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Dissemination and Persistence of *bla*$_{CTX-M-9}$ Are Linked to Class 1 Integrons Containing CR1 Associated with Defective Transposon Derivatives from Tn*402* Located in Early Antibiotic Resistance Plasmids of IncHI2, IncP1-α, and IncFI Groups

Ângela Novais,[1] Rafael Cantón,[1,2] Aránzazu Valverde,[1] Elisabete Machado,[1,3] Juan-Carlos Galán,[1,2] Luísa Peixe,[3] Alessandra Carattoli,[4] Fernando Baquero,[1,2] and Teresa M. Coque[1,2]*

Hospital Universitario Ramón y Cajal, IMSALUD, Madrid, Spain[1]; Unidad de Resistencia a Antibióticos y Virulencia Bacteriana, Consejo Superior de Investigaciones Científicas (CSIC), Madrid, Spain[2]; Faculdade de Farmácia, Universidade do Porto, Porto, Portugal[3]; and Istituto Superiore di Sanità, Roma, Italy[4]

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This study analyzes the diversity of In60, a class 1 integron bearing CR1 and containing *bla*$_{CTX-M-9}$, and its association with Tn*402*, Tn*21*, and classical conjugative plasmids among 45 CTX-M-9-producing clinical strains (41 *Escherichia coli* strains, 2 *Klebsiella pneumoniae* strains, 1 *Salmonella enterica* strain, and 1 *Enterobacter cloacae* strain). Forty-five patients in a Spanish tertiary care hospital were studied (1996 to 2003). The diversity of In60 and association of In60 with Tn*402* or mercury resistance transposons were investigated by overlapping PCR assays and/or hybridization. Plasmid characterization included comparison of restriction fragment length polymorphism patterns and determination of incompatibility group by PCR-based replicon typing, sequencing, and hybridization. CTX-M-9 plasmids belonged to IncHI2 (n = 26), IncP-1α (n = 10), IncFI (n = 4), and IncI (n = 1) groups. Genetic platforms containing *bla*$_{CTX-M-9}$ were classified in six types in relation to the In60 backbone and in eight subtypes in relation to Tn*402* derivatives. They were associated with Tn*21* sequences when located in IncP-1α or IncHI2 plasmids. Our study identified *bla*$_{CTX-M-9}$ in a high diversity of CR1-bearing class 1 integrons linked to different Tn*402* derivatives, often to Tn*21*, highlighting the role of recombination events in the evolution of antibiotic resistance plasmids. The presence of *bla*$_{CTX-M-9}$ on broad-host-range IncP-1α plasmids might contribute to its dissemination to hosts that were not members of the family *Enterobacteriaceae*.

The reasons driving the recent dramatic worldwide dissemination of CTX-M-producing microorganisms are far from understood. Chromosomal β-lactamase genes from different *Kluyvera* species are considered the ancestors of each of the five CTX-M groups described thus far (CTX-M-1, -2, -8, -9, and -25; http://www.lahey.org/studies/webt.htm). Mobilization of *bla*$_{CTX-M}$ genes to other bacterial genera seems to have occurred by recombinatorial events mediated by CR1 (a Common Region that includes a putative recombinase named orf513), IS*Ecp1*, or phage-related elements (3, 19, 20, 28). Although dissemination of specific strains or mobile genetic elements has been documented, the general lack of information about the complete genetic context of *bla*$_{CTX-M}$ genes precludes coming up with a reliable hypothesis about the causes determining their successful spread (3, 5, 19).

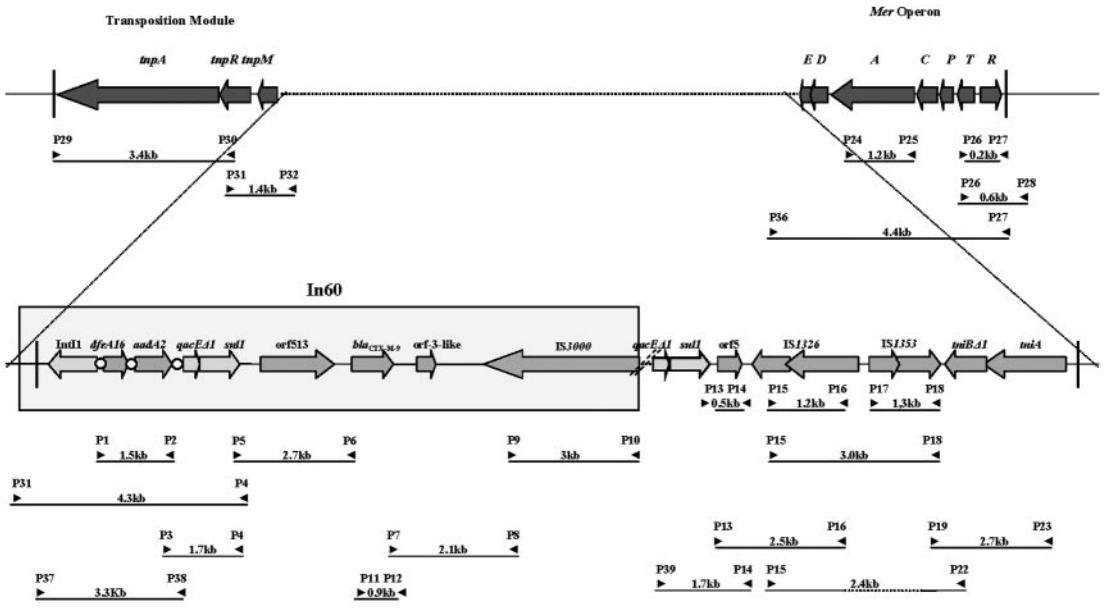
The *bla*$_{CTX-M-9}$ gene has been found associated with a class 1 integron bearing CR1 (34), which has a modular structure consisting of the conserved segments 5′CS and 3′CS flanking variable gene cassette arrays, CR1, genes that do not resemble gene cassettes, and a second copy of the 3′CS designated 3′CS2 (32, 42). Variations of In60, the integron harboring *bla*$_{CTX-M-9}$, have been reported (14, 34), but the complete genetic environment to which In60 or their variants are associated in their turn remain unknown. To date, only a few class 1 integrons bearing CR1 are fully characterized (In6, In34, and In117) (30, 32, 42). They have been found harbored by Tn*21*-like transposons and, in the case of In34, also by an early antibiotic-resistant conjugative plasmid (32). Indeed, our work supports the hypothesis that the spread of CTX-M enzymes takes advantage of the wide availability in nature of old plasmids, already present in the preantibiotic era, as well as old mercury resistance transposons and classic integrons (5, 12, 21, 25, 31).

