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Cooccurrence of Multiple AmpC β-Lactamases in *Escherichia coli*, *Klebsiella pneumoniae*, and *Proteus mirabilis* in Tunisia

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Over a period of 40 months, plasmid-mediated AmpC β-lactamases were detected in Tunis, Tunisia, in 78 isolates (0.59%) of *Escherichia coli*, *Klebsiella pneumoniae*, and *Proteus mirabilis*. In 67 isolates, only one *ampC* gene was detected, i.e., $bla_{\text{CMY-2-type}}$ ($n = 33$), bla_{ACC} ($n = 23$), bla_{DHA} ($n = 6$) or bla_{EBC} ($n = 5$). Multiple *ampC* genes were detected in 11 isolates, with the following distribution: $bla_{\text{MOX-2}}$, $bla_{\text{FOX-3}}$, and $bla_{\text{CMY-4/16}}$ ($n = 6$), $bla_{\text{FOX-3}}$ and $bla_{\text{MOX-2}}$ ($n = 3$), and $bla_{\text{CMY-4}}$ and $bla_{\text{MOX-2}}$ ($n = 2$). A great variety of plasmids carrying these genes was found, independently of the species and the *bla* gene. If the genetic context of $bla_{\text{CMY-2-type}}$ is variable, that of $bla_{\text{MOX-2}}$, reported in part previously, is unique and that of $bla_{\text{FOX-3}}$ is unique and new.

AmpC β-lactamases are cephalosporinases that are poorly inhibited by clavulanic acid (1). AmpC production is one of the mechanisms of resistance to β-lactams in enterobacteria, conferring resistance to all β-lactams except fourth-generation cephalosporins and carbapenems (2). The genes encoding these enzymes are chromosome or plasmid borne (3).

Most plasmid-borne AmpC (pAmpC) β-lactamase genes are derived from chromosomal genes that have been mobilized onto plasmids. Based on sequence similarities with species-specific AmpC enzymes, pAmpC variants are classified into five evolutionary groups: the CIT variants (CMY-2 types) originating in *Citrobacter freundii*, the *Enterobacter* sp. EBC variants (ACT-1 type, MIR-1), the *Morganella morganii* DHA variants, the *Hafnia alvei* ACC variants, and the *Aeromonas* sp. FOX and MOX variants (1, 2). Several genetic elements are involved in the mobilization of pAmpC β-lactamase genes, e.g., the insertion sequences IS*26* (CMY-13), IS*Ecp1* (CMY-2-type, ACC-1-type), and IS*CR1*, associated with complex integrons (DHA-1 type) (1, 2).

The geographic scattering of the different types of pAmpC shows that the CMY-2 type is the most frequent, particularly in Europe (e.g., in France, Spain, Italy, and Turkey [4–7]), in Canada, Argentina, and Tunisia (8–10), and in Korea and China (11, 12). These pAmpC β-lactamases were detected among *Enterobacteriaceae*, especially in *Escherichia coli*, *Klebsiella pneumoniae*, *Klebsiella oxytoca*, *Salmonella enterica*, and *Proteus mirabilis*, and also in naturally AmpC-producing species such as *Enterobacter cloacae*, *Enterobacter aerogenes*, and *C. freundii* (13–15).

The pAmpC genes have also been reported in animals. Indeed, in the United States, CMY-2-producing *E. coli* strains have been found among canine clinical isolates (16). Clinical disease associated with AmpC-producing *E. coli* in dogs in Australia was first reported in 2006 (17). More recently, a survey of clinical isolates of *E. coli* from dogs and horses in the Netherlands revealed that 2% of them were AmpC producers (8). Furthermore, a Swedish study has described CMY-2-producing *E. coli* isolated from broilers (18). Such enzymes have been found in *Klebsiella* isolates from companion animals in Italy (19). In Tunisia, CMY-2-producing *E. coli* isolates have been recovered from healthy food animals and feces from healthy pets (20, 21).

Cooccurrence of multiple pAmpC β-lactamases in a single strain of *Enterobacteriaceae* has not been reported previously but has been observed in a Tunisian collection of various species of this family of bacteria. The present study was conducted to characterize the strains and plasmids carrying multiple pAmpC genes and to explore the genetic context of these genes in an attempt to explain this new phenomenon.

MATERIALS AND METHODS

Bacterial strains, antimicrobial susceptibility, and synergy testing. A total of 11,393 strains from three enterobacterial species. i.e., *E. coli* ($n = 7{,}504$), *Klebsiella* sp. ($n = 2{,}905$) and *P. mirabilis* ($n = 984$), were collected at the clinical microbiology laboratory of Charles Nicolle hospital, Tunis, Tunisia, from January 2006 to April 2009. Only 107 nonduplicate isolates (36 *E. coli*, 58 *Klebsiella*, and 13 *P. mirabilis* isolates) resistant to third-generation cephalosporins, with negative double-disc synergy tests (DDST), were investigated. Susceptibility to antibiotics was determined using standard disc diffusion on Mueller-Hinton agar (Bio-Rad, Marnes-la-Coquette, France), and MICs were determined using standard agar dilution on the same medium according to the CLSI recommendations (22). Moreover, all isolates were phenotypically screened for the production of extended-spectrum β-lactamases (ESBL) using DDST (23) with and without cloxacillin (250 mg/liter) (24).

