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bla$_{CTX-M}$ Genes in *Escherichia coli* Strains from Croatian Hospitals Are Located in New (*bla*$_{CTX-M-3a}$) and Widely Spread (*bla*$_{CTX-M-3a}$ and *bla*$_{CTX-M-15}$) Genetic Structures[▽]

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CTX-M-producing *Escherichia coli* isolates from three Croatian hospitals were analyzed. All *bla*$_{CTX-M-15}$ genes and one *bla*$_{CTX-M-3a}$ gene resided in widely spread IS*Ecp1* transposition modules, but other *bla*$_{CTX-M-3a}$ genes were in a new configuration with two IS*26* copies, indicating a new event of gene mobilization from a *Kluyvera ascorbata* genome. The study confirmed the role of the *E. coli* ST131 clonal group with IncFII-type plasmids in the spread of *bla*$_{CTX-M-15}$ and of IncL/M pCTX-M3-type plasmids in the dissemination of *bla*$_{CTX-M-3a}$.

The rapid spread of CTX-M extended-spectrum β-lactamases (ESBLs) has been one of the recent spectacular changes in ESBL epidemiology (7, 9, 24, 32). CTX-Ms are derivatives of *Kluyvera* species β-lactamases (7, 29, 32), and mobilization of *bla*$_{CTX-M}$ genes has occurred frequently (4), with the essential assistance of IS*Ecp1* and IS*CR1* elements, commonly found at their 5′ flanks (29, 32). These elements may transpose with downstream DNA fragments, which in the case of IS*Ecp1* requires an alternative inverted right repeat (IRR) to form the 3′ end of the transposition module (29). Such IRRs are behind β-lactamase genes in *Kluyvera ascorbata* chromosomes, including one inside *orf477*, which follows the β-lactamase genes directly (22, 31). Structure details of the modules, like the IS*Ecp1*–*bla*$_{CTX-M}$ distance and the 3′-end position, are markers of particular mobilizations. More flexible are the plasmids in which they reside; however, it seems that successful dissemination of some *bla*$_{CTX-M}$ variants greatly depends on their locations on specific molecules of different incompatibility groups (13, 17, 18, 27). The *bla*$_{CTX-M-15}$ gene is linked to IncFII or IncI1 plasmid families worldwide (13, 18, 27), while the diffusion of *bla*$_{CTX-M-3}$ was attributed to IncL/M, IncN, or IncA/C plasmids (3, 6, 17, 18, 27, 33). The most recent data also underline the role of the spread of particular clones, mostly of *Escherichia coli* clones with CTX-M-15 (23, 40).

This study revealed a high diversity of the context of *bla*$_{CTX-M-3/-15}$ genes in *E. coli* from Croatia and confirmed the importance of specific clones and plasmid types in their spread.

Eleven *E. coli* isolates were identified between 2002 and 2005 in three hospitals: the Clinical Hospital Center in Zagreb, Croatia (center Z1) ($n = 5$); the Sisters of Mercy Hospital in Zagreb (center Z2) ($n = 1$); and the University Hospital in Split, Croatia (center S) ($n = 5$) (Table 1). The partial data for isolates from center S were published previously (38). The all isolates tested positive for ESBLs by the double-disk test (19). MICs of β-lactams, determined by broth microdilution according to the CLSI guidelines (12), showed typical ESBL-mediated patterns, with some variation between centers (Table 2). Conjugation was performed as described previously (16), with *E. coli* A15 Rif^r as a recipient and cefotaxime (2 μg/ml) and rifampin (rifampicin) (256 μg/ml) as selection agents. Transfer of resistance to non-β-lactams was tested by disk diffusion (12). All isolates from center Z1 produced transconjugants with ESBLs and resistance to aminoglycosides, co-trimoxazole, and tetracycline (Table 1), differing from transconjugants of isolates from center S (38). Isolate 49 from center Z2 did not mate.