On this basis, we have analyzed the diversity of In60 and its association with Tn*402*, Tn*21*, and classical conjugative plasmids in clinical enterobacterial isolates identified in our institution since its first isolation in 1996 through 2003 by using different PCR methods (8; this study) designed on the basis of available sequences in the GenBank database.

MATERIALS AND METHODS

Bacterial strains and epidemiological background. Seventy CTX-M-9-producing clinical isolates (66 *Escherichia coli* isolates, 2 *Klebsiella pneumoniae* isolates, 1 *Enterobacter cloacae* isolate, and 1 *Salmonella enterica* isolate) from 45 patients at Ramón y Cajal Hospital, a 1,200-bed university teaching hospital in northern Madrid, Spain, were studied (1996 to 2003). Isolates from the same individual showing identical susceptibility profiles and isolated within the same month were excluded in order to avoid overrepresentation of particular strains. Patients were located in medical wards (49%), intensive care units (9%), and surgical wards (2%), and 40% were outpatients. Isolates were recovered from urine (60%), blood (11%), wound exudate (11%), rectal swab (7%), sputum (4%), and other

* Corresponding author. Mailing address: Servicio de Microbiología, Hospital Universitario Ramón y Cajal, Carretera de Colmenar, km. 9.1, Madrid 28034, Spain. Phone: 34-91-336 83 30. Fax: 34-91-336 88 09. E-mail: mcoque.hrc@salud.madrid.org.



			Amplification of PCR products with the following specific primers					Presence of orfs related to class 1 integrons			
Type	Subtype	Isolates	P1-P2	P3-P4	P5-P6	P7-P8	P9-P10	IS*1326*	IS*1353*	IS*6100*	orf5
A	1	n=12	+	+	+	+	+	+	+	-	+
	2	n=4	+	+	+	+	+	+	-	-	+
	3	n=2	+	+	+	+	+	-	-	-	+
	4	n=15	+	+	+	+	+	-	-	-	-
	5	n=1	+	+	+	+	+	-	-	+	+
	6	n=1	+	+	+	+	+	-	-	+	-
B	3	n=3	+	+	+	+	-	-	-	-	+
	4	n=1	+	+	+	+	-	-	-	-	-
C	1	n=1	+*	+	+	+	+	+	+	-	+
	2	n=1	+*	+	+	+	+	+	-	-	+
	7	n=1	+*	+	+	+	+	+	+	-	-
D	8	n=1	+*	+	+	-	-	+	+	+	-
E	4	n=1	-	-	+	+	+	-	-	-	-
F	4	n=1	-	-	-	+	+	-	-	-	-

FIG. 1. Strategy for characterization of $bla_{CTX\text{-}M\text{-}9}$-containing elements on the basis of In60, Tn*402*, and Tn*21* sequences. The locations of the primers used are indicated by gray arrows. Vertical bars symbolize inverted repeats of the integron (gray) or Tn*21* (dark gray). Small white circles represent 59-bp elements of the corresponding gene cassettes. Known In60 sequence is shown within the gray rectangle. Preliminary classification of the $bla_{CTX\text{-}M\text{-}9}$ elements is shown in the table at the bottom of the figure and was performed in relation to the In60 backbone by an overlapping PCR assay and screening of sequences related to Tn*402* such as orf5, IS*1326*, IS*1353*, and IS*6100* (not shown) by PCR and/or hybridization. Linkage of these sequences with Tn*21* was performed by an overlapping PCR assay in a subset of representative isolates. PCR with primers P15 and P22 was performed for isolates lacking IS*1353*; the dotted line indicates the absence of this region. *, PCR product had molecular size higher than expected.

samples (7%). Species identification and preliminary susceptibility testing were performed by using the automated PASCO (Difco, Detroit, MI) or WIDER (Fco. Soria Melguizo, Madrid, Spain) systems. Susceptibility analysis to non-β-lactam antibiotics (gentamicin, tobramycin, amikacin, streptomycin, kanamycin, sulfonamide, trimethoprim, tetracycline, chloramphenicol, ciprofloxacin, and nalidixic acid) was performed by the disk diffusion method following CLSI (formerly NCCLS) guidelines (27). For the purposes of this work, strains with intermediate susceptibility were considered resistant. Characterization of $bla_{CTX\text{-}M\text{-}9}$ was performed by isoelectric focusing, PCR, and further sequencing (24).