E. coli ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, and *Staphylococcus aureus* ATCC 25923 served as controls for MIC determinations. Rifampin-resistant *E. coli* J-53 and azide-resistant *E. coli* C600 were used as recipients in conjugation, and *E. coli* DH10B (Invitrogen SARL, Cergy-Pontoise, France) was used in cloning and transformation experiments.

E. coli CFT073 (O6 K1 H7) and *E. coli* J96 (O4 K6 H6) were used as reference strains for the detection of virulence factors (25). Strains carry-

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ing FOX-3 and MOX-2 genes (26, 27) were used as positive controls for detection of FOX and MOX producers with PCR.

Molecular characterization of β-lactamases. Total DNA preparations for PCR were obtained by thermolysis of the isolates. Initial identification was obtained with primers of a multiplex PCR protocol for the detection of *ampC* families as described by Pérez-Pérez et al. (3). Confirmation was obtained using simplex reactions with primers CF-1/CF-2, FOX MF/MR (20), and MOX MF/MR (MOX-MF, 5′-GCTGCTCAAGG AGCACAGGAT-3′; MOX-MR, 5′-CACATTGACATAGGTGTGGTGC-3′) for bla_{CMY}, bla_{FOX}, and bla_{MOX}, respectively. Amplicons of bla_{AmpC} were purified and sequenced. Primers OT3, OT4, and OXA-1 (forward and reverse) were used to amplify putative bla_{TEM-1} and bla_{OXA-1} genes, respectively.

Epidemiological studies. Total DNA was obtained with the QIAamp DNA minikit (Qiagen, Courtaboeuf, France) to assess epidemiological relationships between isolates producing plasmid-mediated cephalosporinases using repetitive-element PCR (rep-PCR) and enterobacterial repetitive intergenic consensus PCR (ERIC-PCR) as previously described (28, 29) and pulsed-field gel electrophoresis (PFGE) of chromosomal DNA digested with the restriction enzyme XbaI (Biolabs).

Determination of phylogenetic groups of *E. coli* was performed using PCR as previously described (30). The *E. coli* clone O25b-ST131 of phylogenetic group B2 was detected using PCR (31). *E. coli* isolates were screened for the presence of 18 genes encoding putative virulence factors characteristic of extraintestinal pathogenic *E. coli* by multiplex PCR as previously described (32, 33, 34). These genes Included *fimH*, *afa*, *sfa/foc*, *papG* (3 alleles), *cnf1*, *sat*, *hlyA*, *iutA*, *iroN*, *fyuA*, *iha*, *kpsMTII*, *ompT*, *malX* (pathogenic island marker PAI II of the CFT073 reference strain), *traT*, and *usp*.

The clonal structure of the *K. pneumoniae* strains was determined by multilocus sequence typing (MLST) based on the allelic diversity of seven loci: *rpoB*, *mdh*, *pgi*, *phoE*, *infB*, *gapA*, and *tonB*. These housekeeping locus fragments of all *K. pneumoniae* isolates were also sequenced.

The primers used for amplifications were designed by Diancourt et al. (35). Sequence types (STs) were assigned at the http://www.pasteur.fr/recherche/genopole/PF8/mlst website.

The *K. pneumoniae* isolates were screened for the presence of nine genes encoding putative virulence factors and six capsular serotypes by PCR as previously described (36, 37, 38).

These genes included *mrkD*, *kpn*, *ycfM*, *entB*, *ybtS*, $iroN_{Kp}$, *rmpA*, *magA*, *allS2*, *wcaG*, and genes encoding K2, K5, K20, K54, and K57 (capsular serotypes).

Transfer of resistance and molecular characterization of plasmids. Mating experiments were performed by mixing equal volumes (1 ml) of exponentially growing cultures of each isolate tested and *E. coli* J-53 or *E. coli* C600 in Trypticase soy broth (Bio-Rad) and incubating the mixture for 3 h at 37°C. β-Lactam-resistant *E. coli* J-53 or *E. coli* C600 transconjugants were selected on Drigalski agar (Bio-Rad) containing rifampin (250 μg/ml) or azide (100 μg/ml) and cefotaxime (2.5 μg/ml).

E. coli DH10B transformants were obtained by electroporation of plasmid DNA extracted with the High Pure plasmid isolation kit (Roche, Mannheim, Germany) in a Gene Pulser II (Bio-Rad) and selected on Drigalski agar supplemented with cefotaxime (2.5 μg/ml). Transfer of β-lactam resistance markers from all clinical isolates studied was attempted by conjugation or transformation.

Replicon typing of the plasmids of the donor strains and the corresponding transconjugants or transformants was performed by PCR under the conditions previously described (39, 40).

Plasmid DNA of the clinical isolates of *P. mirabilis*, *E. coli*, and *K. pneumoniae* and *E. coli* transconjugants was extracted by alkaline lysis, as previously described (41), to estimate plasmid number and size. RP4 (54 kb), pCFF04 (85 kb), and pIP173 (126.8 kb) were used as reference plasmids.

