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The CTX-M-15-producing *Escherichia coli* diffusing clone belongs to a highly virulent B2 phylogenetic subgroup

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***Objectives*: A clone of CTX-M-15-producing *Escherichia coli* has recently been reported to be spreading through Europe and Africa. The aim of this work was to thoroughly characterize this clone.**

***Materials and methods*: Representative isolates of this clone were subjected to multilocus sequence typing, O typing, virulence gene detection, adhesion assay on human cells, biofilm production assay and mouse lethality assay.**

***Results*: The clone: (i) belongs to a unique B2 phylogenetic subgroup encompassing the pyelonephritogenic diffusely adhering EC7372 strain; (ii) exhibits a specific O25b molecular subtype; (iii) is identical to the *E. coli* clone O25:H4-ST131 producing CTX-M-15; (iv) produces biofilm; and (v) is highly virulent in mice despite lacking classical extraintestinal pathogenicity islands (except for high pathogenicity island) and the *afa/dra* gene.**

***Conclusions*: The CTX-M-15-producing *E. coli* diffusing clone is associated with a high level of antibiotic resistance and with high virulence, showing that, under certain selective pressures, the previously observed trade-off between resistance and virulence may not apply.**

Keywords: *E. coli*, ESBLs, virulence, clonal spread, B2 phylogenetic group

Introduction

Phylogenetic analyses show that *Escherichia coli* strains fall into four main phylogenetic groups (A, B1, B2 and D).[1] Although virulence determinants are considered to be mobile, a link between strain phylogeny and virulence has been reported.[1] Virulent extraintestinal strains belong mainly to B2 group and, to a lesser extent, D group, whereas most commensal strains belong to A and B1 groups.[1] The prevalence of antimicrobial resistance is lower among strains belonging to B2 phylogenetic group, suggesting a trade-off between resistance and virulence.[2]

In recent years, the epidemiology of extended-spectrum β-lactamases (ESBLs) in *E. coli* has changed radically: (i) novel ESBLs such as CTX-M enzymes have replaced classical TEM and SHV-type ESBLs in many countries[3] and (ii) infections by these ESBL-producing *E. coli* are described both in hospitals and in the community.[3] CTX-M-15 β-lactamase, which belongs to the CTX-M-1 group, was first described in 2001, but is now found worldwide in *E. coli* strains, notably during outbreaks in Canada, France, the UK, Spain and Tunisia.[3] We showed the spread of a CTX-M-15 β-lactamase-producing *E. coli* clone in Paris, Tunis and Bangui.[4]

To better understand the causes of the evolutionary success of the CTX-M-15 β-lactamase-producing *E. coli* diffusing clone, we reconstructed its phylogenetic history and characterized its virulence *in vitro* and *in vivo*.

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Materials and methods

E. coli strains

Four representative isolates of the multiresistant CTX-M-15-producing *E. coli* diffusing clone were selected from the panel used in our previous work (Table 1).[4] Two were recovered from Tenon hospital in Paris (TN03 and TN34), and the other two were isolated in Tunis, Tunisia (strain TU) and in Bangui, Central African Republic (strain CAF).[4] One isolate from France [TNN (TE2)] of the recently published CTX-M-15 clone belonging to the ST131 using the Achtman schema was also used for comparison.[5] For the multilocus sequence typing (MLST) analysis, 21 B2 and 1 D strains as well as *Escherichia fergusonii* were used.[6] *In vitro* and *in vivo* experiments were performed on a subset of the above-cited strains. In addition, the TN03 transformant in *E. coli* DH10B (EpTN03) producing the CTX-M-15 enzyme was used as a control in the biofilm experiment.

Phylogenetic grouping and MLST

The phylogenetic group of the *E. coli* isolates was determined with the multiplex PCR-based method.[7] MLST was achieved by analysis of the concatenated sequences (5901 bp) of six essential genes (*trpA*, *trpB*, *pabB*, *putP*, *icd* and *polB*), with *E. fergusonii* as the outgroup, and processed by the maximum likelihood method in the PHYML program.[7]

Virulence factors

The isolates were screened for 21 genes encoding putative virulence factors [*neuC* (K1 antigen), *sfa/foc*, *iroN*, *iutA*, *iha*, *papC*, *papG* (I, II, and III alleles), *hlyC*, *cnf1*, *hra*, *sat*, *ire*, *usp*, *ompT*, *ibeA*, *malX*, *fyuA*, *irp2*, *traT*, *afa/dra* and *ftr1*] by PCR with primers as previously described.[7] Primers for *ftr1*, ftr1up (5′-GATCAGCGC CACAATTAC-3′) and ftr1low (5′-CAGTGGAGTCTGCTGCTC-3′), were used at an annealing temperature of 55°C to generate a PCR product of 153 bp.