TABLE 1. Oligonucleotides used in this study

Oligonucleotide	Primer	Oligonucleotide sequence	GenBank accession no.	Positions	Reference(s)
1	5′-CS	5′-GGC ATC CAA GCA GCA AG-3′	AF174129	1236–1252	42
2	aadA2R	5′-TGA CTT GAT GAT CTC GCC-3′	AF174129	2709–2692	This study
3	aadA2F	5′-GCT GGC CGT GCA TTT GTA CG-3′	AF174129	2013–2032	This study
4	sul 1-R	5′-GC AAG GCG GAA ACC CGC G-3′	AF174129	3704–3687	This study
5	sul 1-F	5′-GCG CGG GTT TCC GCC TTG GGA-3′	AF174129	3687–3703	This study
6	bla_{CTXM-9}-Rf	5′-CCG TTG CAC TCT CTT TGT CA-3′	AF174129	6359–6339	This study
7	bla_{CTXM-9}-Fr	5′-GGC TTC AGC GGC GAG AAT CAT-3′	AF174129	7175–7195	This study
8	TnpA-R	5′-C GCT CAA TCG AGG GAT ATT TAC-3′	AF174129	9313–9292	This study
9	TnpA-F	5′-GTA AAT ATC CCT CGA TTG AGC G-3′	AF174129	9292–9313	This study
10	IS3000 R	5′–GCC GTC TGT GGC CTC CAG-3′	AF174129	12383–12366	This study
11	bla_{CTXM-9}-F	5′-GT GAC AAA GAG AGT GCA ACG G-3′	AF174129	6339–6359	24
12	bla_{CTXM-9}-R	5′-ATG ATT CTC GCC GCT GAA GCC-3′	AF174129	7195–7175	24
13	orf5F	5′-CGA TAT CGA CGA GGT TGT GC-3′	AF071413	7712–7730	42
14	orf5R	5′-AGT TCT AGG CGT TCT GCG–3′	AF071413	8157–8140	42
15	IS1326R	5′-ACT GTC ATA GCG GTT CAC GTT-3′	AF071413	9141–9161	42
16	IS1326F	5′-TAC CGG GTC TTA TGA CCG AGT-3′	AF071413	10357–10337	42
17	IS1353R	5′-ACA CTA CGG CAG CTG GGA TA-3′	AF071413	10830–10849	This study
18	IS1353F	5′-TGC AGC ATT GTC TTG CGA GCA-3′	AF071413	12113–12093	This study
19	IS1353Rf	5′-TGC TCG CCA GAC AAT GCT GCA-3′	AF071413	12093–12113	This study
20	IS6100 F	5′-GGC TCT GTT GCA AAA ATC GTG AAG-3′	AY463797	4669–4692	13
21	IS6100 R	5′-GGC TCT GTT GCA AAG ATT GGC-3′	AY463797	5548–5528	13
22	tniBΔ1F	5′-AT CAT CGA CCT GTC CCA CCT-3′	AF071413	13201–13182	42
23	tniARF	5′-TCG TGC GGA GAT CAT CAG TCC-3′	AF071413	14821–14801	42
24	merA1	5′-ACC ATC GGC GGC ACC TGC GT-3′	AF071413	17597–17578	22
25	merA5	5′-ACC ATC GTC AGG TAG GGG AAC AA-3′	AF071413	16360–16382	22
26	merT1	5′-CCA GGC AGC AGG TCG ATG CAA G-3′	AF071413	19055–19076	22
27	merR1	5′-GCG GAT TTG CCT CCA CGT TGA-3′	AF071413	19278–19260	22
28	Tn21IR/38	5′-GGG CAC CTC AGA AAA CGG AAA-3′	AF071413	19669–19649	42
29	IR1Tn21F	5′-GGG TCG TCT CAG AAA ACG G-3′	AF071413	1–38	42
30	TnpR-R	5′-CCG TGG TGG TGC ATA GCA T-3′	AF071413	3394–3376	This study
31	TnpR-F	5′-ATG CTA TGC ACC ACC ACG G-3′	AF071413	3376–3394	This study
32	intF1	5′-GGG TCA AGG ATC TGG ATT TCG-3′	AF071413	4775–4755	42
33	intR1	5′-ACA TGC GTG TAA ATC ATC GTC G-3′	AF071413	4312–4333	42
34	5′-CSR	5′-CT TGC TGC TTG GAT GCC-3′	AF174129	1252–1236	42
35	3′-CS	5′-AAG CAG ACT TGA CCT GAT-3′	AF174129	2813–2830	42
36	tniAR	5′-GGA CTG ATG ATC TCC GCA CGA-3′	AF071413	14801–14821	42
37	IRIn2F	5′-TTT CAG AAG ACG GCT GCA CTG-3′	AF071413	4046–4066	This study
38	qacEΔ1B	5′-CAA GCT TTT GCC CAT GAA GC-3′	AF174129	3132–3113	This study
39	qacEΔ2	5′-ATC GCA ATA GTT GGC GAA GT-3′	AF174129	2906–2926	This study
40	orf513R	5′-C TCG CTT GAG GCG TTG CAT-3′	AF174129	5791–5773	This study
41	IS26F	5′-AGC GGT AAA TCG TGG AGT GA-3′	AF205943	324–344	This study
42	IS26R	5′-AG GCC GGC ATT TTC AGC GTG-3′	AF205943	960–979	This study

Clonal analysis. Genetic relationships among isolates were established by pulsed-field gel electrophoresis (PFGE) as previously described (18, 40). The assignation of phylogenetic groups among *E. coli* isolates was performed by the classic multiplex PCR assay of Clermont et al. (10) amplifying *chuA* and *yjaA* genes and using an anonymous DNA fragment (*TspE*4C2) which has been found to be specific as phylogenetic group markers.

Conjugation of $bla_{CTX-M-9}$ elements. It was tested by broth and/or filter mating at a 1:10 donor/recipient ratio using *E. coli* K-12 strain BM21R (nalidixic acid and rifampin resistant, lactose fermentation positive, and plasmid free) as a recipient (24). Selection was performed on MacConkey agar plates containing cefotaxime (1 mg/liter) and rifampin (100 mg/liter). Conjugation plates were incubated at both 24°C and 37°C and analyzed at 5 h and 24 h.

Analysis of plasmids. The content and size of the plasmids carrying $bla_{CTX-M-9}$ were determined on *E. coli* transconjugants (or wild-type strains in the absence of transfer) by the technique described by Barton et al. (2, 41). Location of $bla_{CTX-M-9}$ genes was assessed by hybridization of I-CeuI-digested genomic DNA with $bla_{CTX-M-9}$ and 16S rRNA gene probes as previously described (23).

Plasmids were classified according to their incompatibility group using the PCR replicon-typing scheme described by Carattoli et al. (8). This assay discriminates 18 types of plasmids of the classical incompatibility groups by the presence of specific genes involved in plasmid maintenance. Positive-control strains were *E. coli* strain DH5α derivatives containing replicons of the different incompatibility groups cloned into a TA cloning vector (8). PCR products were sequenced in order to confirm the specificity of the method and to analyze similarities with well-characterized plasmids. Correspondence of the replicons amplified with plasmids containing $bla_{CTX-M-9}$ was validated when probes for both $bla_{CTX-M-9}$ and a given replicon hybridized with the same plasmid band. Plasmids of the same size and the same incompatibility group were digested with different restriction enzymes in order to establish their relationship.