The isolates were classified into major *E. coli* phylogroups by using the PCR-based approach (11). All isolates from centers Z1 and Z2 and two from center S (isolates 32 and 86) were classified in the virulent phylogroup B2, while the remaining isolates from center S were classified into the commensal phylogroup A (Table 1). Pulsed-field gel electrophoresis (PFGE) was performed as described by Kaufmann (21) and interpreted according to the guidelines of Tenover et al. (37). All isolates from center Z1 produced identical XbaI PFGE patterns (Table 1), while of the others, only the two B2 isolates from center S, 32 and 86, were related to each other, as shown previously (38). Representative isolate 52 from center Z1 and all isolates from hospitals Z2 and S were analyzed by multilocus sequence typing (39); the Internet database (www.mlst.net) was used for assigning sequence types (STs). These isolates had different STs (Table 1), all of which were new combinations of known alleles. The only similar allelic profiles were those of the related B2 isolates 32 and 86 (ST1038 and ST1039, respectively). Some STs were single-locus variants of STs found previously, with ST1035 of isolate 52 from center Z1 being a single-locus variant of ST131. Accumulating data document the global spread of the *E. coli* ST131 clone with CTX-M-15, observed so far in nine countries in Europe, North and South America, and Asia (13; www.mlst.net). The Croatian outbreak strain from center Z1 seems to represent the same pandemic lineage.

β-Lactamases were profiled by isoelectric focusing, as described previously (5). All isolates from center Z1 and their

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TABLE 2. Antimicrobial susceptibilities of clinical isolates

Isolate	MIC (μg/ml) of[a]:															
	AMP	AMC	PIP	TZP	CTX	CTX + CLA	CRO	CAZ	CAZ + CLA	FEP	FOX	IPM	MEM	AMK	GEN	CIP
16	>1,024	4	256	2	64	0.125	>1,024	1	0.125	128	4	0.125	0.06	64	32	32
32	>1,024	8	>1,024	16	>1,024	0.06	>1,024	4	0.5	2	16	0.25	0.06	16	>128	0.125
86	>1,024	8	>1,024	>128	>1,024	0.125	>1,024	8	0.5	512	16	0.25	0.06	4	>128	0.125
36	>1,024	8	>1,024	4	512	0.06	>1,024	4	0.25	256	8	0.25	0.125	2	1	0.125
100	>1,024	8	>1,024	4	128	0.06	>1,024	4	0.5	32	8	0.5	0.25	>128	>128	8
Z1 isolates	>1,024	32	>1,024	>128	>1,024	0.5	>1,024	16–32	1–2	64	16	1	0.125–0.25	>128	>128	>128
49	>1,024	1	64	8	128	0.06	128	0.25	0.06	8	2	0.06	0.06	≤0.03	0.125	≤0.03
E. coli ATCC 25922	4	4	2	2	0.06	≤0.03	0.06	0.25	0.125	≤0.03	4	0.125	≤0.03	1	0.5	≤0.03

[a] Abbreviations: AMC, amoxicillin (amoxicilline) with clavulanate; AMK, amikacin; AMP, ampicillin; CAZ, ceftazidime; CIP, ciprofloxacin; CLA, clavulanate; CRO, ceftriaxone; CTX, cefotaxime; FEP, cefepime; FOX, cefoxitin; GEN, gentamicin; IPM, imipenem; MEM, meropenem; PIP, piperacillin; TZP, piperacillin with tazobactam. Antimicrobial powders were from Pliva, Zagreb, Croatia (amoxicillin, ampicillin, ceftazidime, and clavulanic acid); Belupo, Koprivnica, Croatia (cefotaxime); AstraZeneca, Zagreb, Croatia (meropenem); Sigma-Aldrich, St. Louis, MO (gentamicin, piperacillin, and tazobactam); and U.S. Pharmacopeia Reference Standards, Rockville, MD (cefepime, cefoxitin, ciprofloxacin, and imipenem). MICs for isolates 16, 32, 36, 86, and 100 were published previously (38).

