

Spread of *bla*_{CTX-M-14} Is Driven Mainly by IncK Plasmids Disseminated among *Escherichia coli* Phylogroups A, B1, and D in Spain[∇]

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Since its first description in 2000, CTX-M-14 has become one of the most widespread extended-spectrum β -lactamases in Spain. In the present *Escherichia coli* multilevel population genetic study involving the characterization of phylogroups, clones, plasmids, and genetic platforms, 61 isolates from 16 hospitalized patients and 40 outpatients and healthy volunteers recovered from 2000 to 2005 were analyzed. Clonal relatedness (XbaI pulsed-field gel electrophoresis [PFGE] type, phylogenetic group, multilocus sequence type [MLST]) was established by standard methods. Analysis of transferred plasmids (I-CeuI; S1 nuclease; restriction fragment length polymorphism analysis; and analysis of RNA interference, replicase, and relaxase) was performed by PCR, sequencing, and hybridization. The genetic environment of *bla*_{CTX-M-14} was characterized by PCR on the basis of known associated structures (ISEcp1, IS903, ISCR1). The isolates were mainly recovered from patients in the community (73.8%; 45/61) with urinary tract infections (62.2%; 28/45). They were clonally unrelated by PFGE and corresponded to phylogenetic groups A (36.1%), D (34.4%), and B1 (29.5%). MLST revealed a high degree of sequence type (ST) diversity among phylogroup D isolates and the overrepresentation of the ST10 complex among phylogroup A isolates and ST359/ST155 among phylogroup B1 isolates. Two variants of *bla*_{CTX-M-14} previously designated *bla*_{CTX-M-14a} ($n = 59/61$) and *bla*_{CTX-M-14b} ($n = 2/61$) were detected. *bla*_{CTX-M-14a} was associated with either ISEcp1 within IncK plasmids ($n = 27$), ISCR1 linked to an IncHI2 plasmid ($n = 1$), or ISCR1 linked to IncI-like plasmids ($n = 3$). The *bla*_{CTX-M-14b} identified was associated with an ISCR1 element located in an IncHI2 plasmid ($n = 1$) or with ISEcp1 located in IncK ($n = 1$). The CTX-M-14-producing *E. coli* isolates in our geographic area are frequent causes of community-acquired urinary tract infections. The increase in the incidence of such isolates is mostly due to the dissemination of IncK plasmids among *E. coli* isolates of phylogroups A, B1, and D.

Since their first description in the late 1980s, plasmid-mediated CTX-M extended-spectrum β -lactamases (ESBLs) have increasingly been reported worldwide in both hospital and community settings and have mainly been associated with *Escherichia coli* isolates, which often cause urinary tract infections (UTIs) (7, 46, 48, 56). The CTX-M enzymes are classified into five groups on the basis of their amino acid sequences, and of these, the CTX-M-1 and CTX-M-9 clusters have been the most frequently reported worldwide (7).

The CTX-M-14 enzyme is, besides CTX-M-9, the most widespread enzyme of the CTX-M-9 group. It was initially identified among *Shigella* clinical isolates from Korea in 1995 (45); and it is now globally disseminated, being endemic in Spain, France, Portugal, the United Kingdom, Korea, China, Hong Kong, Taiwan, Thailand, Japan, Canada, and the United States (9, 22, 23, 25, 26, 32–34, 36, 38, 46, 49, 56). Although

specific strains have caused community outbreaks in Canada and Japan (38, 46, 49), an apparently high degree of clonal diversity of CTX-M-14-producing *E. coli* isolates is described in most of the studies (9, 23, 32, 33, 38, 45, 46). The dissemination of *bla*_{CTX-M-14} via plasmids has also been reported (15, 38, 39).

The *bla*_{CTX-M-14} enzyme is thought to be the precursor of the CTX-M-9 cluster, and several times it seems to have been mobilized from *Kluyvera* chromosomes to plasmids (4). Analysis of specific isolates identified two variants of *bla*_{CTX-M-14} on genetic platforms containing either ISEcp1 or ISCR1 sequences, highlighting the different pathways for the acquisition and evolution of this gene (2, 4, 17, 39). Hitherto, studies describing the occurrence of this ESBL have analyzed only a limited number of isolates at the molecular level. However, a comprehensive multilevel analysis of the whole genetic context in areas of endemicity, including a full description of the *E. coli* phylogenetic groups, clones, plasmids, and genetic platforms involved in the dissemination of *bla*_{CTX-M-14}, has not been performed.

CTX-M-14, initially detected in 2000 in Spain, constitutes one of the most prevalent CTX-M enzymes in that country and

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TABLE 1. CTX-M-14-producing *E. coli* strains (2001 to 2005)