Characterization of integrons carrying $bla_{CTX-M-9}$. Class 1 integrons are associated with defective transposons of the Tn*402* family that differ by the presence and type of insertion sequences located downstream of 3′CS and within the truncated *tni* module (IS*1326* and/or IS*1353* are associated with the In0-In2-In5-In31 lineage, and IS*6100* is associated with the In4 lineage) (31, 33). Thus, the characterization of $bla_{CTX-M-9}$ integrons included both analysis of In60 backbone structure by an overlapping PCR assay and screening of sequences related to Tn*402* derivatives (orf5, IS*1326*, IS*1353*, and IS*6100*) by dot blot hybridization and/or PCR, further linked by an overlapping PCR assay (Fig. 1). Isolates lacking orf5 were screened for the presence of the entire transposition module of Tn*402* (*tniR-tniQ-tniB-tniA*) which has been detected among contemporary plasmids of different incompatibility groups as IncP-1α and/or different mercury resistance transposons as Tn*5058* (25, 39). Control strains for In0, In2, and In4 integrons and Tn*21* were kindly provided by Hatch Stokes (Macquarie University, Sydney, Australia).

Integrons containing $bla_{CTX-M-9}$ were classified as types that indicate In60 basic diversity (designated by capital letters) and subtypes that reflect the content

TABLE 2. Epidemiological features (clinical, clonal, plasmid, and integron data) of CTX-M-9-producing *Enterobacteriaceae* isolates at the Ramón y Cajal Hospital shown in relation to plasmid types (1996 to 2003)

Plasmid Inc group[a]	Approximate plasmid size (kb)[b]	Replicon type by PCR[c]	Type[d]	Subtype[d]	Isolate[e]	No. of patients	Species	Ward[f]	Specimen source	*E. coli* phylogenetic group	Date (mo/yr)	Presence of[g]: IS*26*	Presence of[g]: *merA*	Antibiotic resistance[h]
P-1α	100	P, I1, FIB	In60-A	1	D47	1[i]	*E. coli*	Outpatient	Wound	A	3/02	+	+	Sm, Su, Tp, Te
P-1α	100	P, I1, FIB	In60-A	1	D72	1[i]	*E. coli*	Int. Med.	Rectal swab	B2	4/02	−	+	Sm, Su, Tp, Te, Cm, Na, Ak
P-1α	100	P, I1	In60-A	1	D80	1	*E. coli*	Nephrology	Urine	B1	5/02	−	+	Sm, Su, Tp, Te, Km
P-1α	100	P, I1, FIB, F	In60-A	1	F18	1	*E. coli*	Gastroent.	Urine	D	10/02	+	+	Sm, Su, Tp, Te, Na, Ak
P-1α	100	P	In60-A	3	EC40	1	*E. coli*	Urology	Urine	A	6/98	−	+	Sm, Su, Tp, Te, Na
P-1α	100	P, I1, FIB, F	In60-A	4	F5	1	*E. coli*	Outpatient	Blood	A	9/02	+	+	Sm, Su, Tp, Te, Na, Gm, Ak
P-1α	100	P, I1	In60-A	4	G34	1	*K. pneumoniae*	Outpatient	Urine		6/03	−	+	Sm, Su, Tp, Te, Km
P-1α	100	P, I1	In60-B	3	C77	1	*E. coli*	Outpatient	Urine	A	9/01	−	+	Sm, Su, Tp, Te
P-1α	100	P, I1, FIB	In60-B	3	E27	1	*E. coli*	Outpatient	Urine	B2	5/02	−	+	Sm, Su, Tp, Te
P-1α	100	P, I1, FIB, F	In60-B	4	EC72	1	*E. coli*	Nephrology	Wound	A	10/00	+	+	Sm, Tb, Su, Tp, Te, Cp, Na, Km, Gm
I1	120[j]	I1	In60-A	5	EC44	1	*E. coli*	Dermatology	Urine	A	1/99	+	+	Sm, Su, Tp, Te, Cp, Na
HI2	280	HI2, FIB, F	In60-A	1	EC28	1	*E. coli*	Nephrology	Blood	B1	10/97	+	−	Sm, Su, Tp, Te, Cm, Cp, Na
HI2	280	HI2, FIB, F	In60-A	1	EC76	1	*E. coli*	Outpatient	Urine	A	12/00	+	−	Sm, Su, Tp, Te, Cm, Cp, Na
HI2	280	HI2, FIB, F	In60-A	1	D4	1	*E. coli*	Outpatient	Urine	B1	10/01	+	−	Sm, Su, Tp, Te, Cp, Na
HI2, F1	320	HI2, FIB	In60-A	1	D36	1	*E. coli*	Gastroent.	Blood	B2	3/02	+	−	Sm, Su, Tp, Na, Cp, Km
HI2	320	HI2, FIB	In60-A	2	EC29	1	*E. coli*	Urology	Urine	B2	3/97	+	+	Sm, Su, Tp, Te, Cm, Na
HI2	270	HI2	In60-A	2	EC63	1	*E. coli*	Urology	Urine	A	4/00	+	+	Sm, Su, Tp, Te, Cp, Na
HI2	290	HI2	In60-A	2	EC62	1	*E. coli*	Nephrology	Urine	A	4/00	+	−	Sm, Su, Tp, Te, Cp
HI2	280	HI2	In60-A	2	EC68	1	*E. coli*	Surgery ICU	Drainage	B1	7/00	+	+	Sm, Su, Tp, Te, Cp, Na
HI2	260	HI2, FIB, F	In60-A	3	EC25	2	*E. coli*	Surgery ICU Urology	Respiratory Urine	D	10/96 12/96	+	+	Sm, Su, Tp, Te, Cp, Na, Km
HI2	280	HI2, F	In60-A	4	EC24	1	*E. coli*	Urology	Urine	A	10/96	+	−	Sm, Su, Tp, Te, Cm, Na, Km
HI2	260	HI2, I1	In60-A	4	EC57	1	*E. coli*	Neurosurg. ICU	Respiratory	A	2/00	+	−	Sm, Su, Tp, Te, Cp, Na, Gm, Km, Tb
HI2	280	HI2, FIB, F	In60-A	4	EC39	1	*E. coli*	Neurosurg. ICU	Catheter	D	2/98	+	+	Sm, Su, Tp, Te
HI2	280	HI2, FIB	In60-A	4	EC41	1	*E. coli*	Outpatient	Urine	A	12/98	+	+	Sm, Su, Tp, Te
HI2	280	HI2, FIB	In60-A	4	EC46	1	*E. coli*	Outpatient	Urine	A	3/99	+	−	Sm, Tb, Su, Tp, Te, Cm, Cp, Na, Km, Gm
HI2	280	HI2, FIB, F	In60-A	4	EC42	1	*E. coli*	Outpatient	Blood	D	8/99	+	−	Sm, Su, Tp, Na
HI2, F1	330[j]	HI2, FIB	In60-A	4	EC66	1	*E. coli*	Outpatient	Urine	B2	5/00	+	−	Sm, Su, Tp, Te, Cm, Na
HI2	290	HI2, Y	In60-A	4	D7	1	*E. coli*	Urology	Urine	B1	11/01	+	−	Sm, Su, Tp, Te, Cm, Cp, Na, Gm
HI2	290	HI2	In60-A	4	F25	1	*E. cloacae*	Outpatient	Urine		10/02	+	−	Sm, Su, Tp, Te
HI2	ND[k]	HI2, FIB	In60-A	4	F34	1	*E. coli*	Nephrology	Urine	B1	11/02	+	−	Su, Tp, Te
HI2	280	HI2	In60-A	4	F38	1	*E. coli*	Nephrology	Wound	B2	12/02	+	−	Sm, Su, Tp, Te
HI2	310	HI2	In60-A	4	H65	1	*S. enterica*	Hematology	Rectal swabs		6/03	+	−	Sm, Su, Tp, Te, Na
HI2	280	HI2, FIA, FIB	In60-A	6	EC69	1	*E. coli*	Outpatient	Urine	D	8/00	+	−	Sm, Su, Tp, Te, Cp, Na
HI2	290	HI2	In60-C	1	EC34	1	*E. coli*	Nephrology	Urine	D	12/97	+	+	Sm, Su, Tp, Te, Cp, Na
HI2	290	HI2, Y	In60-C	2	EC33	1	*E. coli*	Gastroent.	Peritoneal	D	11/97	+	+	Sm, Su, Tp, Te, Cm, Cp, Km, Na, Gm, Tb
HI2	260	HI2, Y	In60-E	4	EC50	1	*E. coli*	Urology	Urine	A	12/99	+	−	Sm
HI2	240	HI2, FIB, F	In60-F	4	D61	1	*E. coli*	Nephrology	Urine	B2	4/02	+	−	Su, Te, Na
FIB	150	FIB, F	In60-A	1	C45	1	*E. coli*	Outpatient	Blood	B1	1/01	+	−	Sm, Su, Tp, Te, Cp, Na
FIB	140	FIB, F	In60-A	1	D10	1	*E. coli*	CV surgery	Wound	A	11/01	+	−	Sm, Su, Tp, Te, Na
FIB	160	FIB, F	In60-C	7	D79	1	*E. coli*	Outpatient	Urine	D	5/02	+	−	Sm, Su, Tp, Te, Cp, Na, Gm, Tb