Genetic context analysis. For molecular cloning, we used total DNA of strain Kp10 for the bla_{MOX} gene extracted using the Qiagen DNeasy blood and tissue kit and plasmid DNA from transconjugant J-53 of *K. pneumoniae* Kp8 for the bla_{FOX} gene extracted with the Qiagen large-construct kit. DNA was partially digested with Sau3A and ligated into the BamHI site of pUC18 (42). The recombinant plasmid was introduced into *E. coli* DH10B by transformation. Transformants were selected on Drigalski agar supplemented with cefotaxime (2.5 μg/ml). Recombinant plasmids were extracted with the High Pure plasmid isolation kit (Roche), insert sizes were estimated after digestion with EcoRI, and initial genetic environments were determined by sequencing using universal M13 primers the first time for recombinant plasmids obtained. Recombinant plasmids were extracted with the High Pure plasmid isolation kit (Roche), and insert sizes were estimated after digestion with EcoRI. Initial analysis of the genetic environments of the *bla* genes borne on recombinant plasmids was done by sequencing using universal M13 primers. The genetic context of bla_{MOX}, bla_{FOX}, and bla_{CMY} of recombinant plasmids and all nine remaining strains was further explored by PCR mapping and sequencing using primers designed for bla_{MOX} (A, B, D, E, and G), bla_{FOX} (H and I), and bla_{CMY} (A, B, C, D, E, F, G, H, I, J, K, and L) (see the supplemental material). Two additional primers were tested for bla_{CMY}: CF2/IS*26-SL2* up (IS*26-SL2* up, 5′-CTGGGTAAAATCCTCAACA-3′) and CF2/IS*Ecp1* (IS*Ecp1*, 5′-AAAAATGATTGAAAGGTGGT-3′) (see the supplemental material).

Nucleotide sequence accession number. The accession number of the genetic context of bla_{FOX-3} is LC072710.

RESULTS AND DISCUSSION

Among the 107 enterobacterial isolates studied, multiplex PCR revealed p*ampC* β-lactamase genes in 78 isolates, whereas in 29 isolates (*E. coli*), no p*ampC* β-lactamase genes were detected. In 67 isolates, only one p*ampC* gene was detected with $bla_{CMY-2-type}$ ($n = 32$), $bla_{ACC-1-type}$ ($n = 23$), $bla_{DHA-1-type}$ ($n = 6$), and bla_{EBC} ($n = 5$); among these 67 isolates, 48 (*K. pneumoniae*) also produced CTX-M-15. Multiplex PCR revealed p*ampC* β-lactamase genes in 78 of the 107 isolates studied but not in 29 isolates of *E. coli*. Single p*ampC* genes were detected in 67 isolates, i.e., $bla_{CMY-2-type}$ ($n = 32$), $bla_{ACC-1-type}$ ($n = 23$), $bla_{DHA-1-type}$ ($n = 6$), and bla_{EBC} ($n = 5$). Among the 67 isolates, 48 *K. pneumoniae* isolates also produced CTX-M-15.

In the 11 remaining strains (5 *E. coli*, 4 *K. pneumoniae*, and 2 *P. mirabilis* strains), at least two p*ampC* genes were detected. At this stage, our study was focused on these 11 strains that harbored multiple pAmpC β-lactamases, as follows: MOX-type, FOX-type, and CMY-2-type enzymes in six isolates (in three *E. coli*, one *K. pneumoniae*, and two *P. mirabilis* isolates), MOX-type and FOX-type enzymes in three isolates (in one *E. coli* and two *K. pneumoniae* isolates), and MOX-type and CMY-2-type enzymes in two isolates (in one *E. coli* and one *K. pneumoniae* isolate). The origin and characteristics of clinical interest of these isolates are summarized in Table 1. They were resistant to all β-lactams except ertapenem, imipenem, and cefepime. MICs of ticarcillin and piperacillin ranged from 512 to >2,048 μg/ml, those of cefotaxime from 256 to >2,048 μg/ml, those of ceftazidime from 128 to 1,024 μg/ml, those of cefoxitin from 64 to 512 μg/ml, and those of cefepime 0.5 to 2 μg/ml.

As determined by sequence analysis, bla_{CMY} genes were bla_{CMY-4} ($n = 6$) and bla_{CMY-16} ($n = 2$), bla_{FOX} genes were all bla_{FOX-3} ($n = 9$), and bla_{MOX} genes were all bla_{MOX-2} ($n = 11$). Seven isolates among the pAmpC producers also produced the broad-spectrum TEM-1 β-lactamase, and one of them produced OXA-1 in addition (Table 2).