TABLE 1. Typing, β-lactamase content, plasmid characteristics, and bla_{CTX-M} gene contexts of the study isolates

Isolate	Center	Date (mo/yr)	Specimen[a]	Typing data			IEF pI(s)[c]	bla_{CTX-M} gene	Plasmid data						bla_{CTX-M} context
				Phylogroup	PFGE type	ST[b]			PstI fingerprint[d]	Replicon (s)[e]	bla_{TEM-1}[e]	bla_{OXA-1}[e]	*aac(6′)-Ib-cr*[e]	Resistance in R^{+}[f]	
16	S	6/2002	Urine	A	I	1036	**8.4**	$bla_{CTX-M-3a}$	A	FII	−	−	−	AK, GM	IS*26*–$bla_{CTX-M-3a}$–IS*26*
32	S	9/2002	BA	B2	IIa	1038	**8.4**, 5.4	$bla_{CTX-M-3a}$	B1	L/M	−	−	−	T	IS*26*–$bla_{CTX-M-3a}$–IS*26*
86	S	4/2003	Urine	B2	IIb	1039	**8.4**, 5.4	$bla_{CTX-M-3a}$	B2	L/M	−	−	−	T	IS*26*–$bla_{CTX-M-3a}$–IS*26*
36	S	7/2002	Urine	A	III	1028	**8.4**	$bla_{CTX-M-3a}$	B1	L/M	−	−	−	T	IS*26*–$bla_{CTX-M-3a}$–IS*26*
100	S	12/2003	Urine	A	IV	1027	**8.4**, **5.4**	$bla_{CTX-M-3a}$	C	L/M	+	−	−	AK, GM, SXT	IS*Ecp1*–$bla_{CTX-M-3a}$
52	Z1	10/2005	Blood	B2	V	1035	**8.9**, **7.4**	$bla_{CTX-M-15}$	D	FII + FIA	−	+	+	AK, GM, SXT, T	IS*Ecp1*–$bla_{CTX-M-15}$
1059	Z1	10/2005	Blood	B2	V	ND	**8.9**, **7.4**	$bla_{CTX-M-15}$	NP	FII + FIA	−	+	+	AK, GM, SXT, T	IS*Ecp1*–$bla_{CTX-M-15}$
1830	Z1	10/2005	Blood	B2	V	ND	**8.9**, **7.4**	$bla_{CTX-M-15}$	NP	FII + FIA	−	+	+	AK, GM, SXT, T	IS*Ecp1*–$bla_{CTX-M-15}$
4274	Z1	11/2005	Blood	B2	V	ND	**8.9**, **7.4**	$bla_{CTX-M-15}$	NP	FII + FIA	−	+	+	AK, GM, SXT, T	IS*Ecp1*–$bla_{CTX-M-15}$
4874	Z1	11/2005	Blood	B2	V	ND	**8.9**, **7.4**	$bla_{CTX-M-15}$	NP	FII + FIA	−	+	+	AK, GM, SXT, T	IS*Ecp1*–$bla_{CTX-M-15}$
49	Z2	10/2004	Urine	B2	VI	1037	8.4	$bla_{CTX-M-3a}$	ND	ND	−	−	−		IS*26*–$bla_{CTX-M-3a}$–IS*26*

[a] BA, bronchial aspirate.
[b] ND, not determined (these isolates were represented by isolate 52).
[c] IEF, isoelectric focusing. Boldface pI values refer to the β-lactamases observed also in transconjugants.
[d] NP, the analysis was not performed (the plasmid was from the transconjugant of isolate 52); ND, not determined due to DNA degradation.
[e] The analysis was performed only on plasmid or total DNA from the transconjugants. +, present; −, absent.
[f] Resistance refers only to non-β-lactam resistance cotransferred with ESBLs to transconjugants. AK, amikacin; GM, gentamicin; SXT, co-trimoxazole; T, tetracycline.