Sample	Phylogenetic group	Patient ward or origin	Origin	MLST ^a	
Clinical (<i>n</i> = 42)	A (<i>n</i> = 13)	Medical (<i>n</i> = 4)	Urine (<i>n</i> = 3)	ST10 complex (ST617, <i>n</i> = 1), ST23 complex (<i>n</i> = 2)	
		Outpatient (<i>n</i> = 9)	Blood (<i>n</i> = 1) Urine (<i>n</i> = 9)	ST168 complex (ST93, <i>n</i> = 1) ST10 complex (<i>n</i> = 3, two of ST617 and one of ST167), ST350 (<i>n</i> = 1), ST155 complex (ST58, <i>n</i> = 1), ST1014 (<i>n</i> = 1)	
	B1 (<i>n</i> = 14)	Medical (<i>n</i> = 3)	Urine (<i>n</i> = 3)	ST359 (<i>n</i> = 2)	
		ICU (<i>n</i> = 1)	Urine (<i>n</i> = 1)	ST155 complex (ST1015, <i>n</i> = 1)	
		Surgical (<i>n</i> = 1)	Wound (<i>n</i> = 1)		
		Outpatient (<i>n</i> = 9)	Urine (<i>n</i> = 9)	ST359 (<i>n</i> = 5), ST155 complex (<i>n</i> = 2, ST58 and ST1017)	
	D (<i>n</i> = 15)	Medical (<i>n</i> = 4)	Urine (<i>n</i> = 4)	ST117 (<i>n</i> = 1), <i>fumC4</i> (<i>n</i> = 1), <i>fumC31</i> (<i>n</i> = 2)	
		ICU (<i>n</i> = 1)	Urine (<i>n</i> = 1)		
		Outpatient (<i>n</i> = 10)	Urine (<i>n</i> = 9), Wound (<i>n</i> = 1)	ST31 complex (<i>n</i> = 1, ST393), ST770 (<i>n</i> = 1), ST1019 (<i>n</i> = 1), <i>fumC4</i> (<i>n</i> = 2), <i>fumC31</i> (<i>n</i> = 1), <i>fumC40</i> (<i>n</i> = 1), <i>fumC45</i> (<i>n</i> = 2) ST624 (<i>n</i> = 1)	
	Fecal (<i>n</i> = 19)	A (<i>n</i> = 9)	Medical (<i>n</i> = 1)		ST10 complex (ST10, <i>n</i> = 1)
Outpatient (<i>n</i> = 8)				ST10 complex (<i>n</i> = 7; 5 ST10, 1 ST48, 1 ST167), ST168 complex (ST93, <i>n</i> = 1)	
B1 (<i>n</i> = 4)		Medical (<i>n</i> = 1)			
		Outpatient (<i>n</i> = 3)		ST155 complex (ST1018, <i>n</i> = 1)	
D (<i>n</i> = 6)		Outpatient (<i>n</i> = 6)			ST69 complex (ST106, <i>n</i> = 1), ST59 (<i>n</i> = 1), <i>fumC31</i> (<i>n</i> = 1), <i>fumC32</i> (<i>n</i> = 1), <i>fumC39</i> (<i>n</i> = 1), <i>fumC40</i> (<i>n</i> = 1)

^a MLST was performed with a selected group of isolates (*n* = 37); amplification of *fumC* was performed for all isolates of phylogroup D. Isolates belonging to either phylogroup A or phylogroup B1 were associated with the ST155 clonal complex. Discrepancies among MLSTs and phylogroups have been described previously (20, 55).

is recovered from hospitalized patients, healthy humans, animals, and environmental samples (5, 12, 22, 53, 54). In the work described here, we analyzed the epidemiological features and multilevel genetic diversity of CTX-M-14-producing organisms from humans with and without hospital exposure to obtain a better understanding of their recent widespread appearance and persistence in our geographic area.

MATERIALS AND METHODS

Bacterial strains. We studied 61 CTX-M-14-producing *E. coli* isolates from 56 individuals living in a suburban area of Madrid, Spain, from 2000 to 2005. They were mainly community-based persons (*n* = 40 [71.4%]) comprising 2 healthy volunteers without previous antibiotic or hospital exposure (53), 3 cohabitants of patients with UTIs caused by CTX-M-14 recovered within 2 weeks of the case detection (54), and 35 outpatients. The hospitalized patients were located in medical wards (*n* = 13 [23.2%]), intensive care units (ICUs; *n* = 2 [3.6%]), or surgical wards (*n* = 1 [1.8%]) (Table 1). The sample represents all CTX-M-14-producing isolates recovered in the Hospital Universitario Ramón y Cajal (39 from urine, 1 from blood, 1 from wound, and 2 from feces) and fecal isolates from two surveillance studies performed in the same city during the same time period (53, 54).

One isolate per patient (or more than one per patient, if different antibiotic resistance patterns were detected in isolates from the same sample or if they showed different PFGE patterns) was selected for further analysis. Bacterial identification and antibiotic susceptibility testing were performed with the semi-automated Wider system (Fco. Soria Melguizo, Madrid, Spain). Analysis of the susceptibilities of the isolates to non-β-lactam antibiotics was performed by the disk diffusion method according to CLSI guidelines (11). The CLSI breakpoints for netilmicin were used for apramycin. Strains showing intermediate susceptibility were considered resistant.

Clonal relatedness. Clonal relatedness was established by pulsed-field gel electrophoresis (PFGE) of XbaI-digested genomic DNA in a CHEF-DRIII device (Bio-Rad, La Jolla, CA) by using electrophoresis conditions of 14°C, 6 V/cm², 10 to 40 s, and 24 h; standard procedures and criteria were followed (41). The assignment of *E. coli* phylogenetic groups was carried out by the multiplex PCR assay previously described by Clermont et al. (10). Representative *E. coli* isolates mainly belonging to phylogroups A and B1 were further characterized by multilocus sequence typing (MLST) by using the standard seven housekeeping loci (<http://www.mlst.net>). The *fumC* genes of *E. coli* isolates belonging to the D phylogroup were analyzed for the presence of a single nucleotide polymorphism (C288T), which is a specific marker for *E. coli* "clonal group A." This clone is disseminated worldwide, it belongs to sequence type 69 (ST69), and it is responsible for community UTIs (50).

MLST data were analyzed by using the eBURST program (version 3; <http://eburst.mlst.net>), which assesses the relationship within clonal complexes. The entire *E. coli* MLST database (2,255 isolates and 1,065 STs; last update, June 2009) was considered.

Transferability and location of *bla*_{CTX-M-14} gene. Transfer was tested by broth and filter mating assays with *E. coli* K-12 strain BM21R (nalidixic acid and rifampin [rifampicin] resistant, lactose fermentation positive, and plasmid free) as the recipient for 6 to 12 h at 30°C and 37°C. Transconjugants were selected on Luria-Bertani agar plates supplemented with cefotaxime (2 mg/liter) and rifampin (100 mg/liter) and were incubated at 37°C for 24 h.