CMY-4 was first identified in a clinical *P. mirabilis* isolate from Tunisia (43), and since then, it has been found throughout the

TABLE 1 Chromosomal characteristics of the 11 *Enterobacteriaceae* harboring multiple plasmid-borne AmpC β-lactamase genes[a]

Isolate	Ward	Specimen	Yr	REP profile	ERIC profile	PFGE profile	PG	ST	Virulence profile	Virulence score	KS
Ec3	ICU	Blood	2005	A1	A1	A1	A	ND	*fimH*, *hlyA*, *iha*, *usp*, *iroN*	5	ND
Ec5	Med	Urine	2008	A2	A2	A2	B2	ST131	*fimH*, *pai*, *fyu A*, *sfa/foc*, *pap G*, *hlyA*, *cnf1*, *iha*, *usp*	9	ND
Ec6	Chir	Pus	2008	A3	A3	A3	D	ND	*fimH*, *iutA*, *hlyA*, *iroN*	4	ND
Ec7	Ped	Urine	2008	A4	A4	A4	A	ND	*fimH*, *fyuA*, *sfa/foc*, *cnf1*	4	ND
Ec11	Uro	Urine	2007	A5	A5	A5	B2	ST131	*fimH*, *pai*, *fyu A*, *sfa/foc*, *iut A*, *hlyA*, *papG*, *afa/dra*, *iha*, *usp*	10	ND
Kp2	ICU	Blood	2005	B1	B1	B1	ND	ST218	*entB*, *ybtS*, *mrkD*, *ycfM*, *kpn*	5	K57
Kp4	Ped	Urine	2008	B2	B2	B2	ND	ST11	*entB*, *ybtS*	2	ND
Kp8	ICU	Urine	2005	B1	B1	B1	ND	ST218	*entB*, *ybtS*, *mrkD*, *ycfM*, *kpn*	5	K57
Kp10	Ortho	Blood	2005	B3	B3	B1	ND	ST218	*entB*, *ybtS*, *mrkD*, *ycfM*, *kpn*	5	K57
Pm1	Uro	Urine	2006	C1	C1	C1	ND	ND	ND	ND	ND
Pm9	ICU	Catheter	2007	C2	C2	C2	ND	ND	ND	ND	ND

[a] Ec, *Escherichia coli*; Kp, *Klebsiella pneumoniae*; Pm, *Proteus mirabilis*; ICU, intensive care unit; MED, medicine; Surg, surgery; Ped, pediatrics; Uro, urology; Ortho, orthopedics; REP, genomic fingerprinting by repetitive-element PCR profiling; ERIC, enterobacterial repetitive intergenic consensus PCR profile; PFGE, pulsed-field gel electrophoresis profile; PG, phylogenetic groups; ST, sequence type; KS, capsular serotype; ND, not determined.

world. CMY-16, a variant of the CMY lineage, which differs from its closest homologue, CMY-4, by a single amino acid substitution (A171S), was first identified in a *P. mirabilis* isolate from Italy (6).

FOX-3 and MOX-2 β-lactamases have been detected in *K. pneumoniae* isolates from Italy (26) and Greece (27), respectively. However, these enzymes were not previously reported in Tunisia; hence, this is the first report of FOX-3 and MOX-2 β-lactamase in this country. To our knowledge, this is also the first report of cooccurrence of various plasmid-borne bla_{AmpC} genes in a single strain and of such cooccurrence in several species of *Enterobacteriaceae*.

Based on the distinct patterns observed with molecular typing (rep-PCR, ERIC-PCR, and PFGE), there was no epidemiological link between the five *E. coli* isolates harboring multiple p*ampC* genes. The phylogenetic study showed that the five *E. coli* isolates belonged to phylogenetic groups B2 ($n = 2$), A ($n = 2$), and D ($n = 1$). We found that the two B2 isolates belonged to the ST131 clone and harbored many virulence determinants leading to the highest virulence scores, i.e., 9 and 10 (Table 1). The ST131 isolates harbored an extended virulence profile and presented a virulence gene panel similar to that of other previously described members of ST131 (44), an ST often involved in the dissemination of CTX-M-15 genes (31). Moreover, a recent study showed that this clone has enhanced metabolic capacities, acts as a potent colonizer of the intestine, and displays the typical features of virulent *E. coli* strains (45).

ERIC and Rep-PCR typing revealed a similarity in the patterns of two *K. pneumoniae* isolates (Kp2 and Kp8) indicating that they are genetically related. In addition, PFGE analysis confirmed the existence of an epidemic clone represented by these strains, distinct from Kp10, while all of them belonged to ST218 (Table 1). This ST has been described recently in *K. pneumoniae* isolates from patients with bacteremia in China (46). Moreover, we found that the clonal *K. pneumoniae* strains had the capsular serotype K57 and showed important virulence profiles including five genes, i.e., *entB*, *mrkD*, *ycfM*, *kpn*, and *ybtS*. The latter is considered a major virulence determinant in *K. pneumoniae* and is required for the synthesis of yersiniabactin, which promotes biofilm formation and a reduction in the killing capacity of innate immune cells by blocking the production of reactive oxygen species (47, 48). Kp4, on the other hand, was a sporadic isolate of ST11, reported to be involved in the dissemination of various β-lactamases, including carbapenemases and pAmpC (49).

Plasmids carrying pAmpC genes were successfully transferred via conjugation and/or electroporation from 10 of the 11 isolates tested. The pAmpC gene transfer experiments showed that no more than one gene was transferred at one time, either bla_{CMY} or bla_{FOX}, indicating that they are probably located on different plasmids. However, bla_{MOX} could not be transferred from any isolate, suggesting its location on the chromosome.