O typing

O typing was performed with the traditional antiserum method at the State Serum Institute, Copenhagen, Denmark. The O types were also determined with a recently described molecular approach based on allele-specific PCR.[8] For the newly described O25b O type, primers rfb.1bis (5′-ATACCGACGACGCCGATCTG-3′) and rfbO25b.r (5′-TGCTATTCATTATGCGCAGC-3′) were used at an annealing temperature of 60°C to generate a PCR product of 300 bp. To define the rfbO25b.r primer, the entire *rfb* cluster was amplified and the 5′ extremity was sequenced as previously described in strains ECOR15, EC7372 and TN03.[8]

Human intestinal cell adhesion assays

Adhesion to Int 407 cells, derived from a human embryonic jejunum and ileum, was assayed as previously described.[9] Three independent adhesion assays were used for each strain.

Biofilm assay

Biofilm formation was examined twice in 60 mL aerated microfermentors, as previously described.[10]

Mouse lethality assay

A mouse model of systemic infection was used to assess the intrinsic virulence of the strains.[1] For each strain, 10 outbred female Swiss OF1 mice (3–4 weeks old, 14–18 g) received a subcutaneous abdominal injection of 10^9 cfu/mL of log-phase bacteria. Mortality was monitored for 7 days.[1]

Animals were maintained and handled according to the guidelines of the French Ministry of Agriculture (approval A 75-18-05).

Results and discussion

The four CTX-M-15 isolates (TN03, TN34, TU and CAF) belong to phylogenetic group B2 (Table 1) and are grouped together by MLST with the pyelonephritogenic diffusely adhering EC7372 reference strain,[6] with a high bootstrap value (Figure 1). These five strains are closely related to the diarrhoeic diffusely adhering DAEC11 and DAEC18 strains,[6] and, to a lesser extent, to the ECOR66 strain (Figure 1). All these strains belong to the B2 phylogenetic subgroup I, which is the more basal (anciently diverged) strain in the B2 phylogenetic group.[7]

All four CTX-M-15 strains were O type O25 in the antiserum technique, but no PCR product was obtained with the O25-specific primer (rfbO25a) used in the recently published allele-specific method.[8] In contrast, two control group B2 strains (ECOR51 and 52) belonging to subgroup II were successfully typed as O25 (O25a) by both approaches (Figure 1). Sequencing of the 5′ extremity of the *rfb* cluster yielded an unknown sequence that we have already observed in strain ECOR15 (phylogenetic group A).[8] We therefore designed a primer specific for this sequence (rfbO25b.r) and confirmed that the four CTX-M-15 isolates, EC7372, DAEC11, DAEC18 and ECOR15 strains, belong to this distinct O25 type (O25b) (Figure 1 and Table 1).

The four clonal isolates did not possess the classical extraintestinal PAIs (PAI I_{CFT073}, PAI II_{J96} and PAI III_{536}),[7] except for the high pathogenicity island (HPI). They also lacked the *afa/dra* gene responsible for the diffusely adhering phenotype (Table 1). The four isolates had the same virulence pattern, except that isolate TN34 harboured *iroN* and isolate TU did not harbour *ftr1*. As the absence of *sfa/foc* in isolate TN34 indicates that *iroN* is plasmid-borne (Table 1), the differences in the virulence gene patterns between the clonal isolates are all due to plasmids.

The MLST analysis with our schema (Figure 1), the PCR-based O typing and virulence genotyping (Table 1) of strain TNN (TE2), a French isolate of the CTX-M-15 O25:H4-ST131 clone, show that our four isolates from France, Tunisia and Central African Republic and those spreading in France, Switzerland, Spain, Portugal, Lebanon, Korea and Canada belong to the same clone.[5] Also, according to the serotype and/or the virulence genotype and/or the phylogenetic group, this worldwide diffusing clone is probably identical to those previously described in the UK, Madrid and Rome.[3,11,12]

As the TN03 strain is closely related to the diffusely adhering DAEC11 and DAEC18 strains, we examined the adhesion of these strains, and the ECOR66 strain, to intestinal cells. Similar to the ECOR66 strain, TN03 was less adherent than the DAEC11 and DAEC18 strains (Table 1). No specific pattern of adherence was observed with strains TN03 and ECOR66,