TABLE 3. Selected primers used in the study

Primer	Sequence (5′→3′)	Orientation[a]	Target sequence(s)	Purpose(s)	Reference
P1C	TCGTCTCTTCCAGA	FW	5′ flank of $bla_{\text{CTX-M-1}}$-like genes	PCR detection and sequencing	16
P2D	CAGCGCTTTTGCCGTCTAAG	REV	3′ flank of $bla_{\text{CTX-M-1}}$-like genes (*orf477*)	PCR detection and sequencing	16
FIA FW	CCATGCTGGTTCTAGAGAAGGTG	FW	Iterons	PBRT	10
FIA RV	GTATATCCTTACTGGCTTCCGCAG	REV	Iterons	PBRT	10
F_{repB} FW	TGATCGTTTAAGGAATTTTG	FW	RNAI/*repA*	PBRT	10
F_{repB} RV	GAAGATCAGTCACACCATCC	REV	RNAI/*repA*	PBRT	10
L/M FW	GGATGAAAACTATCAGCATCTGAAG	FW	*repA*, *-B*, *-C*	PBRT	10
L/M RV	CTGCAGGGGCGATTCTTTAGG	REV	*repA*, *-B*, *-C*	PBRT	10
TEM-C	CCCCGAAGAACGTTTTC	FW	bla_{TEM}	PCR detection	25
TEM-E	TCGTCGTTTGGTATGGC	REV	bla_{TEM}	PCR detection	25
OXA-1F	ACACAATACATATCAACTTCGC	FW	$bla_{\text{OXA-1}}$	PCR detection	35
OXA-1R	AGTGTGTTTAGAATGGTGATC	REV	$bla_{\text{OXA-1}}$	PCR detection	35
qac2	GACTGAGCATGACCTTGCG	FW	*aac*(*6′*)*-Ib* and *aac*(*6′*)*-Ib-cr*	PCR detection, ARMS	1
qac3-Ib	GTACACGGCTGGACCATC	REV	*aac*(*6′*)*-Ib*	PCR detection, ARMS	This work
qac3-Ib-cr	GTACACGGCTGGACCATA	REV	*aac*(*6′*)*-Ib-cr*	PCR detection, ARMS	This work
ISEcp1L1	CAGCTTTTATGACTCG	FW	IS*Ecp1*	PCR detection	This work
ALA-5	CCTAAATTCCACGTGTGT	REV	IS*Ecp1* IRR	PCR detection	This work
IS26LF	GTCGGTGGTGATAAAC	FW	IS*26*	PCR detection	This work
IS26RR	GTTAGCGATGAGGCAG	REV	IS*26*	PCR detection	This work
ALA-4	CTATCCGACAAGGGAG	FW	IS*Ecp1 tnpA*, 3′ part	PCR mapping and sequencing	2
ALA-3	TTTGCGCATACAGCGGCACAC	REV	$bla_{\text{CTX-M-1}}$-like genes, 5′ part	PCR mapping and sequencing	2
P1A	GGCGATCCGCGTGATACCAC	FW	$bla_{\text{CTX-M-1}}$-like genes, 3′ part	PCR mapping	3
orf477-IRR	CCTGGGACCTACGTG	REV	*orf477*	PCR mapping	17
orf477–27	CAACGTCTGGCTATTGCCATG	REV	*orf477*	PCR mapping	This work
IS26LR	GTTTATCACCACCGAC	FW or REV[b]	IS*26*	PCR mapping	This work
CTX-M-F1	CCGCTGATTCTGGTCACTT	FW	$bla_{\text{CTX-M-1}}$-like genes	PCR mapping and sequencing	This work
CTX-M-F2	GCGCATAGTCATCGGCAG	FW	*orf477*	PCR mapping and sequencing	This work
tnpAF6	GGTATGCGTTTTGTCAC	REV	Transposase pseudo-ORF	PCR mapping and sequencing	This work
tnpAR3	GTCTGCTGCTCCAGAAG	FW	Transposase pseudo-ORF	PCR mapping and sequencing	This work