FIB	160	FIB, F	In60-D	8	E79	1	*E. coli*	Urology	Urine	D	8/02	+	–	Sm, Su, Tp, Te, Na, Ak, Gm, Km, Tb
NI[f]	70	Y, B/O	In60-A	1	C59	1	*E. coli*	Outpatient	Urine	A	4/01	+	–	Su, Tp, Te, Cp, Na, Gm, Km, Tb
NI	ND	B/O	In60-A	1	D34	1	*E. coli*	Outpatient	Urine	D	3/02	+	+	Sm, Su, Tp, Te, Cm, Cp, Na
NI	120	Y	In60-A	4	EC99	1	*E. coli*	Outpatient	Rectal swabs	D	5/02	+	–	Sm, Su, Tp, Te, Cp, Na, Km
NI	120		In60-B	3	Kp40	1	*K. pneumoniae*	Gastroent.	Wound		11/99	+	+	Sm, Su, Tp, Te, Cp, Na, Km

[a] Incompatibility group of plasmids harboring $bla_{CTX-M-9}$ determined by PCR, hybridization, and in some cases, sequencing of the replicon.
[b] Plasmid size determined by hybridization of I-CeuI- or S1 nuclease-digested genomic DNA of *E. coli* BM21R transconjugants (or wild type if failed transfer) with an intragenic $bla_{CTX-M-9}$ probe.
[c] Replicons hybridizing in the same band as that of $bla_{CTX-M-9}$ are underlined.
[d] Types and subtypes of the $bla_{CTX-M-9}$ integron were defined in Fig. 1.
[e] Transferability of $bla_{CTX-M-9}$ is indicated by underlining.
[f] Int. Med., Internal medicine; Gastroent., gastroenterology; ICU, intensive care unit; Neurosurg., neurosurgery; CV, cardiovascular surgery.
[g] +, present; –, absent.
[h] Antibiotic resistance profile corresponding to the original strain; antibiotic resistance patterns of transconjugants are underlined. Chloramphenicol, nalidixic acid, and ciprofloxacin were not tested in the transconjugants. Sm, streptomycin; Gm, gentamicin; Tb, tobramycin; Km, kanamycin; Ak, amikacin; Su, sulfonamide; Tp, trimethoprim; Te, tetracycline; Cp, ciprofloxacin; Na, nalidixic acid; Cm, chloramphenicol.
[i] Isolates recovered from the same patient.
[j] Chromosomal location of $bla_{CTX-M-9}$.
[k] ND, not determined since DNA extraction repeatedly failed.
[l] NI, not identified.

of orf5, IS*1326*, IS*1353*, and IS*6100* (designated by numbers) (Fig. 1). The presence of IS*26*, often associated with bla_{CTX-M} genes and plasmids of some incompatibility groups (19, 17, 38) was screened by PCR using the primers listed in Table 1.

Analysis of flanking sequences of the integron carrying $bla_{CTX-M-9}$. Since class 1 integrons bearing CR1 have been linked to mercury resistance transposons (33), we tested the occurrence of *merA*, a gene that is highly conserved in a variety of these genetic elements (22). The presence of backbone structures associated with Tn*21* subgroup transposons was investigated by a overlapping PCR assay based on Tn*21* and Tn*1696* sequences (GenBank accession numbers AF071413 and AY223253, respectively; 31) in a subset of isolates containing *merA* and representing different In60 variants (see above) (Fig. 1).