The chromosomal and/or plasmid location of *bla*_{CTX-M-14} genes was assessed by the hybridization of I-CeuI-digested genomic DNA with *bla*_{CTX-M-14} and 16S rRNA gene probes by using the following electrophoresis conditions: 5 to 25 s for 23 h and 60 to 120 s for 10 h, 14°C, and 6 V/cm² (41). Transfer and hybridization were performed by standard procedures. Labeling and detection were carried out with an AlkPhos commercial kit, according to the manufacturer's instructions (Amersham Life Sciences, Uppsala, Sweden).

Plasmid characterization. Plasmid DNA was obtained by using a QIAGEN plasmid midikit (Qiagen, Hilden, Germany). Plasmids were preliminarily classi-

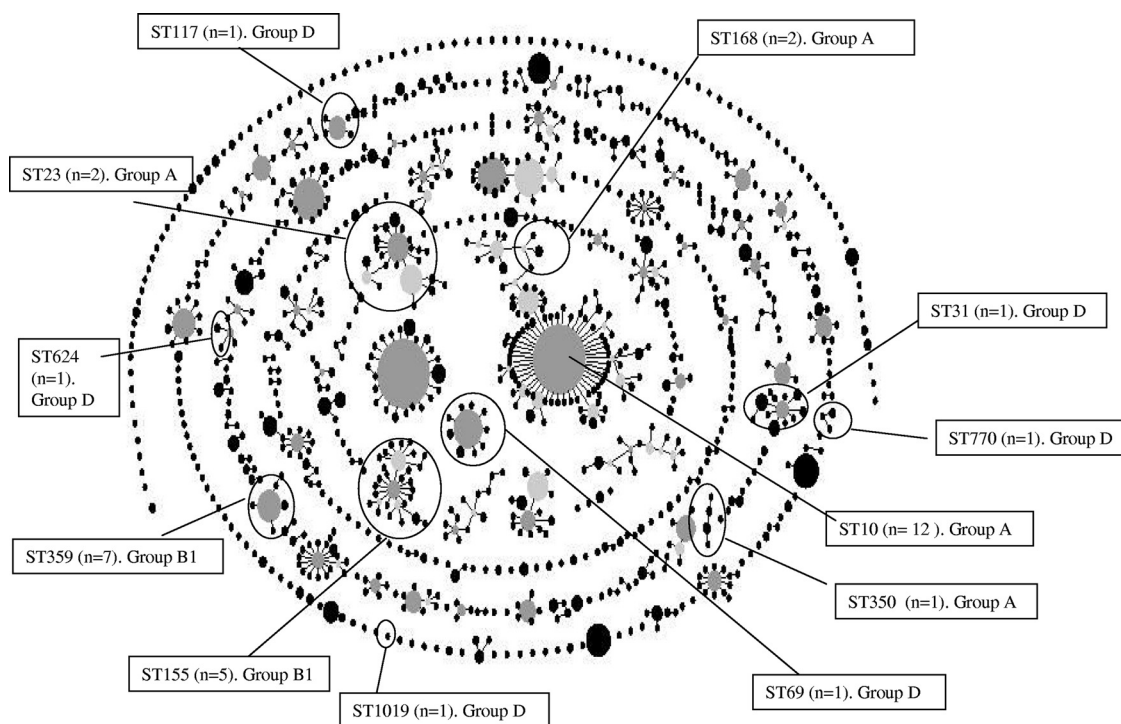


FIG. 1. Snapshot of clusters of *E. coli* populations from the *E. coli* MLST database with related and unrelated STs. The STs identified in this study are represented by circles: dark gray circles, founders of each clonal complex; light gray circles, subgroups of the founder; and black circles, a node connection between two STs reflects a single-locus variation between them. This diagram does not show the genetic distance between unrelated STs and clonal complexes.

fied according to their incompatibility group by using the PCR-based replicotyping (PBRT) scheme described by Carattoli et al. (8). Plasmid size and content were determined by hybridization of S1 nuclease-digested genomic DNA (41) with probes specific for *bla*_{CTX-M-14}. Confirmation of the replicon content was also undertaken by hybridization of PstI-digested plasmid DNA with probes specific for both *bla*_{CTX-M-14} and *rep* amplified by PCR from different samples. The relationship among plasmids was determined by comparison of their DNA patterns generated after digestion with the EcoRI, PstI, and HpaI enzymes and electrophoresis in 1% agarose at 100 V for 4 h.

Plasmids preliminarily classified as members of the IncI complex (those that yielded an amplification product by using primers specific for incompatibility group I (IncI), IncK, and/or IncB/O) by the PBRT method) were further studied (8). We analyzed the sequences corresponding to RNA interference (RNAi; also named *inc*, which encodes a segment of RNA of approximately 70 bp that regulates replication by repressing the expression of the replicase gene) and also to the replication and the relaxase proteins of a subset of representative isolates. Identification of RNAi was performed by analysis of the amplicons obtained by the PBRT method. Identification of the replication proteins was carried out by analysis of the PCR products obtained with primers RNAI (5'-ATGCGCCAT AAGGCATTCA-3') and REPZA (5'-AGTCGC TTCAGATGGTCAT-3') and an amplification protocol consisting of 1 cycle at 94°C for 5 min and 35 cycles at 94°C for 1 min, 54°C for 1 min, and 72°C for 2 min, followed by a final step at 72°C for 10 min. Identification of plasmid relaxases was performed by PCR with primers MOB_{P12} forward (5'-GCAAAAGATGACACTGAYCCYGT TTT-3') and MOB_{P12} reverse (5'-AGCGATGTGGATGTGAAGGTTAT CHGTRTC-3') and conditions of 30 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s. The amplified DNA segments were then sequenced in both orientations (1, 19).

