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Multiple acquisitions of CTX-M plasmids in the rare D_2 genotype of *Escherichia coli* provide evidence for convergent evolution

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Over the last decade, CTX-M enzymes have become the most prevalent extended-spectrum β-lactamases (ESBLs) worldwide, mostly in *Escherichia coli*, causing a major health problem. An epidemiological relationship has been established between a rare genotype of *E. coli*, the D_2 genotype, and the presence of CTX-M genes. We investigated this striking association by exploring the genetic backgrounds of 18 D_2 genotype CTX-M-producing strains and of the plasmids encoding CTX-M enzymes. The 18 strains had different genetic backgrounds, as assessed by multilocus sequence and O typing, and were associated with various plasmids bearing diverse CTX-M genes. The region encompassing the genetic marker of the D_2 genotype (TSPE4.C2) was not correlated with the presence of CTX-M genes. CTX-M-producing D_2 strains had far fewer virulence factors than a control group of 8 non-ESBL-producing D_2 strains, and an inverse relationship was found between the number of co-resistances associated with the CTX-M gene and the number of virulence factors found in the strain. These findings provide evidence for multiple acquisitions of plasmids carrying CTX-M genes in different D_2 genotype strains. They strongly suggest that convergent evolution has occurred, and indicate that there has been selection for the association of a specific genetic background of the strain and the CTX-M gene. This fine-tuning of the relationship between the D_2 genotype and CTX-M genes presumably increases the fitness of the strain, indicating a role for the host cell in the acquisition and dissemination of CTX-M genes.

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INTRODUCTION

Escherichia coli is widespread in the environment and is a component of the intestinal microbiota of most warm-blooded animals, including humans (Bettelheim, 1997a). *E. coli* is also a major cause of extraintestinal infections in humans, being the major agent of urinary tract infections and one of the most common agents of bacteraemia (Bettelheim, 1997b; Eykyn *et al.*, 1990; Russo & Johnson, 2000). Isolates of the species can be assigned to four main phylogenetic groups: A, B1, B2 and D. A triplex PCR-based method has been developed for assigning strains to these four phylogenetic groups. Based on the presence or absence of two genes (*chuA* and *yjaA*) and an anonymous DNA fragment (TSPE4.C2), this method yields seven different genotypes: A_0 and A_1 (for phylogenetic group A), B1, D_1 and D_2 (for phylogenetic group D), and $B2_2$ and $B2_3$ (for phylogenetic group B2) (Clermont *et al.*, 2000; Escobar-Paramo *et al.*, 2004b). A link between strain phylogeny and virulence has been reported. Most virulent extraintestinal strains belong to group B2 or, less frequently, group D, whereas most commensal strains belong to groups A and B1 (Johnson *et al.*, 2001; Picard *et al.*, 1999).

The production of extended-spectrum β-lactamases (ESBLs) is a major mechanism of resistance to third-

Abbreviations: ESBL, extended-spectrum β-lactamase; HPI, high pathogenicity island; MLST, multilocus sequence typing; PAI, pathogenicity island; ST, sequence type; VF, virulence factor.

Supplementary figures showing a population genetics analysis based on MLST data and a schematic representation of the genetic environment of bla_{CTX-M} genes from the 18 ESBL-producing D_2 *E. coli* isolates studied, and a supplementary table of PCR primers are available with the online version of this paper.

generation cephalosporins, and plasmid-encoded ESBLs are mostly of the TEM, SHV or CTX-M types (Paterson & Bonomo, 2005). Over the last decade, CTX-M enzymes have replaced TEM and SHV mutants as the most prevalent ESBLs worldwide, with *E. coli* as a major host (Bonnet, 2004; Livermore *et al.*, 2007; Paterson & Bonomo, 2005). They have been reported in both hospital and community settings (Paterson & Bonomo, 2005; Pitout *et al.*, 2005). They have also been detected in pets and farm animals, products of the food chain and sewage (Carattoli *et al.*, 2005b; Smet *et al.*, 2008; Warren *et al.*, 2008). CTX-M enzymes are diverse, with over 50 enzymes, phylogenetically grouped into five clusters on the basis of sequence homology (www.lahey.org/Studies) (Barlow *et al.*, 2008). They evolved from *Kluyvera* spp. chromosomal β-lactamases by gene transposition and mutation (Canton & Coque, 2006). Various genetic elements may be involved in the mobilization of $bla_{\text{CTX-M}}$: insertion sequences such as IS*Ecp1* or IS*CR1*, which is contained in a class 1 integron, and phage-related sequences, as identified surrounding $bla_{\text{CTX-M-10}}$ in isolates from Spain (Canton & Coque, 2006; Eckert *et al.*, 2006; Oliver *et al.*, 2005; Toleman *et al.*, 2006). These IS elements appear to be associated with specific $bla_{\text{CTX-M}}$ genes. For example, CTX-M-14 and CTX-M-15 are associated with IS*Ecp1*, and CTX-M-9 is associated with IS*CR1*. Genes encoding CTX-M enzymes have been disseminated over different continents through epidemic plasmids and/or particular epidemic strains (Paterson & Bonomo, 2005). However, the driving force behind the recent worldwide dissemination of CTX-M-producing micro-organisms and the emergence of these strains in the community remain unclear.

We previously showed that although ESBLs are present on mobile elements, a complex interaction seems to occur between the type of ESBL produced and the chromosomal genetic background of the strain (Branger *et al.*, 2005). The acquisition, expression and maintenance of genes encoding ESBLs seem to depend on interactions between the type of ESBL, phylogenetic background (strain phylogenetic group), the intrinsic virulence of the strain and the presence of associated fluoroquinolone resistance. ESBLs are not randomly distributed among *E. coli* clones. SHV- and, to a lesser extent, TEM-type ESBLs have been preferentially observed in B2 phylogenetic group strains, whereas CTX-M-type enzymes are associated with the D_2 genotype (*chuA*$^+$ *yjaA*$^-$ TSPE4.C2$^+$). Indeed, strains of the D_2 genotype produce only CTX-M-type enzymes, and tend to have fewer virulence factors and to be more resistant to fluoroquinolones than B2 strains (Branger *et al.*, 2005). Furthermore, the D_2 genotype was found to account for 8.5 % of ESBL-producing strains, whereas it is one of the least frequent genotypes in a worldwide collection of 5749 animal, human and environmental non-ESBL-producing strains, accounting for only 2.9 % of all strains (E. Denamur & D. M. Gordon, unpublished data) (Chi-squared test between D_2 ESBL-producing strains and D_2 non-ESBL-producing strains, $P<0.001$).

In this study, we investigated the striking genetic relationship between the D_2 genotype and CTX-M enzymes by studying in detail the chromosomal genetic background of the strains and the genetic background of the plasmids encoding the CTX-M enzymes. Our findings provide insight into the respective roles of the host cell and plasmid in the acquisition and dissemination of CTX-M genes.