Sequencing of integron-specific PCR products. Sequencing of the amplified DNA fragments corresponding to different functional modules of the genetic elements containing $bla_{CTX-M-9}$ (integrase, 5′CS1-3′CS1, 3′CS1-orf513, 3′CS2-*tniA*, and *tniB-tniA*) was performed for selected isolates by using ABI Prism 377 automated sequencer (Applied Biosystems PE, Foster City, CA). Nucleotide sequences were compared with sequences in the GenBank and EMBL databases by using the BLASTN local alignment search tools. Information about primers used for sequencing can be supplied on request.

DNA methodology. Overlapping PCR assays were performed in volumes of 50 μl under the following conditions: 1.5 mM $MgCl_2$, 0.2 mM of each deoxynucleoside triphosphate, 0.1 μM of each primer, and 1.5 units of *Taq* DNA polymerase (AmpliTaqGold; PE Applied Biosystems, Norwalk, Conn.) for 12 min at 94°C; 35 cycles, with 1 cycle consisting of 1 min at 94°C, 1 to 2 min at 56 to 65°C, and 1 to 3 min at 72°C, and a final step of 10 min at 72°C for standard PCR assays; and 2.5 mM $MgCl_2$, 5% dimethyl sulfoxide (when necessary), 0.1 μM of each primer, and 2.5 units of Takara LA *Taq* polymerase (Takara Bio Inc., Shiga, Japan) for 1 min at 94°; and 35 cycles, with 1 cycle consisting of 20 s at 96°C, 1 min at 55 to 64°C, and 3 min at 72°C, followed by a final step of 10 min at 72°C, for long PCRs (>3 kb). DNA transfer and hybridization were performed by standard procedures (35). All probes were generated by PCR from the appropriate DNA controls as templates using the primers listed in Table 1. Labeling and detection were carried out using ECL kits following the manufacturer's instructions (Amersham Life Sciences, Uppsala, Sweden). PFGE was performed as described previously (18, 40) using the following conditions: 5- to 25-s pulses for 23 h and 60- to 120-s pulses for 10 h, 14°C, 6 V/cm^2 (I-Ceu-I) and 10- to 40-s pulses for 24 h, 14°C, 6 V/cm^2 (XbaI).

RESULTS

CTX-M-9-producing isolates have a heterogeneous epidemiological background. Forty-five isolates with different PFGE types (41 *E. coli* isolates, 2 *K. pneumoniae* isolates, 1 *S. enterica* isolate, and 1 *E. cloacae* isolate) from 45 patients were studied. One *E. coli* isolate was recovered from two individuals, and two different *E. coli* isolates were obtained from a single patient. The *E. coli* phylogenetic groups A and B1 (more related to animal or commensal strains) were found at a higher proportion than were groups D and B2 (associated with extraintestinal pathogenic *E. coli*) among CTX-M-9-producing isolates (56% versus 44%). Most of the strains were resistant to sulfonamides (98%), trimethoprim (96%), streptomycin (96%), tetracycline (93%), and nalidixic acid (73%); a lower percentage showed resistance to ciprofloxacin (49%), gentamicin (20%), kanamycin (29%), and chloramphenicol (22%). Interestingly, resistance to sulfonamide, trimethoprim, or streptomycin that was expected to be related to the In60 integron was not expressed in all transconjugants despite the presence of the corresponding genes in the plasmid (only 41%, 79%, and 71% of transconjugants were resistant to sulfonamide, trimethoprim, or streptomycin, respectively). The epidemiological background of the strains is shown in Table 2.

The $bla_{CTX-M-9}$ gene is often located in IncHI2, IncP1-α, and IncFI conjugative plasmids. Transfer of cefotaxime resistance to *E. coli* BM21R was achieved by conjugation in 76% of the strains. We identified $bla_{CTX-M-9}$ on plasmids ranging from

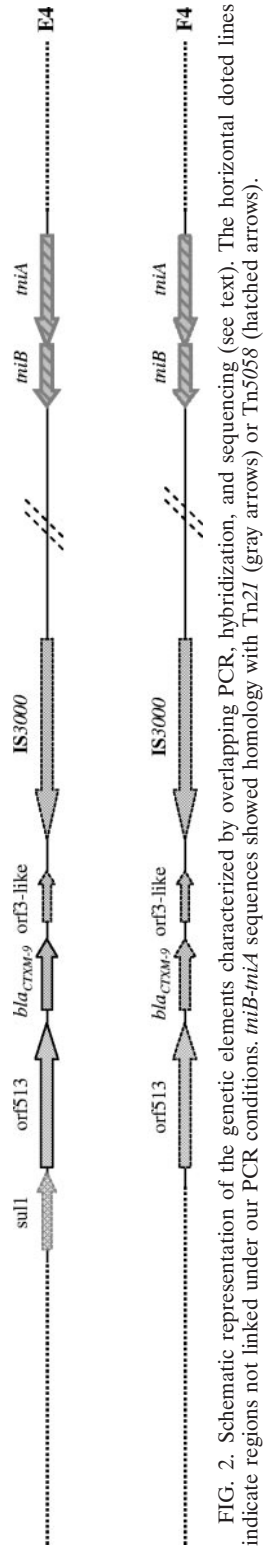


FIG. 2. Schematic representation of the genetic elements characterized by overlapping PCR, hybridization, and sequencing (see text). The horizontal doted lines indicate regions not linked under our PCR conditions. *tniB-tniA* sequences showed homology with Tn*21* (gray arrows) or Tn*5058* (hatched arrows).

approximately 70 to 320 kb and belonging to incompatibility groups IncHI2 ($n = 26$), IncP-1α ($n = 10$), IncFI ($n = 4$), and IncI ($n = 1$).