Analysis of *bla*_{CTX-M-14} genetic environment. As *bla*_{CTX-M-14} has been found to be associated with different genetic environments, we tested for the presence of sequences associated with known genetic platforms: *ISEcp1*, *IS903*, *int11*, and *ISCR1* (2, 17, 39). Oligonucleotides corresponding to different regions of *ISEcp1* and *IS903* were used in order to detect elements containing Δ *ISEcp1* and/or Δ *IS903*, as described previously (17). The link of class 1 integrons containing *bla*_{CTX-M-14} to Tn21-like transposons was screened for by PCR mapping with the primers and conditions previously described (41).

Nucleotide sequence accession numbers. The sequences corresponding to the plasmids described in this paper were assigned the indicated GenBank (NCBI) accession numbers: pRYC105, GQ892052; pRYC106, GQ892053; pRYC109, GQ892054; pRYC110, GQ892050 and GQ892051.

RESULTS AND DISCUSSION

***E. coli* isolates producing CTX-M-14 belong to phylogroups A, B1, and D and are mostly associated with UTIs.** The 61 CTX-M-14-producing isolates included in this study were classified into 60 different PFGE patterns and were similarly distributed among phylogenetic groups A, D, and B1 (22, 21, and 18 isolates, respectively, representing 36.1%, 34.4%, and 29.5% of the isolates included in the study, respectively). Most of the CTX-M-14-producing *E. coli* strains of phylogroups A and B1 belonged to particular clonal complexes; phylogroup A isolates clustered in the ST10 complex (12/18 of the isolates analyzed) and also in the ST168 complex (2/18) or the ST23 complex (2/18), while phylogroup B1 isolates grouped either in the ST359 complex (7/12) or the ST155 complex (5/12). Analysis of the *fumC* sequences from *E. coli* isolates belonging to phylogroup D showed a high degree of diversity (Table 1). One strain with the C288T single polymorphism specific for uropathogenic *E. coli* (UPEC) clonal group A (CGA) was identified as ST106, which fits within the ST69 clonal complex. The other isolates studied corresponded to ST59, ST117, ST393, ST624, ST770, and ST1019 (Fig. 1; Table 1).

The isolates studied were frequently associated with UTIs ($n = 39$, 64%) and were resistant to nalidixic acid (73.8%;

45/61), tetracycline (72.1%; 44/61), sulfonamides (60.6%; 37/61), trimethoprim (59.0%; 36/61), and streptomycin (57.3%; 35/61) and were less frequently resistant to chloramphenicol (36.1%; 22/61), ciprofloxacin (21.3%; 13/61), spectinomycin (19.7.3%; 12/61), tobramycin (9.8%; 6/61), apramycin (8.2%; 5/61), and gentamicin or amikacin (4.9% each; 5/61). A few isolates of phylogroups A ($n = 6$), B1 ($n = 5$), and D ($n = 7$) exhibited the multiresistant phenotype ACSSuTTP (indicating resistance to ampicillin, chloramphenicol, streptomycin, sulfonamides, tetracycline, and trimethoprim, respectively), which has recently been associated with a genetic island that comprises genes encoding antibiotic resistance and virulence in particular *E. coli* clones causing UTIs (UPEC strains) (30). Interestingly, phylogroup A and D isolates showing the ACSSuTTP phenotype were also resistant to fluoroquinolones (data not shown).

Our collection contained a large number of community *E. coli* isolates of phylogroups A and B1, besides those of group D, unlike the population structure of CTX-M-14-producing isolates from other countries, which mainly comprises phylogroups D and B2 (23, 46, 47, 49). It is of note that the majority of the UPEC isolates also belonged to phylogenetic groups B2 and D, with some *E. coli* isolates recently recognized as being globally disseminated UPEC clones, such as ST131, ST69, ST14, ST73, and ST95 (6, 28, 50). Even though groups A and B1 of *E. coli* have rarely been involved in extraintestinal infections, they are prevalent as commensal organisms in both animals and humans and are frequently found to be enterotoxigenic, enterohemorrhagic, and, sometimes, enteroaggregative, with each pathotype possessing a unique combination of virulence traits eventually linked to particular successful clones (18, 24, 52, 55). The *E. coli* ST10 complex is overrepresented among isolates of phylogroup A, being frequently identified among fecal isolates from healthy people, enterotoxigenic strains from community-based persons, and more recently, ESBL producers causing UTIs (29, 44, 52, 55; this study). Similarly, some group B1 *E. coli* clones are increasingly found to be ST155 or ST359 and have recently been isolated from Spanish, Portuguese, and Brazilian outpatients with UTIs (37, 55; unpublished results). These group A and B1 clones might reflect new UPEC strains that have emerged. These strains are independently associated with the presence of *bla*_{CTX-M-14} and may influence the successful spread of certain ESBL genes. Their high degree of epidemicity is also reflected by their high rate of transmission among household contacts of patients with UTIs (54). The selection and spread of particular STs within these phylogroups might be influenced by the acquisition of specific genetic traits that enhance their colonization ability (24, 30, 50).

The *bla*_{CTX-M-14} gene is located on IncK and IncHI2 plasmids. The *bla*_{CTX-M-14} gene was located on plasmids exhibiting various transfer properties (*bla*_{CTX-M-14} was transferred by conjugation in 75.4% of the isolates) and hybridizing with probes specific for narrow-host-range incompatibility group IncI and/or IncK (94.9%; 37/39) and to a much lesser extent IncHI2 (5.1%; 2/39).