METHODS

Bacterial strains. We analysed 18 CTX-M β-lactamase-producing *E. coli* strains with a D_2 genotype. Thirteen strains were from a previous study (Branger *et al.*, 2005) and five strains were taken from our personal collection. The strains produced CTX-M-type enzymes belonging to the three principal CTX-M clusters: five strains produced cluster 1 CTX-Ms (3 CTX-M-1 and 2 CTX-M-15), two strains produced cluster 2 CTX-Ms (CTX-M-2) and 11 strains produced cluster 9 CTX-Ms (3 CTX-M-9 and 8 CTX-M-14) (Table 1). The strains studied were recovered between 1997 and 2004 from different areas of France: Paris (3 hospitals) and Amiens. Sixteen strains were implicated in infections (urinary tract infection, 12; bacteraemia, 2; pus production from miscellaneous infections, 2) and two strains were isolated from faeces colonization.

For genotypic comparison with non-ESBL-producing strains, we used strains of the *E. coli* reference (ECOR) collection, which is known to be representative of the genetic diversity of the species (Ochman & Selander, 1984). All the D strains of the ECOR collection (10 D_1 and 2 D_2 genotype strains) were analysed, together with three B2, three B1 and two A phylogenetic group strains (Fig. 1). Three strains of the D_1 genotype, the complete genome sequence of which is available from GenBank (UMN026, IAI39 and 042), and 6 D_2 strains from our personal collection (Table 1) were also studied. The D_2 strains from our collection were selected to ensure diversity of the country of origin (USA and France), commensal and pathogenic status, and year of isolation (1980s and 2000s). In addition, strains 536, CFT073, UTI89, APECO1 from phylogenetic group B2, Sakai and EDL933 from phylogenetic group E, IAI1 and 55989 from phylogenetic group B1 and HS and K-12 MG1655 from phylogenetic group A, for which the genome is publicly available, were used for analysis of the TSPE4.C2 region. Lastly, *Escherichia fergusonii*, the closest relative of *E. coli*, was used as an outgroup.

Antimicrobial susceptibility. Susceptibility to antimicrobial agents was determined by the disk diffusion method on Mueller–Hinton agar (Bio-Rad), according to the guidelines of the Antibiogram Committee of the French Society for Microbiology (www.sfm.asso.fr). The following antibiotics were tested: ampicillin, streptomycin, kanamycin, gentamicin, tobramycin, netilmicin, amikacin, tetracycline, minocycline, ciprofloxacin, chloramphenicol, trimethoprim and sulfonamide.

β-Lactam resistance transfer assays. Mating experiments were performed, as described previously (Eckert *et al.*, 2004), with *E. coli* J53-2 Rf^r as the recipient strain. Transconjugants were then selected on Mueller–Hinton agar plates containing rifampicin (250 mg l^{-1}) and cefotaxime (2.5 mg l^{-1}). For transformation, plasmid DNA, isolated with the Nucleobond BAC 100 kit (Macherey-Nagel), was transferred by electroporation into *E. coli* EP-Max10B competent cells (Bio-Rad) with a MicroPulser, according to the manufacturer's instructions (Bio-Rad). Transformants were incubated for 1 h at 37 °C and plated on Mueller–Hinton agar supplemented with cefotaxime (2.5 mg l^{-1}).

Table 1. Characteristics of the D_2 *E. coli* strains studied

Strain	Origin	CTX-M cluster	CTX-M type	MLST D subgroup	TSPE4.C2 type	PCR O type	Virulence factors*																
							sfa/foc	*iroN*	*aer*	*iha*	*papC*	*papG*	*hlyC*	*cnf1*	*hra*	*sat*	*ireA*	*usp*	*ompT*	*ibeA*	*malX*	*fyuA*	*irp2*	*traT*
TN46	Urine	1	1	VI	1	O11	−	+	+	−	−	−	−	−	−	−	−	−	−	−	−	+	+	−
523	Urine	1	1	V	2	ND	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+
658	Rectal swab	1	1	V	2	ND	−	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+
637	Urine	1	15	IX	1	O2	−	−	+	−	−	−	−	−	−	−	−	−	+	−	+	−	−	+
473	Hepatic pus	1	15	III	2	O2	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+
505	Urine	2	2	IX	1	O2	−	−	+	−	−	−	−	−	−	−	−	−	−	−	+	−	−	+
TN19	Urine	2	2	VI	1	ND	−	+	+	−	−	−	−	−	−	−	−	−	−	−	−	+	+	+
501	Urine	9	9	II	2	O86	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	+	+	+
666	Urine	9	9	II	2	O86	−	−	+	−	−	−	−	−	+	−	−	−	−	−	−	+	+	+
670	Rectal swab	9	9	II	2	O86	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	+	+	+
513	Blood	9	14	IX	1	O1	−	−	−	−	−	−	−	−	−	−	−	−	+	−	+	+	+	−
375	Pus	9	14	I	2+IS	O102	−	−	+	−	−	−	−	−	−	−	−	−	−	−	+	+	+	+
TN13	Blood	9	14	I	2	O102	−	−	+	+	−	−	−	−	−	+	−	−	−	−	+	+	+	+
TN21	Urine	9	14	I	2	O102	−	−	+	+	+	II	+	−	−	+	−	−	−	−	+	+	+	+
TN48	Urine	9	14	I	2	O102	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	+
499	Urine	9	14	I	2	O2	−	−	+	−	−	−	−	−	−	+	−	−	−	−	+	+	+	−
715	Urine	9	14	II	2	O1	−	−	+	−	−	−	−	−	+	+	−	−	−	−	−	+	+	+
506	Urine	9	14	VI	1	ND	−	+	+	−	+	−	−	−	−	−	−	−	+	−	−	+	+	+
LBC7a	Stool	−	−	IX	1	O1	−	+	+	−	−	−	−	−	−	−	−	−	+	−	+	+	+	+
ECOR49	Stool	−	−	I	2	O2	−	−	+	+	+	II	−	−	−	+	−	−	+	−	+	+	+	+
ECOR50	Urine	−	−	I	2	O2	−	−	+	+	+	II	−	−	+	+	+	−	+	−	+	+	+	+
F376	Stool	−	−	I	2	O2	−	−	+	+	+	II	+	−	+	+	−	−	+	−	+	+	+	+
JGM51	Urine	−	−	I	2	O2	−	−	+	+	+	II	+	−	+	+	−	−	+	−	+	+	+	+
LBC6a	Stool	−	−	I	2	O2	−	−	+	+	+	II	−	−	−	+	−	−	+	−	+	+	+	+
Py199	Urine	−	−	I	2	O2	−	−	+	+	+	II	−	−	−	+	−	−	+	−	+	+	+	+
Py9	Urine	−	−	I	2	O2	−	−	+	+	+	II	+	−	+	+	−	−	+	−	+	+	+	+

ND, Not determined.

*Pathogenicity islands and plasmid-borne genes are boxed as in the paper by Bingen-Bidois *et al.* (2002): white boxes, PAI I_{CFTO73}; light grey boxes HPI; dark grey boxes, plasmid.