Table 1. Principal characteristics of the *E. coli* strains studied

Strain	Origin	Phylogenetic group	Serotype PCR	Serotype CM[a]	Virulence factors *neuC* (K1)	*sfa/foc*	*iroN*	*iutA*	*iha*	*papC*	*papG*	*hlyC*	*cnf1*	*hra*	*sat*	*ire*	*usp*	*ompT*	*ibeA*	*malX*	*fyuA*	*irp2*	*traT*	*afa/dra*	*ftr1*	Adherence to Int 407 cell line[b]	Production of biofilm[c]	Mouse lethality assay[d]
TN03	urine	B2	O25b	O25	−	−	−	+	+	−	−	−	−	−	+	−	+	+	−	+	+	+	+	−	+	0.28	1.76	10
TN34	urine	B2	O25b	O25	−	−	+	+	+	−	−	−	−	−	+	−	+	+	−	+	+	+	+	−	+	ND	2.98	10
TU	blood	B2	O25b	O25	−	−	−	+	+	−	−	−	−	−	+	−	+	+	−	+	+	+	+	−	−	ND	ND	10
CAF	urine	B2	O25b	O25	−	−	−	+	+	−	−	−	−	−	+	−	+	+	−	+	+	+	+	−	+	ND	ND	10
EpTN03		A	ND	ND	ND	ND	−	−	−	ND	ND	ND	ND	ND	−	ND	−	−	ND	−	−	−	−	ND	+	ND	<0.05	ND
TNN (TE2)	urine	B2	O25b	O25	−	−	−	+	+	−	−	−	−	−	+	−	+	+	−	+	+	+	+	−	+	ND	ND	ND
EC7372	urine	B2	O25b	O25	−	−	−	+	+	+	II	+	−	−	+	−	+	+	+	+	+	+	+	+	+	ND	ND	10
DAEC11	faeces	B2	O25b	O25	−	−	−	+	+	−	−	−	−	−	+	−	+	+	+	+	+	+	+	+	+	0.75	ND	10
DAEC18	faeces	B2	O25b	O25	−	−	−	+	+	−	−	−	−	−	+	−	+	+	+	+	+	+	+	+	+	0.96	ND	10
ECOR66	faeces	B2	O4	O4	+	+	+	−	−	+	III	−	−	+	−	−	−	+	+	+	+	+	−	−	+	0.21	ND	10

ND, not done.

[a]CM, serotyping by conventional method.

[b]The number of adherent cfu per monolayer is expressed as a percentage of the number of cfu in the inoculum. Three independent adhesion assays were used for each strain.

[c]Mature biofilms that had formed after 48 h of incubation on the removable glass slide were resuspended in 5 mL of M63B1 minimal medium; absorbance was read at 620 nm.

[d]Expressed as the number of killed mice of the 10 inoculated. Data of EC7372 and ECOR66 strains are from Le Gall *et al.*[7]

PAI I_{CFT073} PAI II_{J96} PAI III_{536} PAI IV_{536} (HPI).