[a] FW, forward; REV, reverse.
[b] This primer, annealing to the 5′ part of IS*26* and directed upstream of the element, was used as a forward primer in PCR with ALA-3 and as a reverse primer in PCR with tnpAR3 in the PCR mapping of IS*26*–$bla_{\text{CTX-M-3a}}$–IS*26* modules.

transconjugants produced β-lactamases with pIs of 8.9 and 7.4, while those from hospitals S and Z2 had enzymes with a pI of 8.4 (Table 1). β-Lactamases with a pI of 5.4 were found in some isolates from center S and in one transconjugant of these isolates (isolate 100). The $bla_{\text{CTX-M}}$ genes were amplified with primers P1C and P2D (Table 3) (16) and sequenced as reported previously (3). The pI 8.9 β-lactamases were CTX-M-15 (20), and the pI 8.4 enzymes were CTX-M-3, specified by the $bla_{\text{CTX-M-3a}}$ allele (16, 41). CTX-M-3 and especially CTX-M-15 belong to predominant CTX-M types in Europe (9, 24, 32).

Plasmid DNA was purified (Plasmid Midi kit; Qiagen, Hilden, Germany) from the transconjugant of isolate 52 from center Z1, from transconjugants of all isolates from center S, and from isolate 49 from center Z2. In PCRs, total DNAs of the other transconjugants from center Z1 were included. Plasmid preparations contained single large molecules. For fingerprinting, they were digested with PstI (Fermentas, Vilnius, Lithuania). Four fingerprints were observed (Table 1): pattern D for the isolate from center Z1 (~150 kb) and patterns A, B, and C for the isolates from center S (the plasmid of the center Z2 isolate degraded). PCR-based replicon typing (PBRT), limited to replicons F1A, F1B, FII, I1, and L/M, was performed according to the method of Carattoli et al. (10). Replicons FII and FIA were detected in plasmids of center Z1 isolates, while among isolates from hospital S, replicon FII correlated with fingerprint A and replicon L/M with B and C (Table 1). None of the replicons tested was found in the center Z2 isolate. The β-lactamase genes $bla_{\text{TEM-1}}$ and $bla_{\text{OXA-1}}$ were identified by PCR (25, 35) (Table 3). The aminoglycoside and quinolone resistance gene *aac*(*6′*)*-Ib-cr* (30) was detected with primer qac2 (1) and two primers with variant 3′ nucleotides, qac3-Ib and qac3-Ib-cr (Table 3); a positive result was obtained with qac2 and qac3-Ib-cr. Plasmids of center Z1 isolates carried $bla_{\text{OXA-1}}$ and *aac*(*6′*)*-Ib-cr*, whereas that of isolate 100 from hospital S contained $bla_{\text{TEM-1}}$ (Table 1). The results obtained indicated that the $bla_{\text{CTX-M-15}}$ gene of the outbreak isolates from center Z1 was located on a plasmid(s) similar to plasmids