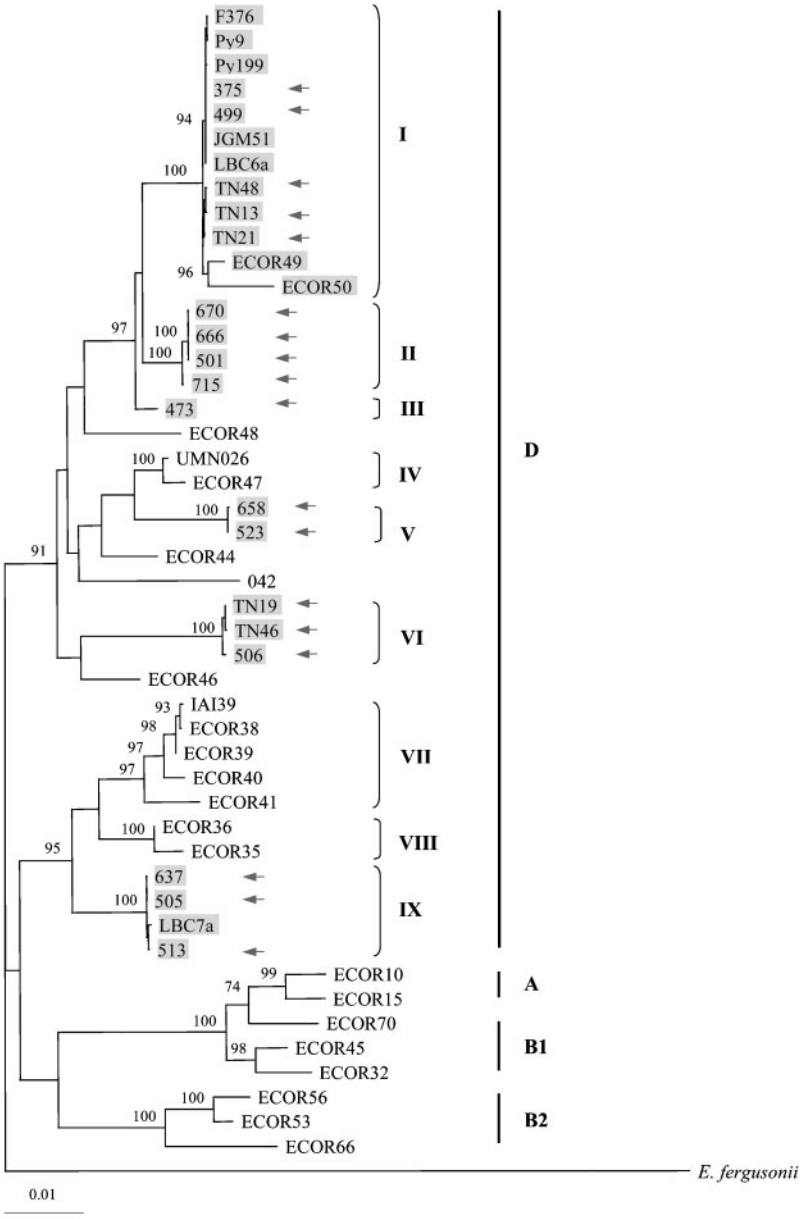

Fig. 1. Phylogenetic tree of the A, B1, B2 and D phylogenetic group strains, reconstructed from the DNA sequences of six housekeeping genes (*trpA*, *trpB*, *pabB*, *putP*, *icd* and *polB*), using the maximum-likelihood procedure. *E. fergusonii* served as an outgroup. Bootstrap values exceeding 70 % are indicated above the nodes. CTX-M-producing strains are indicated by an arrow. D_2 genotype strains (*chuA*$^+$ *yjaA*$^-$ TSPE4.C2$^+$) are boxed in grey. Other D group strains display the D_1 genotype (*chuA*$^+$ *yjaA*$^-$ TSPE4.C2$^-$).

Plasmid analysis. Plasmid DNA was purified from *E. coli* transconjugants and transformants by an alkaline lysis procedure, with the Qiagen Large-Construct kit. DNA restriction fragment length polymorphisms were analysed after the electrophoresis in 0.8 % agarose gels of plasmid DNA cleaved with *Eco*RI or *Hpa*I. Southern hybridization analysis was carried out with a $bla_{\text{CTX-M-9}}$ probe (Southern, 1975) and a digoxin labelling kit (Roche). The approximate size of the plasmids was estimated by comparing the bands obtained after restriction with *Hpa*I or *Eco*RI with DNA markers. Plasmids were also assigned to incompatibility groups according to a PCR replicon typing scheme (Carattoli *et al.*, 2005a) capable of detecting 18 plasmid replicons frequently found in the *Enterobacteriaceae*. The FII replicon was detected with primers CA1 and OP1 (Osborn *et al.*, 2000). Positive controls were kindly provided by Alessandra Caratolli (Instituto Superior di Sanita, Rome, Italy). DNA sequence variations in portions of *finO*, *traY*, *traD* and *repA* in F-related plasmids were also examined by PCR and sequencing, as described by Boyd *et al.* (1996). Replicon typing was also used to type the total plasmid content of the parental strains.

Characterization of the genetic environment of $bla_{\text{CTX-M}}$ genes. The presence of sequences surrounding $bla_{\text{CTX-M}}$ was investigated by overlapping PCR assays with a subset of specific primers, as described by Eckert *et al.* (2006). Overlapping amplification products were sequenced by the classical Sanger method and the nucleotide sequences obtained were compared with the sequences contained in the GenBank and EMBL databases. TEM and SHV β-lactamase genes were detected with specific primers, as described previously (Branger *et al.*, 2005), and were subsequently sequenced.

Strain molecular typing. Pulsed-field gel electrophoresis (PFGE) was performed with a CHEF-DRII system (Bio-Rad), using genomic DNA digested with *Xba*I, as described previously (Branger *et al.*, 1997). Fragments were separated at 6 V cm^{-1} and 14 °C, with pulse

time of 5–50 s for 21 h. The DNA patterns obtained on PFGE were analysed with Gel Compar software (Applied Maths). A similarity matrix was created with the band-based Dice similarity coefficient, and the unweighted pair group method with arithmetic mean (UPGMA) was used to cluster the strains.

Multilocus sequence typing (MLST) was carried out with the nucleotide sequence data of six housekeeping genes (*trpA*, *trpB*, *putP*, *icd*, *polB* and *pabB*), as described previously (Le Gall *et al.*, 2007). Data were analysed in two ways. (i) Phylogenetic analysis was performed with the concatenated sequences of the six genes, using the maximum-likelihood method in PHYML (Guindon & Gascuel, 2003) with *E. fergusonii* as the outgroup. (ii) An analysis of allele-assigned data was also carried out, to give the same weight to recombination and mutation events. The different sequences at each locus were treated as alleles, making it possible to define each strain by its allelic profile (sequence type, ST), corresponding to the alleles present at each of the six loci. The allelic profile data were used to construct a minimum-spanning tree with Prim's algorithm, to determine the links between STs (www.pubmlst.org/analysis).

Virulence factor (VF) and O typing. The presence of 18 *E. coli* extraintestinal virulence genes [*sfa/foc*, *iroN*, *aer*, *iha*, *papC*, *papG* (I, II and III alleles), *hlyC*, *cnf1*, *hra*, *sat*, *ireA*, *usp*, *ompT*, *ibeA*, *malX*, *fyuA*, *irp2*, *traT*] was investigated by PCR, with the primers reported in previous studies (Bingen-Bidois *et al.*, 2002; Le Gall *et al.*, 2007). The presence of these genes can be used to infer whether one or more of four *E. coli* pathogenicity islands (PAIs) [PAI I_{CFT073}, PAI II_{j96}, PAI III_{536}, high pathogenicity island (HPI)] and plasmids bearing *aer* and/or *iroN* are present in strains, as reported by Bingen-Bidois *et al.* (2002).