We found that pAmpC genes were carried on plasmids of different incompatibility groups. The bla_{CMY} genes were carried on conjugative Inc A/C ($n = 2$), Inc FIA ($n = 2$), Inc FIB ($n = 1$), Inc Q ($n = 1$), or Inc FIIK ($n = 1$) plasmids, and bla_{FOX} genes were located on Inc FIA ($n = 2$), Inc A/C ($n = 2$), or Inc Q ($n = 1$) plasmids (Table 2). Our findings are consistent with previous reports noting the predominance of Inc A/C replicons among plasmids carrying bla_{CMY} (34). Accordingly, the Inc A/C replicon is the most commonly reported worldwide (39). The fact that pAmpC genes are carried by different plasmid types promotes their rapid spread among *Enterobacteriaceae* (28, 50). Furthermore, we found that pAmpC genes were generally located on plasmids of various sizes. Analysis of DNA extracted according to Kado and Liu (41) revealed that *K. pneumoniae* isolates Kp2, Kp4, and Kp8 harbored two large plasmids (>130 kb) and a third smaller one (ca. 50 kb), while *E. coli* isolate Ec7 harbored only one large plasmid (>130 kb) and one of smaller one (50 kb). The Kp10 isolate and its corresponding transformant harbored one large plasmid (>130 kb) (Fig. 1). For the remaining *E. coli* and *P. mirabilis* isolates, we obtained no clear results with the procedure of Kado and Liu (41). Our results are in agreement with reports of pAmpC genes found on plasmids of sizes varying from 7 to 180 kb (2).

Two plasmids of the present study carrying CMY-4 and FOX-3 genes belonged to the Q1 group. These replicons are characterized by their relatively small size (5 to 10 kb), their ability to be mobilized by several self-transmissible plasmids, and their broad host range, making them highly promiscuous (51).

Analysis of the genetic environment of bla_{MOX-2} by cloning and

TABLE 2 Plasmid characteristics of the 11 *Enterobacteriaceae* harboring multiple plasmid-borne AmpC genes and exploration by PCR mapping[a]

Isolate	Resistance pattern	bla_{AMPC}	bla_{TEM-1}, bla_{OXA-1}	RT	PCR mapping result																				
					bla_{MOX}					bla_{FOX}		bla_{CMY}														
					A	B	D	E	G	H	I	A	B	C	F	G	H	I	J	K	L	D	E	CF2/IS*26*-*SL2* up	CF2/IS*Ecp1* commune
Ec3	GM, TM, NET, TE, C, NA, SXT	FOX-3, MOX-2		A/C	+	+	+	+	+	+	+														
Ec5	GM, TM, NA, OFX, CIP	CMY-4, FOX-3, MOX-2	TEM-1	FIA, FIB	+	+	+	+	+	+	+	−	−	+	+	−	−	−	−	−	+	+	+	−	−
El Ec5	TM	CMY-4		FIA																					
Ec6	TE	CMY-4, FOX-3, MOX-2	TEM-1	A/C, FIB	+	+	+	+	+	+	+	−	+	+	+	+	+	+	−	−	−	−	−	−	−
El Ec6	TE	CMY-4		FIB																					
Ec7	TE	CMY-4, MOX-2	TEM-1	Q	+	+	+	+	+			−	−	−	−	−	−	−	−	−	−	−	−	−	−
Tc Ec7		CMY-4	TEM-1	Q																					
Ec11	TM, NET, AN, NA, CIP, SXT, OFX	CMY-16, FOX-3, MOX-2	OXA-1	FIA	+	+	+	+	+	+	+	−	+	+	−	−	−	−	−	−	−	−	−	−	−
El Ec11	TM, SXT	CMY-16	OXA-1	FIA																					
Kp2	GM, TM, NET, TE, C, SXT	FOX-3, MOX-2		A/C, FIIK	+	+	+	+	+	+	+														
Tc Kp2	TM, TE, SXT	FOX-3		A/C																					
Kp4	TE, SXT	CMY-16, FOX-3, MOX-2	TEM-1	Q	+	+	+	+	+	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Tc Kp4A		FOX-3		Q																					
El Kp4B	TE, SXT	CMY-16	TEM-1	-																					
Kp8	GM, TM, NET, TE, SXT	FOX-3, MOX-2		A/C, FIIK	+	+	+	+	+	+	+														
Tc Kp8	TM, TE, SXT	FOX-3		A/C																					
Kp10	GM, TM, NET, TE, SXT	CMY-4, MOX-2	TEM-1	A/C, FIIK	+	+	+	+	+			−	+	+	+	+	−	−	−	−	−	−	−	−	−
El Kp10	TE, SXT	CMY-4		FIIK																					
Pm1	TE, C, NA, OFX, CIP, SXT	CMY-4, FOX-3, MOX-2	TEM-1	A/C, FIA	+	+	+	+	+	+	+	−	+	+	+	+	+	+	−	−	−	−	−	−	
Tc Pm1A	C, SXT	CMY-4		A/C																					
Tc Pm1B	TE	FOX-3		FIA																					
Pm9	TM, NET, AN, TE, SXT	CMY-4, FOX-3, MOX-2	TEM-1	A/C, FIA	+	+	+	+	+	+	+	−	+	+	+	+	−	−	−	−	−	−	−	−	−
Tc Pm9A	TM, TE, SXT	CMY-4	TEM-1	A/C																					
Tc Pm9B		FOX-3		FIA																					

[a] Ec, *Escherichia coli*; Kp, *Klebsiella pneumoniae*; Pm, *Proteus mirabilis*; Tc, transconjugant; El, electroporant; RT, replicon type; GM, gentamicin; TM, tobramycin; NET, netilmicin; AN, amikacin; C, chloramphenicol; NA, nalidixic acid; OFX, ofloxacin; CIP, ciprofloxacin; SXT, trimethoprim-sulfamethoxazole; TE, tetracycline.