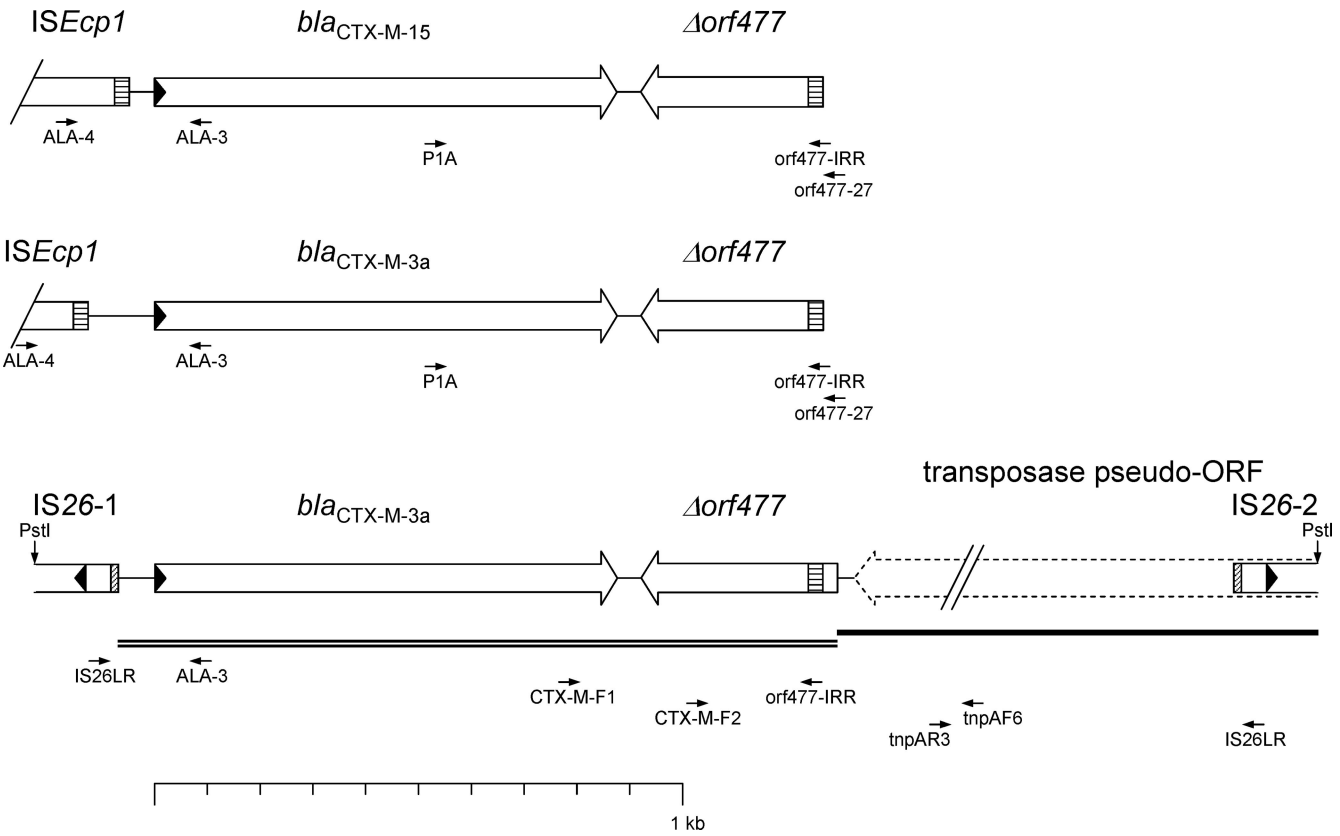

FIG. 1. Schematic representation of the genetic structures identified in the study: the IS*Ecp1*–$bla_{CTX-M-15}$ (top) and IS*Ecp1*–$bla_{CTX-M-3a}$ (middle) transposition modules and the IS*26*–$bla_{CTX-M-3a}$–IS*26* element (bottom). The structures of the IS*Ecp1* modules are based on the sequence data from references 8 and 17. The scheme of the IS*26*-associated locus is based on sequences of the plasmidic PstI fragments (4,553 bp) cloned in this work. The double horizontal bar indicates a fragment homologous to *K. ascorbata* DNA (31), while the single thick bar indicates a fragment homologous to the *A. baumannii* AYE sequence (15). The broken line used to draw the arrow of the transposase pseudo-ORF symbolizes its disruption by the nonframe deletion. Striped boxes refer to the IRL and IRR segments of IS*26* and IS*Ecp1* elements, respectively, including the alternative IS*Ecp1* IRR located within *K. ascorbata orf477* (in the IS*26*-associated structure shown only for the comparison of Δ*orf477* fragments in the modules). Black triangles refer to ATG codons of bla_{CTX-M} and IS*26 tnpA* genes. PCR primers shown in the diagrams are those that were used for PCR mapping of the bla_{CTX-M}-carrying modules.

