMP profiles of *E. coli* strains. OMPs were profiled by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Lanes 1 to 4 correspond to *E. coli* clinical isolates 1 to 4, respectively, and *E. coli* JM109 is a reference strain. *E. coli* isolates JF703 and JF701, expressing, respectively, OmpC or OmpF alone, are from reference 12. The molecular mass marker (M) and corresponding sizes (in kilodaltons) are indicated on the left. Horizontal arrows on the right indicate positions of the OMPs OmpC, OmpF, and OmpA.

Conclusions. This report indicates that an imipenem-containing regimen may select for high-level imipenem resistance in *E. coli*. The conjugative property of the resistance plasmid explains its ability to transfer the β-lactam resistance marker to either clonally related (isolates 1 and 2) or non-clonally related (isolates 2 and 3) *E. coli* isolates. The plasmid encoded a cephalosporinase CMY-2 that is widespread in *Enterobacteriaceae* in human and animal isolates, especially in the United States (7, 10, 11, 18) and associated with TEM-type β-lactamase genes (7, 9, 10, 11, 20). Difference in susceptibility to several β-lactams (cefepime and moxalactam) between two unrelated CMY-2-positive *E. coli* isolates (isolates 2 and 3) may result from difference in another OMP, OmpA (Fig. 1).

This report indicates that plasmid-mediated cephalosporinases may constitute a reservoir of antibiotic resistance (18, 20), enhancing the probability to select for carbapenem resistance in *E. coli*. In those cases, use of carbapenems, including the novel commercially available ertapenem versus the cephalosporinase-resistant cefepime and cefpirome, is debatable. We showed as well that in vivo-acquired nucleotide substitutions in the OmpF gene contributed to resistance to carbapenems, as suggested by in vitro experiments (15). Selection of *E. coli* isolates with different antibiotic resistance patterns from bile duct parallels another study showing that molecular evolution of antibiotic resistance may have occurred in multiple liver cysts infected with *E. coli* (14).

From a general point of view, this case illustrates that high-level resistance to broad-spectrum β-lactams may occur in en-

terobacterial isolates responsible for chronic or recurrent infections from a two-step mechanism that arose successively: i.e., horizontal gene transfer and chromosomal mutations. Management of such infections may benefit from "on-line" surveillance of molecular mechanisms of resistance.

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