O type was determined with a molecular approach based on allele-specific PCR (Clermont *et al.*, 2007). In addition to the published set of primers, which identify 12 O types frequently found in strains causing bacteraemia, we also designed O-specific reverse primers for determining O11, O86 and O102 type: rfbO11.r (5′-CGCGTCGAACAGCACTTTAC-3′), rfbO86.r (5′-CGTTGTTAATAATTCTGAATGCG-3′) and rfbO102.r (5′-TACCCATGATGGTACTGGTG-3′). With the forward primer gndbis.f (5′-ATACCGACGACGCCGATCTG-3′), these primers gave PCR products of 394, 361 and 550 bp, respectively.

Determination of the TSPE4.C2 region. The TSPE4.C2 fragment is situated within a gene encoding a putative lipase esterase. This gene is flanked by the *yiiD* and *yiiE* genes, encoding a putative transferase and a putative transcriptional regulator, respectively (Gordon *et al.*, 2008). The region around TSPE4.C2 was investigated by PCR walking followed by sequencing with the primers shown in Supplementary Table S1 (available with the online version of this paper). The regions sequenced in D_2 strains were compared with the available genome sequences of strains of phylogenetic group D (042, UMN026 and IAI39), B1 (IAI1 and 55989), B2 (536, APECO1, CFT073 and UTI89), E (Sakai and EDL933) and A (HS and K-12 MG1655). A phylogenetic analysis was carried out with the maximum-likelihood protocol of PHYML (Guindon & Gascuel, 2003) and the sequences of the putative lipase esterase genes of the genotype D_2 strains studied, the phylogenetic group B1 strains (ECOR32, ECOR45, IAI1 and 55989) and the phylogenetic group B2 strains (ECOR56, ECOR66, 536, APECO1, CFT073 and UTI89).

RESULTS

MLST analysis

MLST analysis was performed with 39 *E. coli* strains from phylogenetic group D, including the 18 CTX-M-producing D_2 strains studied, eight strains representative of other *E. coli* phylogenetic groups and *E. fergusonii* as an outgroup. Maximum-likelihood method-based phylogenetic studies on the concatenated sequences of six housekeeping genes showed that phylogenetic group D consists of two major groups of at least nine subgroups, with bootstrap values exceeding 90 % (I–VI for the first major group, and VII–IX for the second major group) (Fig. 1 and Table 1). A population genetics approach was also used for analysis of the MLST data, to avoid possible artefacts due to recombination events. Designation of the different sequences of each of the housekeeping genes as alleles showed that the strains belonged to 41 STs and that the genotype D_2 strains belonged to 32 STs (Supplementary Fig. S1, available with the online version of this paper). Prim's algorithm was used to analyse the links between STs and showed that phylogenetic group D could be divided into the same nine subgroups defined above by the phylogenetic approach (Fig. S1). Thus, genotype D_2 strains were clearly not monophyletic, as they belonged to six subgroups (I, II, III, V, VI and IX), the first five belonging to the first major group and the sixth belonging to the second major group. These six subgroups consisted exclusively of D_2 genotype strains. Subgroups I and IX included both CTX-M-producing and -sensitive strains, whereas the other four subgroups contained only CTX-M-producing strains (Fig. 1).

PFGE analysis

The strains were further analysed by PFGE to exclude epidemiological relationships between strains. All the patterns obtained were distinct (differing by more than 8 bands), displaying less than 80 % similarity. Cluster analysis identified no defined group of strains as producing a particular type of CTX-M. CTX-M-producing D_2 strains were randomly distributed among the non-CTX-M-producing group D strains (data not shown) and no correlation was observed between the MLST subgroups and the various clusters obtained by PFGE. This analysis confirmed the high level of chromosomal polymorphism among CTX-M-producing D_2 strains.

O type determination and virulence factor content

Among the CTX-M-producing D_2 strains, we identified five different O types, and four strains could not be typed by PCR. The O2 type was found in four strains (22 %), O102 in four strains (22 %), O86 in three strains (16 %), O1 in two strains (11 %) and O11 in one strain (5.5 %) (Table 1). By contrast, O2 predominated among the non-CTX-M-producing D_2 strains (eight of nine strains).

PCR was used to detect 18 accessory traits involved in extraintestinal virulence. All but two of the 18 CTX-M-producing strains had different virulence patterns (Table 1). The number of VFs ranged from 1 to 10 (mean VF score 4.4), with *aer*, *fuyA*, *irp2* and *traT* the most frequently detected (72, 72, 72 and 83 %, respectively). Thirteen of the

18 CTX-M-producing strains possessed HPI (*fuyA* and *irp2*), but only one strain, TN21, possessed the classical extra-intestinal PAI I_{CFT073} (*aer*, *iha*, *papC*, *hlyC*). Both *aer* and *iroN* were present in four strains, indicating that *aer* was plasmid-borne in these strains. No correlation was found between the type of CTX-M and the presence of virulence traits.

TSPE4.C2 region characterization

Two types of TSPE4.C2 region were found in genotype D_2 strains (Fig. 2 and Table 1). (i) Type 1 was found in six CTX-M-producing strains and only one non-producing strain. This region was 2397 bp long and included, downstream from *yiiD*, a sequence encoding a putative lipase esterase (of 925 bp) carrying TSPE4.C2, followed by sequences encoding three conserved hypothetical proteins, named CHP3, CHP4 and CHP6, of 312, 219 and 220 bp in length, respectively. This structure is identical to that found in the *E. coli* 536 strain of phylogenetic group B2 and in the *E. coli* IAI1 and 55989 strains of phylogenetic group B1. (ii) Type 2 was more frequent, being found in 12 CTX-M-producing strains and seven non-producing strains. The type 2 region was 5184 bp long and characterized by the presence of an additional sequence encoding two conserved hypothetical proteins, named CHP1 and CHP2, of 993 and 915 bp in length, respectively, between *yiiD* and the putative lipase esterase gene. These genes are identical to those found, at the same position, in the *E. coli* 042, UMN026, and IAI39 strains of phylogenetic group D and in the *E. coli* Sakai and EDL933 strains of phylogenetic group E. CHP1 was also found alone in the *E. coli* HS and K-12 MG1655 strains of phylogenetic group A. In one CTX-M-producing strain, strain 375, an insertion sequence, IS*200*, was found upstream from the CHP1 sequence (Fig. 2). Downstream from the putative lipase esterase gene, the type 1 and 2 sequences displayed several differences. A 274 bp conserved sequence encoding a hypothetical protein, named CHP5, was found to have replaced CHP6 in the type 2 sequence. The structure downstream from the putative lipase esterase gene in the type 2 sequence was similar to that in the *E. coli* APECO1, CFT073 and UTI89 strains of phylogenetic group B2. An association was found between the MLST subgroup and TSPE4.C2 region type. The strains of MLST subgroups VI and IX had type 1 regions, whereas the strains of MLST subgroups I, II, III, V had type 2 regions (Table 1).