observed worldwide [replicons FII and FIA, bla_{OXA-1}, *aac(6′)-Ib-cr*, resistance to co-trimoxazole and tetracycline] (13, 18, 27). The plasmid with $bla_{CTX-M-3a}$ in isolate 100 from hospital S resembled plasmids spreading in Poland (pCTX-M3-type) and Bulgaria (replicon L/M, bla_{TEM-1}, resistance to aminoglycosides and co-trimoxazole) (3, 17, 33).

The presence of IS*Ecp1* and IS*26* was studied by PCR and hybridization. The elements were amplified with primers ISEcp1L1 and ALA-5 and primers IS26LF and IS26RR, respectively (Table 3). In hybridization, the $bla_{CTX-M-3a/15}$ genes were included as well. PstI-digested plasmid DNA was blotted onto a Hybond-N+ membrane and hybridized sequentially with bla_{CTX-M}, IS*26*, and IS*Ecp1* probes (34), using the ECL labeling and detection system (Amersham Biosciences, Little Chalfont, United Kingdom). IS*Ecp1* was identified in plasmids of all isolates from center Z1 and only in isolate 100 from center S of the others, and the IS*Ecp1* and bla_{CTX-M} probes hybridized to single and the same PstI bands (results not shown). The IS*26* PCR was positive with each DNA, and all plasmids tested had multiple bands hybridizing with the IS*26* probe. In plasmids of isolates 16, 32, 36, and 86 from center S, IS*26* hybridized to bands of ~4.5 kb which also contained their $bla_{CTX-M-3a}$ genes.

The location of IS*Ecp1* upstream from $bla_{CTX-M-3a/-15}$ genes was analyzed for all isolates containing IS*Ecp1* with primers ALA-4 and ALA-3 (2) and sequencing of the amplicons. The 3′ ends of the transposition modules were mapped with primer P1A (2) and two reverse primers hybridizing with *K. ascorbata orf477* (Table 3; Fig. 1). Primer orf477-IRR matches the alternative IS*Ecp1* IRR, whereas orf477-27 anneals just further downstream (from bla_{CTX-M}) (17, 31). In isolates from center Z1, IS*Ecp1* was distant by 49 bp from $bla_{CTX-M-15}$, while in isolate 100 from center S, it resided 128 bp from $bla_{CTX-M-3a}$. In the mapping of the 3′ ends, only the PCR with primers P1A and orf477-IRR worked in all these cases, indicating that both modules terminated at the IS*Ecp1* IRR within *orf477*. The $bla_{CTX-M-15}$ gene of the outbreak isolates from center Z1 was located in the structure (Fig. 1) originally identified in the IncFII plasmid pC15-1a from Canada (8) and later in other studies (14, 24, 27). The module with $bla_{CTX-M-3a}$ in isolate 100 from center S was identical to the mobile element of pCTX-M3-type plasmids in Poland (3, 17) and seen also in France (14) (Fig. 1).