Plasmids of the IncI complex. Twenty-seven of the 29 plasmids characterized were similar in size (80 to 90 kb), could be amplified with primers included in the PBRT assay specific for both the IncI and the IncK groups (but rarely with primers

specific for the IncB/O group), and hybridized with probes specific for the *inc* regions of these plasmid groups. They were classified into four types on the basis of their restriction fragment length polymorphism patterns, with three of them (pRYC105, pRYC108, and pRYC109) showing very similar profiles that differed by only a few bands ($n = 1$ to 3). pRYC105 was the predominant plasmid variant and was recovered from inpatients, outpatients, and healthy volunteers from 2000 to 2005. Nowadays, it is still detected in the Hospital Universitario Ramón y Cajal and other geographical areas (data not shown). pRYC105, pRYC108, and pRYC109 showed 99% to 100% sequence homology to *inc*_R387, RepA_pSERB1/R387, and NikB_pSERB1/R387 (GenBank accession no. M93063 for plasmid R387 and GenBank accession no. AY686591 for plasmid pSERB1) and were classified as plasmids of the IncK group. The entire 1.2-kb replication region of pRYC105 (*inc*, *repY*, *repA/repZ*, *ori*) from different isolates was sequenced and showed 99% sequence homology at the nucleotide level with the nucleotide sequence of R387. Nevertheless, analysis of the sequence corresponding to the PCR product obtained with IncI-specific primers showed 99 to 100% homology with the RNAi regions of plasmids pTN38148, pSL476_91, and pCVM29188 (GenBank accession nos. NC_011514, NC_011077, and CP01118, respectively), which indeed belong to the IncI group (Fig. 2 and 3). The probes generated from the PCR products corresponding to *inc*_R387/pSERB1 and *inc*_pSL476_91 hybridized with a single band of PstI-digested plasmid DNA from different isolates. Although cross hybridization can occur when these probes are used (13), the presence of two different sequences identical to the *inc* regions of plasmids belonging to the IncI and IncK groups was demonstrated in the isolates analyzed, indicating the presence of two different *inc* sequences located close together.

The fourth type, pRYC106, showed a different restriction fragment length polymorphism pattern with sequences identical to *inc*_pSL476_91, RepA_pSL476_91, and NikB_pSERB1 (GenBank accession no. CP001118 for plasmid pSL476_91 and GenBank accession no. AY686591 for plasmid pSERB1; Fig. 2) and it was considered a mosaic plasmid of the IncI complex. Despite the multiresistance phenotypes of the *E. coli* hosts, transconjugants containing these plasmids conferred resistance only to cephalosporins (Table 2). The results of the analysis of their DNA sequences corresponding to the RNA antisense molecules involved in the control of replication, the replicases, and the relaxases are shown in Fig. 2 and 3.

The plasmid analytic approach used in this work permits not only the discrimination between IncI and IncK plasmids but also allows the detection of recombinant plasmids containing either the replication or the conjugation systems of these groups, which seem to be frequent in nature. Some examples are recently described plasmids pSC138 (GenBank accession no. NC_006856) and pO113 (GenBank accession no. NC_007365), which contain only conjugation systems related to the IncI group; pCooKm (GenBank accession no. CR942285), which contains a replication region and a conjugative region related to those of the incompatibility plasmid groups I and F, respectively; and p026-Vir (GenBank accession no. NC_012487.1), which contains two replicases homologous to different IncI plasmids and two relaxases highly similar to those of IncK plasmids. Even though the presence of