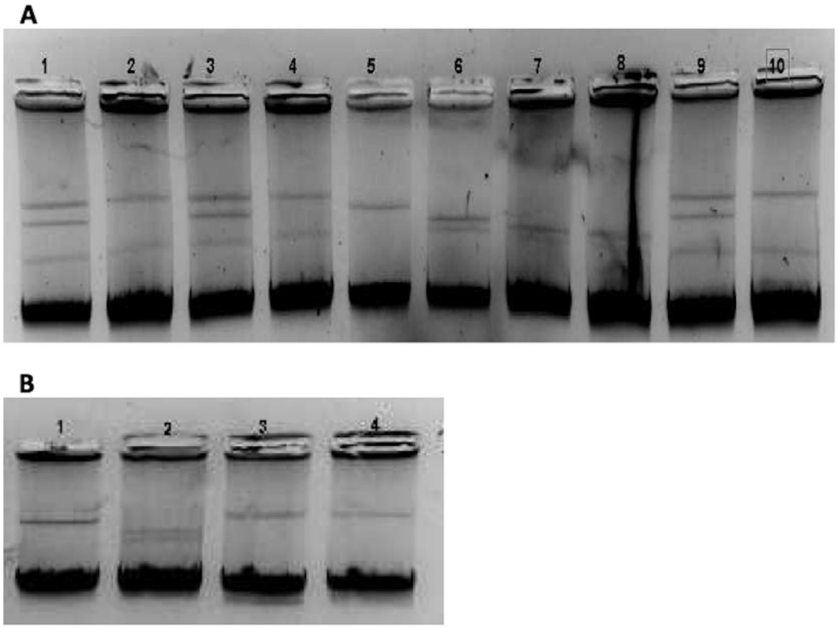

FIG 1 Plasmid analysis. (A) Lane 1, Kp2; lane 2, transconjugant (Tc) Kp2; lane 3, Kp4; lane 4, Tc Kp4 B; lane 5, pIP173 (126 kb); lane 6, RP4 (55 kb) and pCFF04 (85 kb); lane 7, Ec7; lane 8, Tc Ec7; lane 9, Kp8; lane 10, Tc Kp8. (B) Lane 1, pIP173 (126 kb); lane 2, RP4 (55 kb) and pCFF04 (85 kb); lane 3, Kp10; lane 4, transformant (Tf) Kp10.

mapping experiments showed that it was very closely related to that of the single reference strain *K. pneumoniae* KOL (accession number AJ276453) reported in 2002 (27). The DNA insert carrying bla_{MOX-2} was sequenced and four additional open reading frames (ORFs) were identified (Fig. 2). The ORFs are on the opposite DNA strand. The first (ORF2, accession number AJ276453) is located 212 bp upstream from bla_{MOX-2} and encodes a putative 375-amino-acid protein 99% identical to a transposase (IS*A1* type) of the IS*4* family. Nine copies of this ORF are found on the chromosome of *Aeromonas media* strain WS (accession number CP007567) and on the multiresistance-encoding plasmid pP2G1 from an *Aeromonas* sp. (accession number HE616910). Two other ORFs are located upstream from IS*A1* and encode two hypothetical proteins of 150 and 129 amino acids, respectively; they are organized in tandem in many plasmids from *Enterobacteriaceae*, *Vibrio* spp., and *Aeromonas* spp. A 612-bp ORF lacking 5′ sequences was identified 1,010 bp upstream from ORF3. It encodes a 204-amino-acid product 100% identical to the putative transposase A of IS*186* of *E. coli* K-12 (accession number X03123).

Exploration of the genetic environment of bla_{FOX-3} of Kp8 revealed the presence of IS*Apu2* upstream from bla_{FOX-3} (Fig. 3). The genetic organization was different from that of the previously published genes bla_{FOX-1}, bla_{FOX-5}, bla_{FOX-6}, and bla_{FOX-7} (accession numbers X77455, CP007732 and DQ478715, AY034848, and HG934082, respectively), suggesting different recombination events, although the insertion sequence IS*Apu2* also seems to play a role in the mobilization of FOX-1 and FOX-7. We noted the presence of a repeat sequence of unknown function (TGCTAA) upstream from bla_{FOX-3}. Using PCR mapping and sequencing, the genetic environment of bla_{FOX-3} in the other strains carrying this