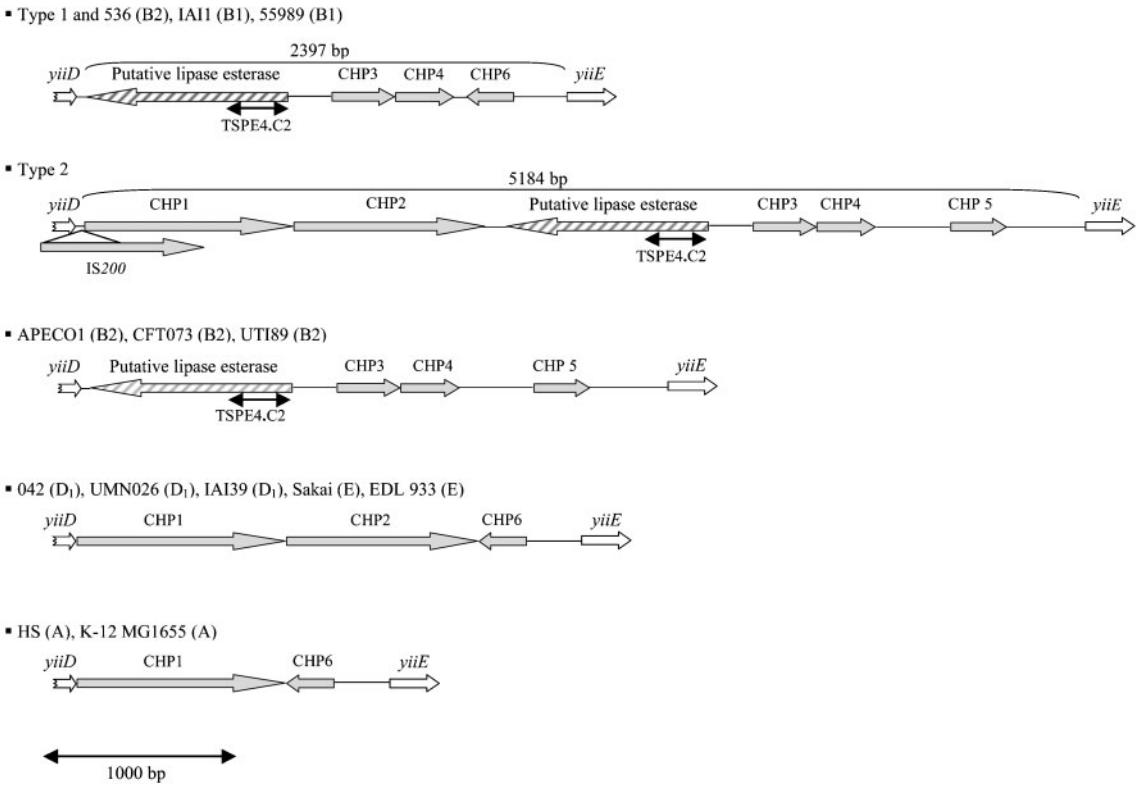

Fig. 2. Genetic organization of the region surrounding the TSPE4.C2 fragment in the D_2 strains studied, and comparison with the organization of available sequences of the same region from *E. coli* strains of genotypes B2 (TSPE4.C2$^+$; 536, APECO1, CFT073 and UTI89), B1 (TSPE4.C2$^+$; IAI1 and 55989), D_1 (TSPE4.C2$^-$; 042, UMN026 and IAI39), E (TSPE4.C2$^-$; Sakai and EDL933) and A (TSPE4.C2$^-$; HS and K-12 MG1655). CHP, Conserved hypothetical protein. The TSPE4.C2 fragment corresponds to the 152 bp PCR product used in the phylotyping method (Clermont *et al.*, 2000).

We investigated the evolutionary history of this region in more detail, by carrying out a phylogenetic analysis of the putative lipase esterase gene. Four major clusters were identified, with high bootstrap values: one for the B2 strains, one for the B1 strains and two for the D_2 strains (Fig. 3). This result was consistent with the phylogenetic history of the species (Gordon *et al.*, 2008). In the D_2 strains, the putative lipase esterase clusters were correlated with the MLST subgroups and the TSPE4.C2 region types. The first cluster included the strains of MLST subgroups I, II, III and V with a type 2 TSPE4.C2 region, whereas the second cluster included the strains of MLST subgroups VI and IX with a type 1 TSPE4.C2 region (Fig. 3). The putative lipase esterase gene of the B2 phylogenetic group strain 536 is separated by a long branch from the remaining B2 strains. This is consistent with the TSPE4.C2 region of strain 536 being a type 1 region, as in the B1 strains, whereas the other B2 strains display a different organization of the TSPE4.C2 region (Fig. 2).

These data suggest that the region bordered by *yiiD* and *yiiE* is a likely hotspot for the insertion/recombination of various protein-encoding sequences of unknown function. The putative lipase esterase gene was probably acquired early in the emergence of the *E. coli* species (this gene is absent from *E. fergusonii*) and subsequently repeatedly lost in various phylogenetic groups/subgroups during the evolution of *E. coli*.

Antibiotic resistance profile

The CTX-M-producing strains had highly heterogeneous co-resistance profiles, displaying resistance to 3–11 of the 12 non-β-lactam antibiotics tested (Table 2). Only the co-resistance profiles of strains 501 and 666 were identical (Table 2). A large proportion of strains were resistant to sulfonamide (83 %), trimethoprim (66 %), tetracycline (66 %) and aminoglycosides (61 %). Fluoroquinolone resistance (ciprofloxacin) was observed in 50 % of the CTX-M-producing strains. Four of the eight non-ESBL-producing D_2 control strains were susceptible to all the antibiotics tested, and four displayed resistance to certain antibiotics, such as ampicillin (3 strains), streptomycin (3 strains), sulfonamide (2 strains), tetracycline (2 strains), minocycline (1 strain) and trimethoprim (1 strain). None of these strains was resistant to quinolones (data not shown).