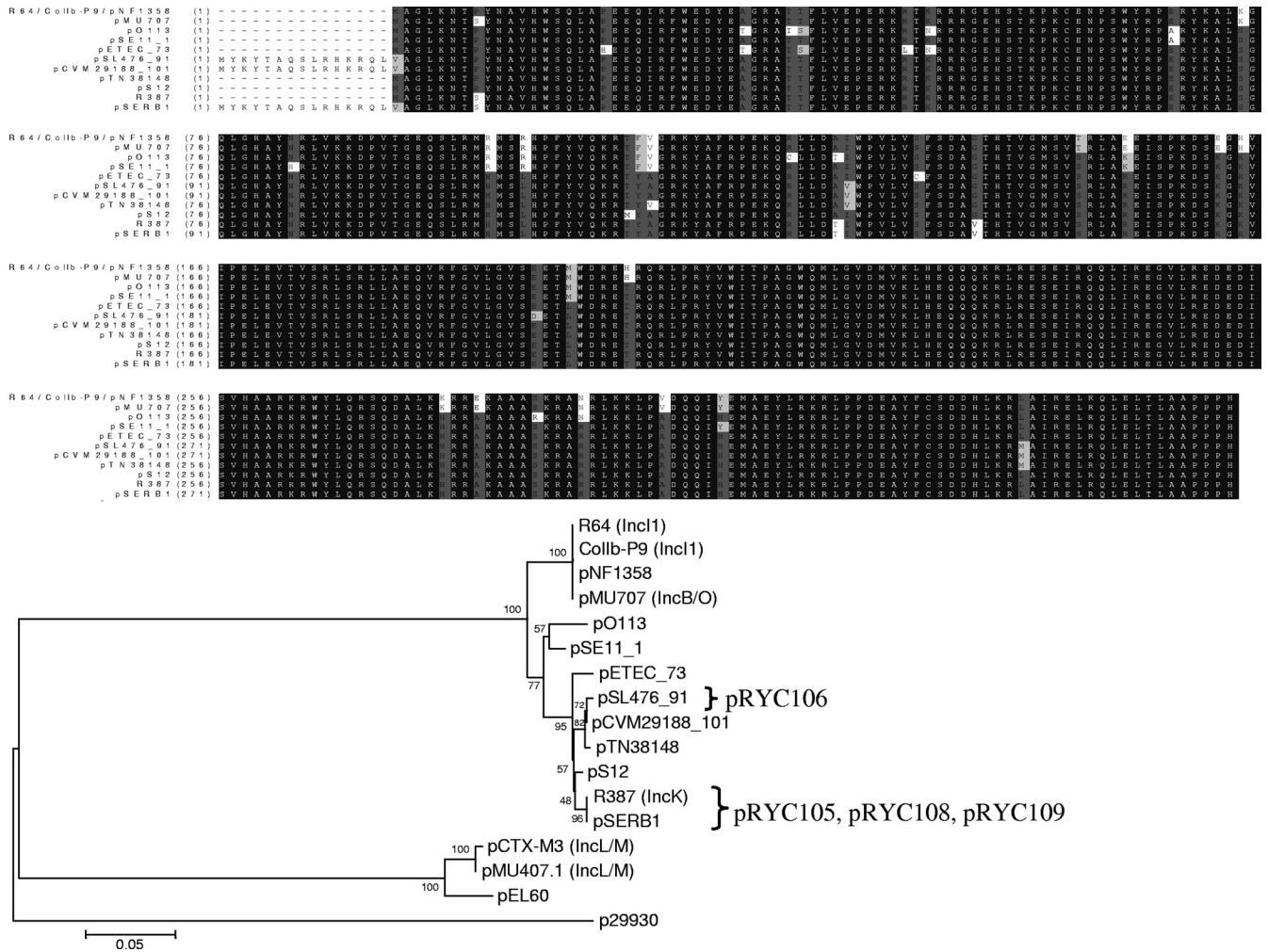


FIG. 2. (Top) Protein sequence alignment of replication proteins amplified from IncI and IncK plasmids. White on black, invariant amino acids; black on dark gray, strongly conserved amino acids; black on light gray, similar amino acids; gray on white, weakly similar amino acids. (Bottom) Phylogeny of replicases from the IncI and IncK plasmids. A neighbor-joining tree was constructed by using the complete deletion and p-distance model and was tested with bootstrap values (1,000 replicates) and by tree of the multiple alignments of complete replicase sequences of several IncI, IncK, and IncL/M plasmids by using MEGA software (version 3.1) (27). The replicase of plasmid p29930 was used to root the tree. Replicases whose protein sequences are 100% identical to those amplified from samples of plasmids pRYC105, pRYC106, and pRYC108 are grouped by keys. The GenBank accession numbers of the proteins used in the phylogeny are as follows: R64, NP_863360; Collb-P9, NP_052449; pNF1358, AAZ05341; pMU707, AAA98176; pO113, YP_308827; pSE11-1, YP_002296026; pETEC_73, YP_001451513; pSL476_91, YP_002043842; pCVM29188_101, YP_002039041; pTN38148, YP_002302249; pS12, BAD72945; pSERB1, AAT94184; pCTX-M3, NP_775034; pMU407.1, AAA87028; pEL60, NP_943262; p29930, CAD58558.

two *inc* sequences that are located close together has not been observed among plasmids of the IncI complex, plasmids of incompatibility groups F and N may contain two *inc/cop* sequences within the same replication region (14, 42). It is worthwhile to highlight the stability of the pRYC105 fingerprints among different *E. coli* lineages over time, which suggests a high degree of adaptation to specific genetic backgrounds. However, the wide distribution of pRYC105 among *E. coli* isolates from community-based persons (53, 54) and food-borne animals (31) is of concern since it creates a reservoir of highly transmissible plasmids in heterogeneous *E. coli* populations that colonize different environments which may be able to evolve toward more virulent variants by recombination, as seems to have oc-

curred with p026-Vir (GenBank accession no. NC_012487.1) and pCooKm (GenBank accession no. CR942285).

IncK plasmids have been isolated from patients with UTIs since the early 1970s (1a, 16, 21, 51). A type IV secretion system that contributes both to plasmid conjugation and to adherence to epithelial cells and abiotic surfaces identified in IncK plasmids such as pSERB1 (GenBank accession no. AY686591) (16) may facilitate the ability of *E. coli* to colonize the intestine and, consequently, enhance the pathogenic profile of specific clones or clonal groups (16, 43). The acquisition of IncK plasmids containing *bla*_{CTX-M-14} by group A and B1 *E. coli* clones could have enhanced their ability to colonize the urinary tract in patients exposed to antibiotics, as highlighted