The results shown above and previously (38) suggested that in most of the isolates from center S, the $bla_{CTX\text{-}M\text{-}3a}$ genes were linked to the IS*26* element(s). The ~4.5-kb PstI plasmid fragments of isolates 16, 32, and 86, hybridizing with IS*26* and $bla_{CTX\text{-}M}$ probes, were cloned in vector pHSG398 (36). *E. coli* DH5α transformants were selected with 2 μg/ml cefotaxime and 25 μg/ml chloramphenicol. The entire inserts were sequenced by primer walking; sequences were analyzed with the Lasergene version 7.1.0 software (DNAStar, Madison, WI) and the NCBI BLASTn option (www.ncbi.nlm.nih.gov). The three fragments had identical sequences, with parts of IS*26* elements at each end (PstI cuts at one site inside IS*26*). The structure of the locus is shown in Fig. 1. The IS*26*-1 and IS*26*-2 elements are directed outside the locus. Upstream from IS*26*-1 there is a 1,362-bp region identical to a chromosomal fragment of *K. ascorbata* strain 69 with $bla_{CTX\text{-}M\text{-}3a}$ (31). The $bla_{CTX\text{-}M\text{-}3a}$ coding sequence starts 69 bp upstream from IS*26*-1 and is followed by a 372-bp fragment of *orf477*. The remaining 3,032-bp region is homologous to a fragment of a large resistance island in the *Acinetobacter baumannii* AYE strain (GenBank accession no. CT025832) (15), containing an open reading frame (ORF) of a putative transposase (position 69382..72822) that overlaps an oppositely oriented IS*26* (position 69153..69972). The cloned plasmidic sequence lacked the 3′ part of IS*26*-2 (661 bp) with the 5′ end of the ORF (432 bp). The transposase ORF-like region differs by 81 nucleotides and by having a 10-bp deletion from the corresponding part of the AYE sequence (97.0% identity), which causes frameshifting and a nonsense mutation, shortening the ORF by 918 of 1,147 codons. Two other homologous sequences matched fragments located downstream of the deletion and not overlapping IS*26*. These were the Tn*1000*-like transposase ORF (1,209 bp [96.8% identity]) from the vicinity of the $bla_{CTX\text{-}M\text{-}10}$ gene (28) and the Tn*5394* transposase gene from plasmid pEP36 (2,740 bp [76.7% identity]) (26). Four pairs of primers (Table 3; Fig. 1) were used for PCR mapping of the loci in the remaining isolate from center S (isolate 36) and in isolate 49 from center Z2, showing the same structure in both isolates.

The IS*26*–$bla_{CTX\text{-}M\text{-}3a}$–IS*26* module of the isolates from center S and center Z2 is the first case of a $bla_{CTX\text{-}M}$ gene flanked by two IS*26* copies. Such configurations are usually mobile (29), which probably also applies to this module residing in different plasmid platforms. It is difficult to judge whether $bla_{CTX\text{-}M\text{-}3a}$ was originally mobilized by IS*26* or, e.g., by IS*Ecp1* followed by secondary IS*26* insertions like those in some other $bla_{CTX\text{-}M\text{-}3a}$ or $bla_{CTX\text{-}M\text{-}15}$ genes (14, 40). However, this $bla_{CTX\text{-}M\text{-}3a}$ gene was mobilized in an event other than those reported so far. It could not have arisen from the pCTX-M3 IS*Ecp1*–$bla_{CTX\text{-}M\text{-}3a}$ module because the *K. ascorbata* DNA continues 27 bp beyond the *orf477* IS*Ecp1* IRR or from the module described in Spain, where IS*Ecp1* is placed 46 bp from $bla_{CTX\text{-}M\text{-}3a}$ (27). Therefore, the known $bla_{CTX\text{-}M\text{-}3a}$ genes arose from at least three mobilizations, strengthening the earlier observation of frequent $bla_{CTX\text{-}M}$ escapes from *Kluyvera* genomes (4). The significance of the transposase pseudo-ORF remains unknown. It might have had transposition functions; however, it is difficult to reveal when and how it was assembled with $bla_{CTX\text{-}M\text{-}3a}$ and whether it played any role in the gene's mobilization or spread.

Nucleotide sequence accession number. The nucleotide sequence of the IS*26*–$bla_{CTX\text{-}M\text{-}3a}$–IS*26* locus of isolate 16 will appear in the EMBL database under accession no. FM213371.

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