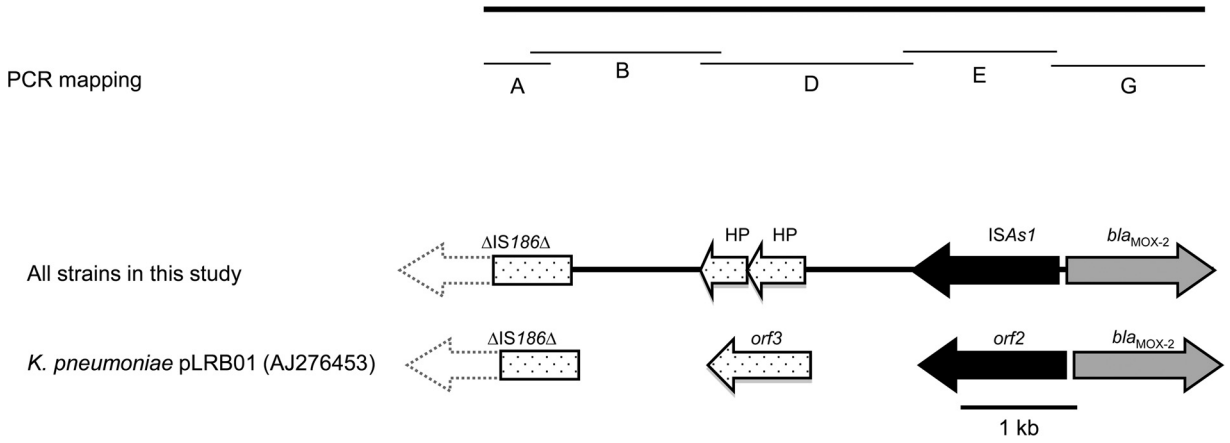

FIG 2 Genetic context of bla_{MOX-2} beta-lactamase genes.

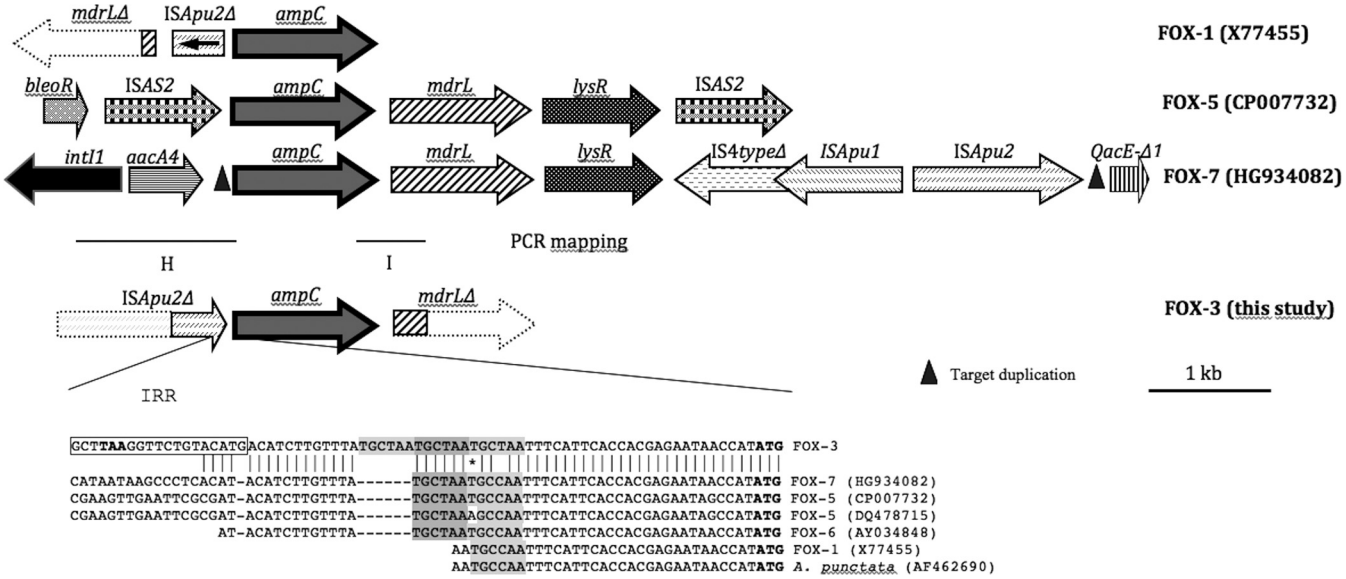

FIG 3 Genetic context of bla_{FOX-3} beta-lactamase genes.

gene was found to be the same. To our knowledge, this is the first report of the genetic environment of bla_{FOX-3}.

In six isolates (Ec6, Pm1, Kp10, Pm9, Ec5, and Ec11) of eight in the present study which carried bla_{CMY}, at least part of IS*Ecp1* was present upstream from the bla_{CMY-2}-like gene (Fig. 4). The region between IS*Ecp1* and the bla_{CMY-2}-like gene had the same structure in only five isolates (Table 2; Fig. 4).

Our findings are consistent with those concerning the genetic environment of $bla_{CMY-2-type}$ genes described previously in which IS*Ecp1* is present (42). It was suggested that IS*Ecp1* could be involved in mobilizing the bla_{CMY-2}-like gene from the *C. freundii* chromosome to plasmids (52, 53).