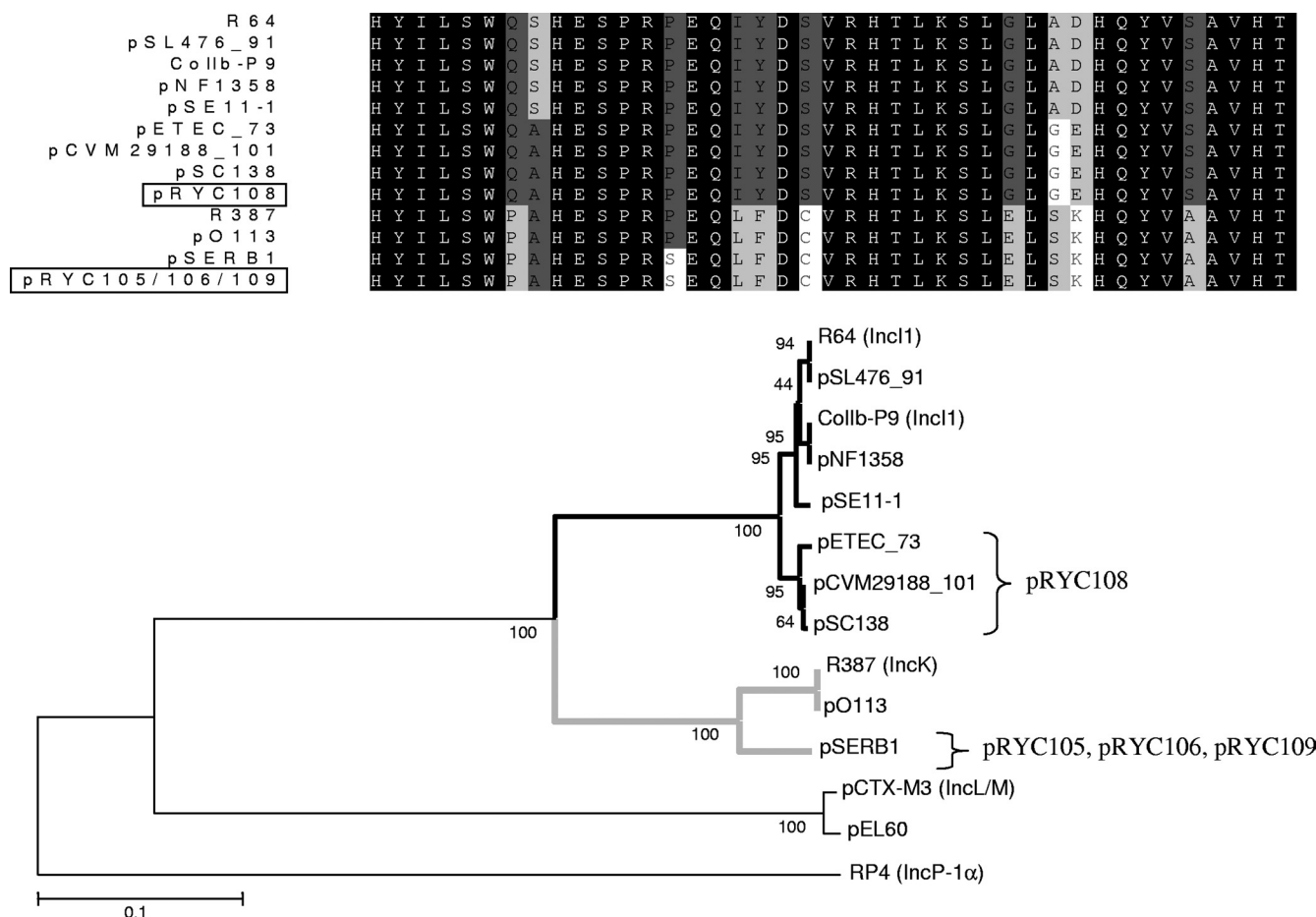


FIG. 3. (Top) Sequence alignment of partial relaxase sequences amplified from CTX-M-14-carrying plasmids pRYC105 and pRYC108 with oligonucleotides specific for IncI1 and IncK plasmids. White on black, invariant amino acids; black on dark gray, strongly conserved amino acids; black on light gray, similar amino acids; gray on white, weakly similar amino acids. (Bottom) Phylogeny of clade MOB_{P12} relaxases from IncI1 and IncK plasmids and an expanded view of clade MOB_{P12} (19). A neighbor-joining tree was constructed by using the pairwise deletion and p-distance model and was tested with bootstrap values (1,000 replicates) and by use of the multiple alignment of the N-terminal 300 amino acids of relaxase proteins of several IncI1, IncK, and IncL/M plasmids by using MEGA software (version 3.1) (27). Relaxase TraI of IncP1-α plasmid RP4 was used to root the tree. Black lines, branches comprising relaxases of IncI1 plasmids; gray lines, relaxases of IncK plasmids. Relaxases whose protein sequences are 100% identical to those amplified from samples of plasmids pRYC105 and pRYC108 are grouped by keys. The GenBank accession numbers of the plasmid relaxases used in the phylogeny are as follows: R64, BAA78021; pSL476_91, YP_002043909; Collb-P9, NP_052501; pNF1358, AAZ05365; pSE11-1, BAG80277; pETEC_73, YP_001451521; pCVM29188_101, YP_002039113; pSC138, YP_209402; pO113, YP_308733; pSERB1, AAT94234; pCTX-M3, AAN87675; pEL60, NP_943241; and RP4, CAA38336.

by the high frequency of fluoroquinolone resistance among CTX-M-14-producing *E. coli* isolates causing UTIs (65.6%).

Plasmids of the IncHI2 group. The *bla*_{CTX-M-14} gene was located on a 290-kb transferable IncHI2 plasmid in two isolates. The sequences corresponding to the replication origin and the relaxase showed homology with R478 (GenBank accession no. BX664015.1), the prototype of the IncHI2 plasmids. Despite the low frequency of IncHI2 plasmids in our series, the role of this plasmid group in the spread of *bla*_{CTX-M-14} must not be underestimated, as it has been reported worldwide, suggesting that it is widespread (39).

The *bla*_{CTX-M-14} enzyme is located in different genetic platforms, supporting different mobilization events. We identified two variants of *bla*_{CTX-M-14} previously designated CTX-M-14a (*n* = 59/61) and CTX-M-14b (*n* = 2/61) (GenBank accession nos. AF252622 and DQ359215, respectively). *bla*_{CTX-M-14a} is the predominant variant described and encodes the enzyme

considered to be the progenitor of the CTX-M-9 cluster also known as CTX-M-18 (4). To date, the *bla*_{CTX-M-14b} enzyme (GenBank accession no. EU274579) has been detected only in Spain and Tunisia (39), and it exhibits three nucleotide changes (A372G, G570A, G702A) in relation to the nucleotide sequence of *bla*_{CTX-M-14a}. The last two are also found in *bla*_{CTX-M-9} (GenBank accession no. AF174129).