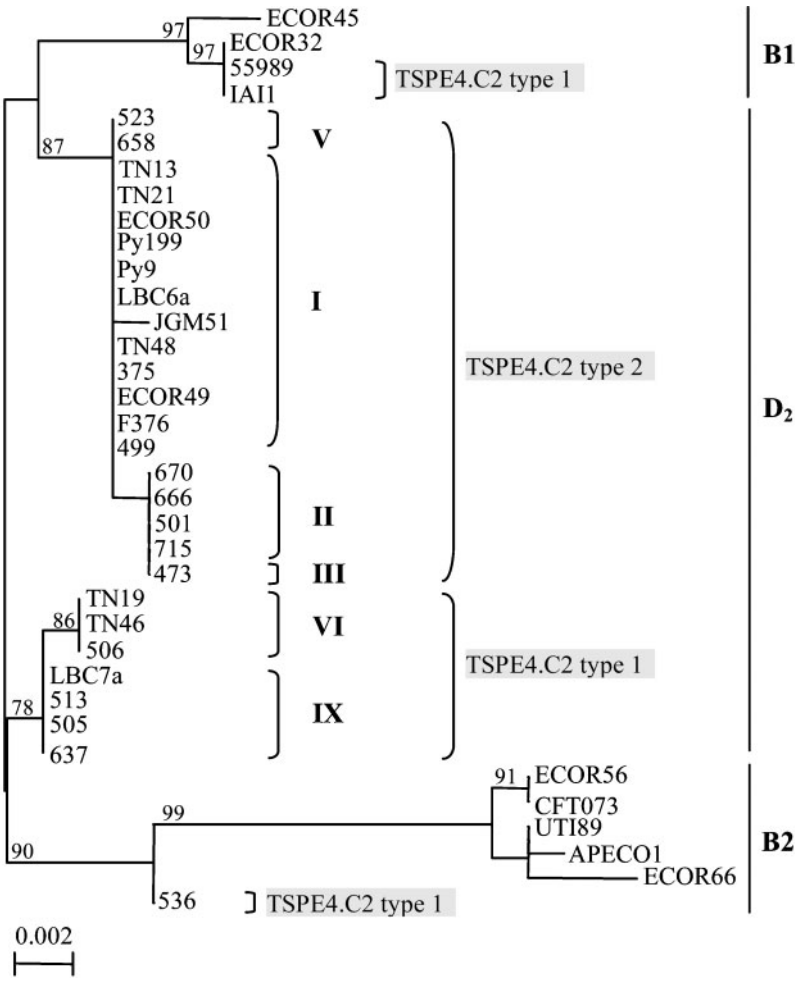

Fig. 3. Unrooted phylogenetic tree of B1 and B2 phylogenetic group strains and of the genotype D_2 strains studied, reconstructed from the DNA sequences of putative lipase esterase genes by using the maximum-likelihood procedure. Bootstrap values exceeding 70 % are indicated above the nodes. Numbers I–IX correspond to the MLST subgroups, as described in Fig. 1 and Table 1.

Table 2. Antibiotic susceptibility and replicon typing of the D_2 *E. coli* strains used in this study, and characteristics of their bla_{CTX-M} plasmids

Strain	CTX-M type	Antibiotic resistance†	bla_{TEM}‡	PCR replicon type§	CTX-M environment‖								Approx. CTX-M plasmid size (kb)¶
					IS*Ecp1* structure					Integron structure			
					bla_{TEM}	IS*Ecp1*	IS*10*	IS*26*	IS*903*	ORF477	*sul1*	IS*CR1*	ORF3	
TN46	1	Te, Ch, Tp, Su		I1-Iy,FIB, FII		+ (XW)				+				90*
523	1	S, Te, Su		N, FIB, FII		+ (XW)		+		+				74
658	1	S, K, Tp, Su	TEM-1	I1-Iy, N, FIB, FII		+ (XW)		+		+				134
637	15	Cp, S, G, T, N, Te, Mn, Ch, Tp, Su	TEM-1	FII	+	+ (W)				+				68
473	15	Cp, S, K, G, T, N, Te, Mn, Ch	TEM-1	FIA, FIB, FII	+	+ (W)				+				116*
505	2	Cp, K, G, T, N, A, Tp, Su	TEM-1	A/C, FII							+	+ (KLU)		113*
TN19	2	S, Te, Tp, Su		HI1, FIB, P, FII							+	+ (KLU)		103*
501	9	S, K, G, T, N, Tp, Su		FIB, FII							+	+ (ZY)	+	161*
666	9	S, K, G, T, N, Tp, Su		FIB, FII							+	+ (ZY)	+	161*
670	9	K, G, T, N, Su		FII							+	+ (ZY)	+	134*
513	14	Cp, S, Ch, Su		A/C		+ (Y)			+					106
375	14	Cp, S, G, T, N, Te, Mn, Tp, Su	TEM-1	FIB, FII, B/O		+ (Y)			+					48*
TN13	14	Cp, Te, Mn, Ch,	TEM-1	I1-Iy, FIB, FII		+ (Y)	+		t					80
TN21	14	Cp, S, Te, Min, Tp, Su	TEM-1	I1-Iy, FIA, FIB, FII		+ (Y)			+					89*
TN48	14	Cp, S, K, G, T, N, A, Te, Ch, Tp, Su	TEM-1	FIB, FII		+ (Y)			+					123*
499	14	Te, Mn, Tp, Su	TEM-1	FIB, FII, K		+ (Y)			t					77
715	14	Cp, G, T, Te, Mn, Ch		FIA, FII		+ (Y)	+		+					60*
506	14	S, K, G, T, Te, Mn, Ch, Tp, Su		FIA, FIB, FII		+ (Y)			t					64*

†Antibiotic resistance profiles corresponding to the original strain; antibiotic resistance patterns of transconjugants or transformants are underlined. Cp, Ciprofloxacin; S, streptomycin; K, kanamycin; G, gentamicin; T, tobramycin; N, netilmicin; A, amikacin; Te, tetracycline; Mn, minocycline; Ch, chloramphenicol; Tp, trimethoprim; Su, sulfonamide.

‡bla_{TEM} detected in the original strain; bla_{TEM} transferred into transconjugants or transformants is underlined.

§PCR replicon types corresponding to the original strain; PCR replicon types of transconjugants or transformants are underlined.

‖CTX-M environment according to Eckert *et al.* (2006): XW (80 bp), W (48 bp) and Y (42 bp) are the sizes of the sequences between bla_{CTX-M} and IS*Ecp1*; KLU (266 bp) and ZY (94 bp) are the sizes of the sequences between IS*CR1* and bla_{CTX-M} (Supplementary Fig. S2). +, Presence; t, truncated IS.

¶An asterisk indicates that plasmids were transferred by electroporation, otherwise plasmids were transferred by conjugation.

The antibiotic resistance profiles of the transconjugants (6 strains) or transformants (12 strains) showed that aminoglycoside, tetracycline, sulfonamide, trimethoprim and/or chloramphenicol resistances were transferred along with bla_{CTX-M} in 11 of the 18 recipient strains (Table 2). Between 2 and 10 resistance traits were transferred, conferring highly diverse antibiotic resistance profiles on the recipient strains. Resistances to sulfonamide and aminoglycosides were the most frequently transferred (Table 2). Quinolone resistance was not transferred in any case. bla_{TEM-1} was detected in nine strains (50 %), and cotransferred with bla_{CTX-M} in five strains. All attempts to amplify SHV genes by PCR were negative.

Relationship between virulence and resistance

The CTX-M-producing strains had fewer VFs than the eight non-CTX-M-producing D_2 control strains (mean VF score of 4.4 versus 10.62, $P<0.001$). Unlike the CTX-M-producing strains, the non-ESBL-producing strains possessed PAIs: all had HPI, and seven of the eight possessed PAI I_{CFT073} (Table 1). Among the CTX-M-producing strains, ciprofloxacin-resistant strains (50 % of the strains) and ciprofloxacin-sensitive strains had similar numbers of VFs (mean VF score of 4.7 versus 4.2, P=not significant). Strains carrying CTX-M plasmids without transferable co-resistance had more VFs than strains carrying CTX-M plasmids with transferable co-resistances, regardless of any other types of antibiotic resistance in the strains concerned (mean VF score of 5.8 versus 3.6, $P=0.012$). The number of co-resistances carried by CTX-M plasmids was inversely proportional to the number of virulence traits detected in the strains (correlation coefficient −0.47, $P=0.047$; Fig. 4). A Mantel test was used for inferring this association. The test was performed with statistical software XLSTAT (Addinsoft) and showed significant association (Pearson's correlation $r=0.161$, $P=0.046$).