Plasmids identified as belonging to the IncHI2 group were recovered from patients in different areas of the hospital and from different sources during an extended period of time (1996 to 2003). They showed variability in molecular size (240 to 320 kb) and in the presence of both *merA* and antibiotic resistance markers (Table 2). The presence of common bands among a variable number of restriction fragment length polymorphism patterns from a representative number of IncHI2 plasmids and the 99% homology (1 nucleotide change) of amplified iteron regions from six IncHI2 plasmids of different sizes and *merA* contents with sequences of pR478 (GenBank accession number BX664015) seem to reflect that they were all derived from a single IncHI2 ancestor plasmid suffering different rearrangement events. A large number of transconjugants containing more than one plasmid (19 out of 26) amplified with primers for the IncHI2 replicons and other Inc replicons (mainly those of IncFI). In two cases, a positive hybridization signal within a single band was obtained with probes for both IncHI2 and IncFI (isolates D36 and EC66), suggesting the formation of cointegrates in the transconjugant. This hypothesis is strengthened by the larger size of the plasmid carried by D36 (320 kb). For EC66, these probes hybridized with a chromosomal fragment of approximately 330 kb (Table 2).

The IncP-1α plasmids were isolated from patients in the hospital from 1998 through 2003, both from outpatients and patients in medical areas, and all had a molecular size of approximately 100 kb (Table 2). The highly related restriction fragment length polymorphism patterns and the 100% homology of the amplified replicon with the sequence of pRK2 (GenBank accession number M20134), the prototype of IncP-1α plasmids, suggest the spread of a single plasmid species. As with the IncHI2 plasmids, the CTX-M-9 IncP-1α plasmids usually coexisted with IncF plasmids in most isolates. Although this plasmid type was frequently detected in CTX-M-9-producing strains, the location of $bla_{CTX-M-9}$ in IncF plasmids was demonstrated in only four cases from 2001 to 2002. These four plasmids, classified as IncFI on the basis of sequences of repFIB and repF PCR products, had a variable molecular size (140 to 160 kb) and did not contain *merA*.

IncI amplicons were also detected among the CTX-M-9 producers of our collection, but there was evidence of $bla_{CTX-M-9}$ location in an IncI plasmid in only one case. We could not identify the Inc plasmid group in four cases: two plasmids of approximately 120 kb, one of approximately 70 kb, and one of undetermined size (Table 2). The results of hybridization with probes for IncP-1α, IncI, IncHI2, and IncFI were all negative in these four cases.

$bla_{CTX-M-9}$ is located in a variety of class 1 integrons containing CR1 and associated with Tn*402* derivatives. Integrons containing $bla_{CTX-M-9}$ were classified as six different In60 variants (A to F) on the basis of the previously described In60 backbone: type A is identical to In60 ($n = 35$), type B has differences in sequences downstream of $bla_{CTX-M-9}$ ($n = 4$), types C and D contain different gene cassette arrays within 5′CS-3′CS1 (*aadA1* or *dfrA12*-orfX-*aadA8*, respectively, versus *dfrA16-aadA2* in In60) ($n = 4$), and types E and F lack the first 5′CS-3′CS ($n = 2$). Subtypes, defined according to the content

of IS*1326*, IS*1353*, IS*6100*, and orf5, reflect association of In60 variants with different Tn*402* derivatives. The distribution of types and subtypes appears in Fig. 1.

A high degree of homology was found among sequences from all of the identified gene cassette arrays: *dfrA16-aadA2* (indistinguishable among all our isolates and those carrying *qnr* from China and North America, GenBank accession number AY259085), *aadA1* (indistinguishable from that of the worldwide disseminated Tn*21*, GenBank accession number AF071413), and *dfrA12*-orfX-*aadA8b* from our E79 isolate (identical to that described in fecal isolates from Australia and clinical isolates from the United Kingdom) (15, 21, 45) (GenBank accession number AM040708). Analysis of the integrase *int*I1 of one representative isolate of each integron type revealed the presence of P_c promoter sequences that correspond to the weak and intermediate versions of the P_c promoter (33).

The 405-bp intergenic region between *sul*1F and orf513 described in other class 1 integrons bearing CR1 as In35, In36, In37, or In117 (GenBank accession numbers AY079169, AY259085, AY259086, and AY162283, respectively) was identified in all cases studied. Although the region immediately downstream of IS*3000* of In60 (Fig. 2) could not be identified, positive hybridization of IS*3000* and Tn*402*/*tni* sequences for specific isolates in the same DNA fragment suggest an eventual linkage between them. A 3′CS2-*tni* region similar to that of In0 (isolates EC29 and EC33) or that of In2 (isolates D72 and EC34) was detected in isolates harboring $bla_{CTX\text{-}M\text{-}9}$ located in different integron types (subtypes 1 and 2). In these cases, sequencing of IRi regions confirmed that they belonged to the In0-In2 lineage of Tn*402* (data not shown). The upstream and downstream regions of subtypes 4, 5, 7, and 8 could not be identified by our overlapping PCR assay. Interestingly, the *tniA-tniB* region was amplified in two isolates of subtype 4 (In60E-4 and In60F-4 corresponding to isolates EC50 and D61) harboring plasmids of the IncHI2 group. These sequences were identical to those of Tn*5058*, a mercury resistance transposon derivative of Tn*5053*, although a genetic linkage of this region with In60 was not established.

Integrons containing $bla_{CTX\text{-}M\text{-}9}$ are frequently associated with transposons of the Tn*21* subgroup. Forty-seven percent of the isolates containing $bla_{CTX\text{-}M\text{-}9}$ studied carried *merA*. The presence of the complete left arm of Tn*21* (*tnpA-tnpR-tnpM*) was demonstrated in isolates harboring different integron platforms (types In60 A-1, B-3, C-1, and C-2) located on plasmids of either the IncP-1α or IncHI2 group. However, the presence of the left branch of Tn*21* was variable in isolates containing the same integron type (isolates KP40 and *E. coli* E27 harboring type In60 B-3). Tn*21-mer* sequences were detected in all isolates carrying $bla_{CTX\text{-}M\text{-}9}$ on an IncP-1α plasmid, in a variable number of isolates carrying IncHI2 plasmid, and were absent in those on IncFI plasmids.