In our study, *bla*_{CTX-M-14a} was associated with either *ISEcp1* in IncK plasmids (*n* = 27) or *ISCR1* in an IncHI2 plasmid (*n* = 1) and IncI-like plasmids (*n* = 3). *bla*_{CTX-M-14b} was linked to *ISCR1* in an IncHI2 plasmid (*n* = 1) or to *ISEcp1* in an IncK plasmid (pRYC109) (*n* = 1). A truncated *IS903* sequence was identified downstream of *ISEcp1-bla* sequences in representative isolates corresponding to each plasmid type. *ISEcp1* was detected 42 nucleotides upstream of both *bla*_{CTX-M-14a} and *bla*_{CTX-M-14b}. It is of note that an identical 42-bp region has also been identified upstream of different genes encoding

TABLE 2. Characteristics of plasmids harboring *bla*_{CTX-M-14}

Inc group	RFLP ^a	No. of isolates	<i>E. coli</i> phylogroup (no. of isolates)	Date	PCR results for replicon ^b	Plasmid size (kb)	<i>bla</i> _{CTX-M-14} variant (no. of isolates)	Genetic environment (no. of isolates)	Antibiotic resistance ^c
IncK	pRYC105	24	A (10)	2001–2005	<u>I1</u> , <u>K</u> (B/O)	80	a	<i>ISECp1</i>	(Ap, Cm, Sm, Su, Tp, Te, Gm, Km, Nt, Na, Cip)
			B1 (7)	2002–2005	<u>I1</u> , <u>K</u>	80	a	<i>ISECp1</i>	(Ap, Cm, Sp, Su, Tp, Tb, Gm, Km, Ak, Ne)
			D (8)	2002–2005	<u>I1</u> , <u>K</u>	80	a	<i>ISECp1</i>	(Cm, Sm, Su, W, Te, Sp, Ne, Nt, Na, Cip)
IncK	pRYC109	1	D	2004	I1, K	80	b	<i>ISECp1</i>	Cm, Sm, Su, Tp, Te, Sp, Cip, Na
IncK	pRYC108	1	A	2002	<u>I1</u> , <u>K</u>	80	a	<i>ISECp1</i>	Te, Na
IncI1	pRYC106	1	D	2003	<u>I1</u> , B/O	80	a	<i>ISECp1</i>	Sm, Su, Tp, Te, Cip, Ne
IncI1	ND	2	A	2005	<u>I1</u>	60,90	a	<i>ISECp1</i> (1), <i>ISCR1</i> (1)	(Cm, Sm, Su, Te, Sp, Cp), Tp
IncI1	ND	4	B1	2002–2005	<u>I1</u>	60–80	a	<i>ISECp1</i>	(Ap, Cm, Sm, Su, Sp, Tp, Te, Gm, Km, Ak, Ne)
IncI1	ND	4	D	2004	<u>I1</u>	70–75	a	<i>ISECp1</i> (2), <i>ISCR1</i> (2)	(Cm, Sm, Su, Sp, Tp, Te, Km, Cp)
IncHI2	pRYC110	2	D (2)	2002–2003	<u>HI2</u>	240	a (1), b (1)	<i>ISCR1</i>	(Cm, Su, Sp, Tp, Na) Sm, Te

^a Plasmid type as determined by RFLP. ND, not determined (corresponding to isolates for which transconjugants were not obtained; the presence of a replicon was inferred by hybridization with specific probes). Sporadic isolates were amplified with primers specific for IncB/O plasmids, the numbers of which are represented in parentheses.

^b Replicon type identified by PCR and sequencing. The replicon types assessed by hybridization are underlined.

^c Abbreviations: Su, sulfonamide; Tp, trimethoprim; Gm, gentamicin; Sm, streptomycin; Sp, spectinomycin; Ne, neomycin; Km, kanamycin; Tb, tobramycin; Nt, netilmicin; Ak, amikacin; Cp, ciprofloxacin; Te, tetracycline; Cm, chloramphenicol. Resistance to antibiotics appearing in parentheses was not present in all isolates.

ESBLs of the CTX-M-9 cluster, such as CTX-M-9, -14, -16, -17, -19, -21, and -24 and Toho-2 (GenBank accession nos. AF174129, AF252622, AY033516, AF454633, AF458080, AJ416346, NC_011617, and D89862, respectively), which would indicate a unique origin for these variants, as suggested previously (4). An integron platform similar to that of In60 which contains *bla*_{CTX-M-9} (GenBank accession no. AF174129) and which is not linked to Tn21 was identified among the five isolates harboring *ISCR1* and *bla*_{CTX-M-14a} or *bla*_{CTX-M-14b} within IncI and IncHI2 plasmids (41). The characterization of similar genetic structures for the *bla*_{CTX-M-14} variants in this and other studies probably reflects in-platform β-lactamase gene mutation-based evolution. This kind of presumed local sequence evolution has been demonstrated for CTX-M enzymes belonging to other phylogenetic clusters, such as *bla*_{CTX-M-1} and *bla*_{CTX-M-32} or *bla*_{CTX-M-3} and *bla*_{CTX-M-15} (40).

The location of *bla*_{CTX-M-14a} and *bla*_{CTX-M-14b} on different genetic platforms linked to *ISECp1* and *ISCR1* on plasmids of different incompatibility groups suggests different mobilization events and subsequent recombinatorial processes among plasmids.

Conclusions. The high frequency of occurrence of the *bla*_{CTX-M-14} enzyme in our geographic area is mainly due to the spread of IncK plasmids among UPEC clones of phylogroups A, B1, and D, with particular STs being overrepresented. The countrywide dissemination of this plasmid may also be suggested on the basis of two Spanish studies which inferred the presence of IncK plasmids among CTX-M-14-producing isolates recovered from different cities by the amplification of specific sequences from a few isolates by the PBRT method (15, 39). However, data about plasmids containing *bla*_{CTX-M-14} from other countries where this ESBL is commonly detected indicate that other incompatibility groups can also be involved in the dissemination of CTX-M-14 (34, 54a). The diversity of genetic platforms containing *bla*_{CTX-M-14} highlights the hypothesis of the existence of different mobilization events for

this gene (4) and the influence of the genetic context on its successful dissemination.

The results obtained by the multilevel approach used in the present study offer an example of how different biological and genetic entities should be simultaneously taken into consideration to explain the local spread of antibiotic resistance (3). This particular complex landscape reflects the efficient interplay between clonal epidemicity, plasmid transfer and maintenance, the capture of resistance genes by particular genetic platforms, and, possibly, bacterial virulence traits in determining the evolution of bacteria at the local level (35).

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