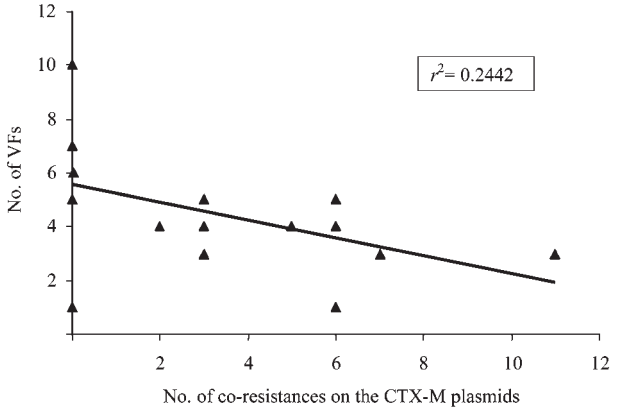

Fig. 4. Relationships between the number of VFs found in the D_2 genotype CTX-M-producing strains and the number of antibiotic co-resistances carried by the CTX-M plasmids. Triangles represent strains; r^2, coefficient of determination.

Total plasmid content analysis

PCR-based replicon typing of the total plasmid content of the parental strains showed that most strains contained an IncF-type plasmid (16 of 18 strains; Table 2), consistent with other reports (Johnson *et al.*, 2007; Sherley *et al.*, 2004). IncF plasmids are widespread in *E. coli* strains and seem to be well adapted to this species (Boyd *et al.*, 1996). All but three of the strains contained multiple replicons (mean 2.5, range 1–4). One of the three exceptions contained an IncA/C replicon and the other two contained an FII replicon.

CTX-M plasmid backbone analysis

All but one of the tranformants/conjugants were also typed by the PCR-based replicon typing method. The plasmids encoding the CTX-M enzymes were of various replicon types (Table 2). Nine strains were of the IncF type (five were FII, three were FIB-FII and one was FIA-FIB-FII). The other eight strains were of the IncN, IncA/C and IncI1-Iy (two strains each) and IncP and IncK (one strain each) types. Restriction analysis was carried out to investigate the relationship between CTX-M plasmids of the same Inc type. The *Eco*RI restriction patterns obtained were all different and independent of Inc type (Fig. 5a). Similar results were obtained with *Hpa*I (data not shown). However, among the IncF plasmids, two plasmids carrying $bla_{CTX-M-14}$ (strains 715 and TN13; lanes 7 and 8 of Fig. 5a) and three plasmids carrying $bla_{CTX-M-9}$ (strains 670, 666, 501; lanes 9, 10, 11 of Fig. 5a) had related restriction patterns, indicating the existence of a common plasmid backbone in each group of strains (backbone A and backbone B, respectively). Analysis of the sequences of the transfer genes *finO*, *traD* and *traY* of the IncF-type plasmids showed a correlation with backbone type (data not shown). Southern blot hybridization with the bla_{CTX-M} probe showed that bla_{CTX-M} was present on *Eco*RI fragments of various sizes between 3.5 and 23 kb (Fig. 5b), confirming the presence of the cefotaxime resistance gene on the plasmids. In backbone A plasmids, bla_{CTX-M} was present on *Eco*RI fragments of about 18 kb, whereas in backbone B plasmids, it was present on *Eco*RI fragments of 23 kb. These results highlight the relationship between plasmids with the same backbone. However, the overall results of the plasmid analysis, for both the parental and recipient strains, indicated a high level of polymorphism in these plasmids.

Exploration of the regions surrounding bla_{CTX-M} genes

We explored the sequences flanking the bla_{CTX-M} gene with a view to identifying genetic structures able to mobilize the ESBL gene in genotype D_2 strains. PCR analysis and sequencing of the surrounding regions showed, as expected, that each type of bla_{CTX-M} was associated with a specific region, as reported by Eckert *et al.* (2006). The

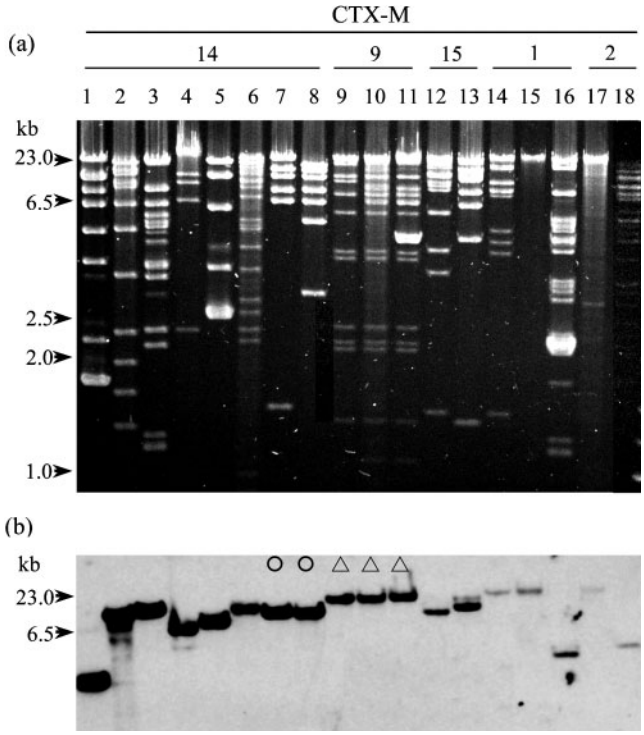

Fig. 5. *Eco*RI restriction patterns (a) and Southern blot hybridization with the bla_{CTX-M} probe (b) of plasmid DNA isolated from *E. coli* transconjugants (T) or electroporated cells (E). Inc groups are shown in parentheses. DNA markers [*Hin*dIII-digested λ DNA (Biolabs) and Euroladder (Eurobio)] — were used to assess the lengths of the DNA fragments. Lanes: 1, E 506 (F); 2, T 499 (K); 3, E TN21 (I1-Iy); 4, T 513 (A/C); 5, E 375 (unknown); 6, E TN48 (F); 7, E 715 (F); 8, T TN13 (F); 9, E 670 (F); 10, E 666 (F); 11, E 501 (F); 12, E 473 (F); 13, T 637 (F); 14, E 658 (N); 15, T 523 (N); 16, E TN46 (I1-Iy); 17, E 505 (A/C); 18, E TN19 (P). ○, Backbone A; △, backbone B.

insertion sequence IS*Ecp1* was identified 42–80 bp upstream from the $bla_{CTX-M-1}$, $bla_{CTX-M-14}$ and $bla_{CTX-M-15}$ genes (Table 2; Supplementary Fig. S2, available with the online version of this paper). Two CTX-M-1-producing strains contained IS*26* upstream from IS*Ecp1*, which was in this case truncated, and two CTX-M-14-producing strains contained IS*10* inserted into IS*Ecp1*. We detected bla_{TEM-1} upstream from IS*Ecp1* in the two $bla_{CTX-M-15}$-producing strains, as described by Eckert *et al.* (2006). IS*903* was found intact in five strains and truncated in three strains, downstream from $bla_{CTX-M-14}$ genes, and ORF477 was found downstream from $bla_{CTX-M-1}$ and $bla_{CTX-M-15}$. As expected, strains producing CTX-M-9 and CTX-M-2 enzymes presented a region corresponding to a class 1 integron complex, which includes IS*CR1* and *sul1*, upstream from $bla_{CTX-M-9}$ and $bla_{CTX-M-2}$ genes at various distances, and ORF3 downstream from $bla_{CTX-M-9}$ and $bla_{CTX-M-2}$ genes (Table 2; Fig. S2). Overall, analysis of the DNA sequence surrounding the bla_{CTX-M} genes showed considerable polymorphism between strains as a function of CTX-M type, as previously reported (Eckert *et al.*, 2006), independent of the genetic background of the plasmid or strain.