All plasmids carrying bla_{CMY} described here shared various structural similarities with the 13-kb type I structure described in *Salmonella enterica* serovar Newport (accession number DQ164214) (Fig. 4) in which bla_{CMY-2} is duplicated. The second copy is adjacent to a partial copy of IS*Ecp1* in the opposite orientation (53). Downstream from the bla_{CMY-2}-like gene in this 13-kb type I structure, several ORFs were detected between the two copies of bla_{CMY-2} (53). Very similar structures were found in isolates Ec6, Pm1, Pm9, and Kp10. However, no isolate harbored two copies of the bla_{CMY-2}-like gene. For Ec6, Pm1, Kp10, and Pm9, the sequence either downstream from *traC* or downstream from *orf1* remains unknown (Fig. 4). In Ec5, as opposed to Ec6, Pm1, Pm9, and Kp10, there were fewer similarities with the 13-kb type I structure. Downstream from the bla_{CMY-2}-like gene were *blc*, *sugE*,

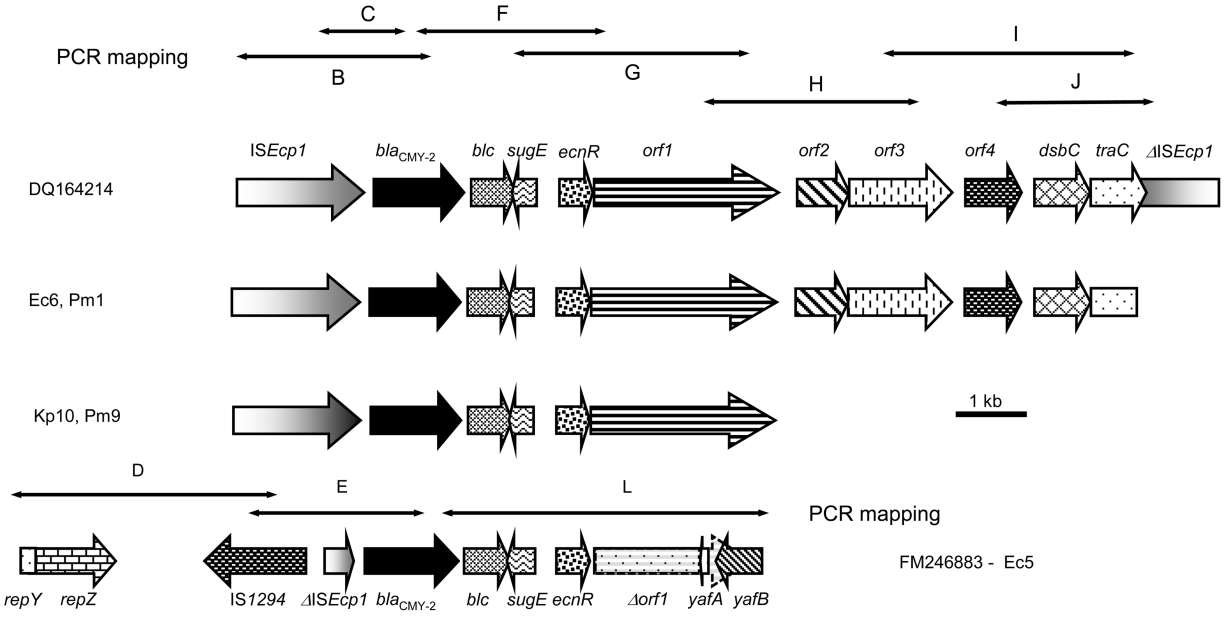

FIG 4 Genetic context of $bla_{CMY-2-type}$ beta-lactamase genes.

ecnR, and *orf1* only, with a truncated *yafA* adjacent in reverse orientation, followed by *yafB*. Both *yaf* genes were part of a segment which is 98% identical to a region of *S. enterica* plasmid pNF1358 (accession number DQ017661) (Fig. 4). This segment is located directly upstream from IS*Ecp1*−bla_{CMY-2} on pNF1358. In Ec5, truncation of IS*Ecp1* was due to the insertion of IS*1294* in the reverse orientation. IS*1294* belongs to the IS*91* family and is a putative transposable element capable of mediating one-ended transposition (www-is.biotoul.fr). Downstream from IS*1294*, a segment comprising the *repY* and *repZ* genes involved in replication initiation is 95% identical to a region of *S. enterica* pNF1358. In Ec11, only IS*Ecp1* was present upstream from the bla_{CMY-2}-like gene, while the rest of the structure remains unknown, suggesting a new genetic environment.

The results of the present study are consistent with those of Barlow and Hall with respect to Ec6, Pm1, Kp10, Pm9, Ec5, and Ec11, in which IS*Ecp1* could be involved in the mobilization of the bla_{CMY-2}-like gene, irrespective of the replicon type and the plasmid backbone (54).

According to Hopkins et al., each replicon type reflects the genetic organization surrounding the bla_{CMY-2}-like gene, with several minor rearrangements, including small insertions or deletions (55). Our findings are not consistent with those of Hopkins et al., since we found in our collection diverse plasmids carrying bla_{CMY-2}-like genes in which the structure of the genetic environment of bla_{CMY} is different even in cases of the same replicon type. In Ec7, bla_{CMY} was carried on an IncQ plasmid. This is the first observation of the gene on such an extremely rare plasmid. The study of the genetic environment of bla_{CMY} carried on IncQ by mapping PCR was negative, suggesting a new genetic structure.

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