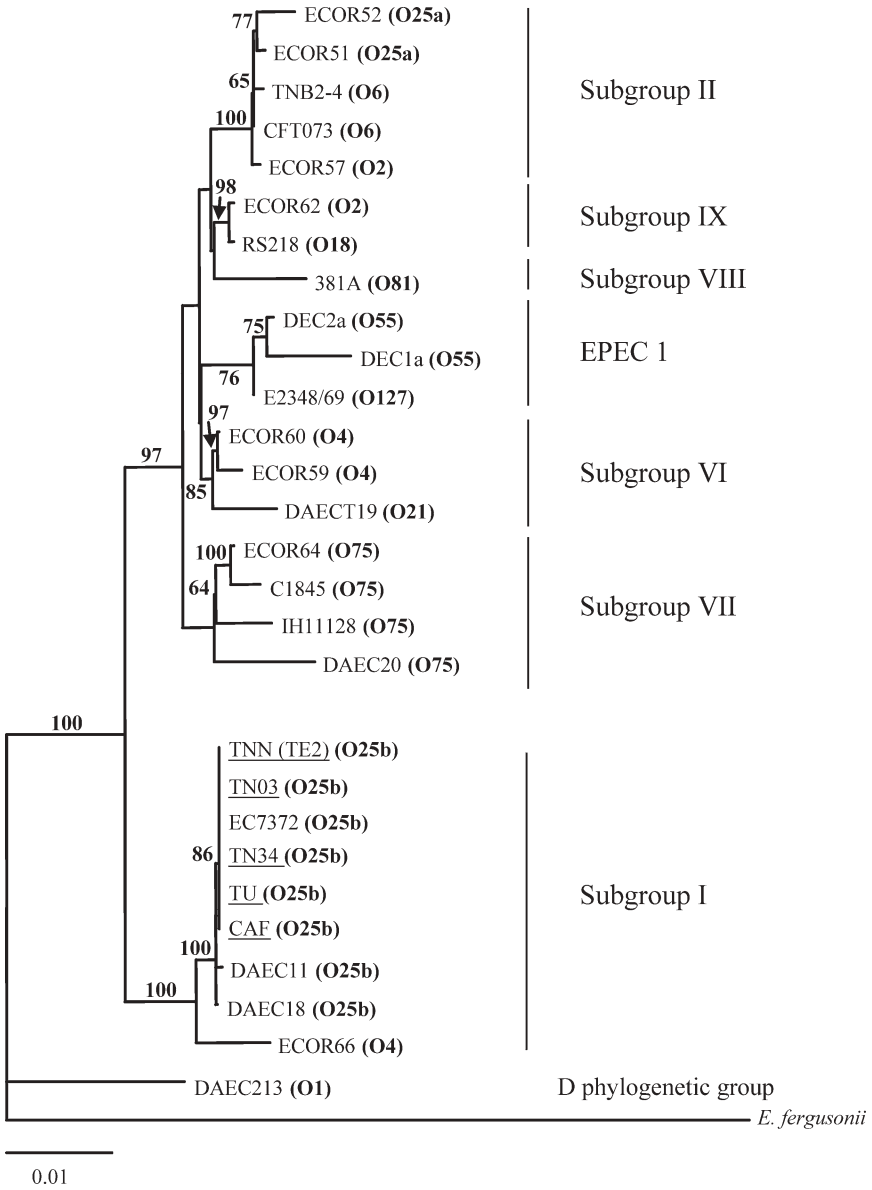

Figure 1. Phylogenetic tree of the B2 and D phylogenetic group strains, reconstructed from the DNA sequences of six essential chromosomal genes (*trpA*, *trpB*, *pabB*, *putP*, *icd* and *polB*) using the maximum likelihood procedure. *E. fergusonii* served as an outgroup. Bootstrap values higher than 50% are indicated above the nodes. Identical topologies were obtained with the neighbour joining and parsimony procedures (data not shown). ESBL producers are underlined. The other strains represent the genetic diversity of the B2 phylogenetic group and are described by Escobar-Páramo *et al.*[6] The O type of the strains is indicated in bold and in parentheses. B2 subgroup numbering is as described by Le Gall *et al.*[7] EPEC1, enteropathogenic *E. coli* group 1.

whereas the DAEC strains showed typical diffuse adherence (data not shown), in keeping with their lack of *afa/dra* (Table 1).

We then examined whether TN03 and TN34 and the transformant EpTN03 could produce a biofilm on a glass support. TN03 and TN34 produced biofilm after 48 h, whereas EpTN03 produced none, showing that this property is not harboured by the plasmid encoding the $bla_{\text{CTX-M-15}}$ gene; see Table 1 and Figure S1 [available as Supplementary data at *JAC* Online (http://jac.oxfordjournals.org/)].

We also tested the four isolates of the CTX-M-15 clone in a mouse model of extraintestinal virulence.[1] Despite the apparently small number of virulence genes, all CTX-M-15 isolates killed all 10 of the 10 inoculated mice, as the pyelonephritogenic antibiotic-susceptible EC7372 strain and ECOR66 strain exhibit several PAIs (Table 1). Interestingly, DAEC11 and DAEC18 also killed all 10 mice, indicating a high extraintestinal potential virulence within strains of subgroup I.

Why is the CTX-M-15-producing *E. coli* clone so successful? Usually, B2 phylogenetic group *E. coli* clinical isolates that do not produce ESBL belong rarely (~2%) to O subgroup O25b (Clermont and Denamur, personal data). In a recent study, O25 isolates were responsible for 38% of the upper urinary tract infection (UTI) in renal transplant patients and were associated with poor prognosis, whereas O25 strains represented $<2\%$ of the isolates from immunocompetent patients with upper UTI.[13] These O25 strains possess P fimbriae *papG* class II and Dr

adhesin, suggesting that they are similar to the EC7372 strain (Table 1). The authors suggested that a unique pattern of O type and adherence factors contributed to acute allograft injury in these renal transplant patients with UTI.[13] Indeed, the EC7372 strain is highly virulent in the mouse model.[7] Surprisingly, although closely related to strain EC7372, our clone did not produce these particular adhesion factors and did not adhere very avidly to epithelial intestinal cells (Table 1). The CTX-M-15 clone isolates produced biofilm: a property not supported by the CTX-M plasmid. This biofilm production could contribute to the long-term persistence of this clone in various environments and to its resistance to antimicrobial agents or disinfectants.[10] Biofilms also confer increased resistance to host immune defences. Anderson *et al.*[14] described the formation, in bladder cells, of intracellular bacterial communities that matured in biofilm-like structures (pods). Recently, it was reported that *E. coli* strains causing recurrent UTI were more likely to produce biofilms and to synthesize yersiniabactin (*fyu*) and aerobactin (*aer/iutA*), like our clone.[15] The CTX-M-15 clone isolates were also highly virulent in the mouse model of extraintestinal virulence, even though they lack several classical virulence genes. This indicates that these isolates possess unidentified virulence genes and that these genes could have a role in clonal spread.

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Transparency declarations

None to declare.

Supplementary data

Figure S1 is available as Supplementary data at *JAC* Online (http://jac.oxfordjournals.org/).

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