DISCUSSION

We describe the association of the CR1 integron containing $bla_{CTX\text{-}M\text{-}9}$ with different Tn*402* derivatives, often associated with Tn*21* and mostly located in early antibiotic resistance IncHI2, IncP1α, and IncFI plasmids. The high diversity found in every functional module of the genetic element containing $bla_{CTX\text{-}M\text{-}9}$ (5′CS-3′CS1 and CR1 regions and the Tn*402-tni* module) was not surprising, since recombinatorial exchange between these regions and the corresponding homologous regions in other elements has been reported. Moreover, the multiplicity of hot spots for recombination (*int*I1, 3′CS, Tn*402-tni* module on class 1 integrons or *res* site and *mer* operon in Tn*21* derivatives) may yield chimeric structures and localized deletions (1, 4, 32, 33, 36–38, 43, 47). Plasticity of gene cassettes was also observed. The *dfrA12*-orfX-*aadA8b* array from our E79 isolate seems to have arisen by recombination events involving the *aadA2* gene from the globally disseminated *dfrA12-orfX-aadA2* array first described in Finland in 1969 (15). Our results are in agreement with other studies and show that the propensity for recombination and genetic exchange in nature seems to be more frequent than previously believed, playing a relevant role in the adaptation of specific plasmids to different cellular environments (9, 29). They also reflect the difficulties in understanding the spread of specific antibiotic resistance genes in the absence of a detailed characterization of their genetic environment, thus hiding the relevance of widespread key genetic elements in gene dispersal (36, 37).

Defective Tn*402* transposons belong to the In0-In2-In5 lineage and are often associated with mercury resistance transposons such as Tn*21* (30–32). To date, such mercury resistance transposons have been detected on narrow-host-range plasmids of incompatibility group F, such as pR100, pC15-1a, pRMH760, and pRSB107, or on IncHI1 plasmids, such as pHCM1 (GenBank accession numbers NC_002134, AY458016, AY123253, AJ851089, and AL513383, respectively; 16, 21, 32, 36–38, 43). Our study highlights the current wide spread of mercury resistance transposons in other groups of early antibiotic resistance plasmids such as IncHI2, IncP-1α, or IncI. IncHI2 plasmids, frequently harboring $bla_{CTX\text{-}M\text{-}9}$ in our study, were first isolated in *Serratia marcescens* in the United States in 1969 and later recovered from environmental and human *Salmonella enterica* serovar Panama isolates from Chile in the 1980s and 1990s (11, 17), but recent reports remain scarce. IncHI2 plasmids possess a large segment named the "principal plasticity zone" that encodes the majority of resistance determinants such as *ter*, *cat*, *aphA*, *mer*, *sil*, *cop*, and Tn7 and also a large number of IS*26* sequences (17). The widespread presence of IS and transposons in these plasmids would enable intra- and interplasmid recombinatorial events and might explain the variability in size and presence of *mer* and resistance markers in IncHI2 plasmids detected in our work. IncP-1α plasmids, also frequently associated with $bla_{CTX\text{-}M\text{-}9}$, were first isolated in 1969 from *Pseudomonas aeruginosa* and enterobacterial clinical isolates from Birmingham, United Kingdom, and they have recently been recovered from wastewater in Germany (39). In our series, IncP-1α plasmids were mainly isolated from community isolates and, unlike those previously described, contained *merA* mostly associated with Tn*21*-like structures. It is of interest to note that IncF plasmids, often linked withTn*21* in the literature (7, 16, 21, 30, 36–38, 43), did not contain sequences related to mercury resistance transposons. IncI plasmids have been shown to contain class 1 integrons and transposases similar to those of Tn*21*, although to our knowledge, the presence of the *mer* operon has not been demonstrated thus far (46). Defective Tn*402* derivatives containing orf5 and IS*6100* (class 1 integrons of In4 lineage, subtype 5) were rare in our collection despite having been previ-

ously found in a variety of plasmids, transposons, and bacterial chromosomes (26, 31). The presence of IS*6100* detected alone (subtype A-5 and A-6) or in combination with IS*1353* and IS*1326* (type D-8) was not surprising, since this insertion sequence can be located in a wide genetic background without major specific associations.

Resistance to sulfonamide, trimethoprim, and streptomycin in wild clinical strains was not detected in a percentage of transconjugants, even though In60 contains gene cassettes presumptively responsible for these resistance phenotypes. The presence of additional genes encoding resistance to these antibiotics in some CTX-M-9-producing isolates as *sulII*, present in most sulfonamide-resistant transconjugants (unpublished results), or Tn*7* (*dhfrA1-sat-aadA1*-orfX) present in some CTX-M-9-producing isolates (24) and also in IncHI2 plasmids (17) might have caused this phenomenon. Other hypotheses, such as gene inactivation or silencing, cannot be discarded and also deserve to be studied further.

CTX-M enzymes remain confined to members of the family *Enterobacteriaceae*, whereas other widely disseminated extended-spectrum beta-lactamases or metallo-β-lactamases have been found in different bacterial families (6, 44). It is tempting to suggest that this species selectivity might be related to the host range of the plasmids involved in their dissemination, but the identification of $bla_{CTX-M-9}$ on plasmids of both narrow host range (IncHI2, IncI, and IncF) and broad host range (IncP-1α group) indicates the necessity of further studies. Finally, the results of this study contribute to increasing the list of fully characterized integrons bearing CR1 (In117, In34, and integrons of epidemic *Salmonella* IncF plasmids) associated in all cases with Tn*21* and highlight the role of mercury resistance transposons frequently located in classical conjugative plasmids in fuelling antibiotic resistance genes (12, 30, 32, 33, 37, 42).

In summary, this study highlights the relevance of classical conjugative plasmids found in early antibiotic-resistant isolates in the dissemination of contemporary antibiotic resistance genes. The presence of similar plasmid backbones containing a high diversity of genetic elements harboring $bla_{CTX-M-9}$ suggests the intraplasmid evolution of these elements by multiple recombinatorial events. The modular plasticity of plasmid-contained mobile genetic elements is of concern, since widely disseminated antibiotic resistance genes located on integrons might be incorporated into a variety of these plasmid-located modular platforms, as previously happened for the SG1 genetic island of *Salmonella* or antibiotic resistance IncF plasmids of *Salmonella* (4, 7). In addition, the presence of $bla_{CTX-M-9}$ on broad-host-range IncP-1α plasmids might contribute to its dissemination to hosts other than *Enterobacteriaceae*.

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