DISCUSSION

CTX-M-producing isolates have spread over the last decade and most are *E. coli* (Paterson & Bonomo, 2005). A previous study investigating the potential relationship of a specific *E. coli* phylogenetic group with a specific ESBL type highlighted an association between CTX-M enzyme production and the D_2 genotype (Branger *et al.*, 2005). We studied this interplay further in terms of bacterial clonality, resistance genes and genetic structures promoting the dissemination of the CTX-M.

D_2 genotype strains are not monophyletic and have a highly heterogeneous chromosomal background

MLST provides a fundamental vision of the population of a species based on polymorphism within conserved housekeeping genes and has the potential to reveal relationships even between isolates found to be different by other molecular methods, including PFGE. MLST (Fig. 1 and Fig. S1) showed that the CTX-M-producing strains of genotype D_2 studied were not monophyletic, belonging instead to several distinct subgroups not specifically related to the production of ESBL, as non-producing strains were also found clustered in these subgroups. The complexity of the PFGE patterns provided evidence of rearrangement events and confirmed a lack of epidemiological relationship between the strains. The different O types and the polymorphism of the virulence profiles were consistent with the evolution of the strains within the subgroups. Thus, each subgroup consisted of strains that had diverged since their origin.

The region encompassing the genetic marker of the D_2 genotype (the TSPE4.C2 fragment) is not correlated with the presence of CTX-M genes

The D_2 genotype, which is extremely rare in natural *E. coli* populations (Gordon *et al.*, 2008), differs from the D_1 genotype in possessing the TSPE4.C2 fragment. As we observed an association of this genotype with the presence of CTX-M among ESBL-producing strains, we hypothesized that a specific region encompassing the TSPE4.C2 fragment might be associated with the CTX-M genes. If this was the case, this region would include genetic elements for the capture and maintenance of CTX-M genes. Alternatively, this fragment might be present on the CTX-M plasmid. However, our experiments did not confirm either of these hypotheses. Using PCR on recipient strains, we checked that the TSPE4.C2 fragment was not transferred (data not shown). Considerable polymorphism of the region encompassing TSPE4.C2 was observed but

was not correlated with the presence of CTX-M: none of the genes found around TSPE4.C2 in the CTX-M-producing D_2 strains was specific to this genotype (Fig. 2 and Table 1). This analysis also provided no additional insight into this question, as all the surrounding genes were of unknown function.

There is no relationship between *bla*$_{CTX-M}$ type, the genetic background of the CTX-M-encoding plasmids and the chromosomal genetic background of the strains

Replicon typing and restriction patterns provided strong evidence that, with the exception of the three *bla*$_{CTX-M-9}$ and two *bla*$_{CTX-M-14}$ mobilized on closely related IncF plasmids (backbone A and backbone B, respectively), all the other *bla*$_{CTX-M}$ were mobilized on various large plasmids of narrow (F, I1-Iy) or broad (A/C, N, P) host range. The CTX-M plasmids were diverse, with a wide range of resistance profiles and no discernible evolutionary lineages. The three plasmids carrying *bla*$_{CTX-M-9}$ (backbone B) were found in strains with certain genetic characteristics in common. All were of the MLST D subgroup II, with a type 2 TSPE4.C2 region and O86 (type found only for these strains in this study), but differences in PFGE patterns and virulence profiles showed that these strains were epidemiologically unrelated. The two plasmids carrying *bla*$_{CTX-M-14}$ (backbone A) were found in strains with different genetic backgrounds: MLST D subgroups I and II. In all other strains, we found no association between the genetic background of the strains, represented by the MLST type, the CTX-M plasmid type or the CTX-M type. There is therefore no co-evolution of the genetic background of the strain with the plasmid bearing the CTX-M genes.

The virulence/resistance trade-off

CTX-M-producing D_2 strains had far fewer virulence factors than non-producing strains. A trade-off between virulence and resistance in *E. coli* has been reported on a number of occasions. Several studies have demonstrated that isolates of *E. coli* resistant to quinolones or fluoroquinolones have fewer virulence factors than susceptible isolates, and seem to have a reduced invasive capacity (Velasco *et al.*, 2001; Vila *et al.*, 2002) and a different phylogenetic background (Johnson *et al.*, 2002). A lack of P fimbriae and haemolysin has also been associated with resistance to other traditional antibiotics, such as ampicillin, tetracycline, sulfonamide and kanamycin (Johnson *et al.*, 1991). We analysed the number and distribution of VFs in CTX-M-producing strains with respect to ciprofloxacin resistance, and observed no difference. Thus, the small number of virulence factors in the CTX-M-producing strains could not be linked to resistance to ciprofloxacin, but rather to the presence of *bla*$_{CTX-M}$ and the other co-resistance markers (Fig. 4). A link was shown between the number of co-resistances associated with *bla*$_{CTX-M}$ and the lack of VFs found in the strain, as if there was antinomy between the acquisition of a CTX-M plasmid with co-resistance and the presence of a genetic background with PAIs. The small number of VFs in the CTX-M-producing strains may be due to the loss of some 'unstable' VFs, such as PAI I_{CFT073}, *ompT* or *sat*, following the acquisition of *bla*$_{CTX-M}$ and/or associated co-resistance markers. The partial or total loss of PAIs, induced by subinhibitory concentrations of quinolones, has been achieved *in vitro*, by SOS-dependent or -independent pathways, in uropathogenic *E. coli* (Soto *et al.*, 2006). Alternatively, CTX-M-encoding plasmids and the associated co-resistance markers may have been preferentially acquired by strains with smaller numbers of extraintestinal virulence genes (Johnson *et al.*, 2005).

Evolutionary implications

We found an association between a rare specific genetic background, the D_2 genotype, exhibiting few VFs, and a plasmid-borne mobile element, the CTX-M gene (Branger *et al.*, 2005). There are two possible explanations for these findings. First, a CTX-M gene may have been acquired, by chance, by a D_2 genotype strain that subsequently diversified and spread. Second, multiple CTX-M gene acquisitions may have occurred in *E. coli* strains. Our data clearly suggest that multiple acquisitions have occurred. The diversity of the *E. coli* strains of D_2 genotype and that of the plasmids bearing the CTX-M gene and CTX-M types are consistent with multiple acquisitions of plasmids carrying CTX-M genes in *E. coli* strains. These findings strongly support a model of convergent evolution with selection for particular associations of genetic background of the strain and the CTX-M gene. This fine-tuning of the D_2 genotype and CTX-M genes presumably increases the fitness of the strain, indicating a role for the host cell in the acquisition and dissemination of CTX-M genes. The molecular mechanisms involved in these epistatic interactions are currently unknown. Epistatic interactions have already been reported in *E. coli* between the genetic background of the strain and its VFs (Escobar-Paramo *et al.*, 2004a) and global regulators, such as CRP (Cooper *et al.*, 2008).

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