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ORIGINAL ARTICLE

Interplay between IncF plasmids and topoisomerase mutations conferring quinolone resistance in the *Escherichia coli* ST131 clone: stability and resistance evolution

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Abstract

The *Escherichia coli* ST131 *H30*-Rx subclone vehicles CTX-M-15 plasmids and mutations in *gyrA* and *parC* conferring multidrug resistance successfully in the clinical setting. The aim of this study was (1) to investigate the relationship of specific topoisomerase mutations on the stability of IncF (CTX-M producing) plasmids using isogenic *E. coli* mutants and (2) to investigate the impact of the IncF-type plasmids present in the *E. coli* clone ST131 on the evolution of quinolone resistance. *E. coli* ATCC 25922 (background strain) and derived mutants encoding specific QRDR substitutions were used. Also, NGS-characterized IncFIA and IncFIB plasmids (encoding CTX-M genes) were included. Plasmid stability was evaluated by sequential dilutions into Luria broth medium without antibiotics for 7 days. Mutant frequency to ciprofloxacin was also evaluated. Moderate differences in the IncF plasmids stability were observed among *E. coli* ATCC 25922 and isogenic mutants. Under our experimental conditions, the fluctuation of bacteria harboring plasmids was less than 0.5-$\log_{(10)}$ in all cases. In the mutant frequency tests, it was observed that the presence of these IncF plasmids increased this value significantly (10–1000-fold). Quinolone resistance substitutions in *gyrA* or *parC* genes, frequently found associated with *E. coli* clone ST131, do not modify the stability of ST131-associated IncFIA and IncFIB plasmids under *in vitro* conditions. IncF-type plasmids present in *E. coli* clone ST131 facilitate the selection of resistance to quinolones. These results are consistent with the clinical scenario in which the combination of resistance to quinolones and beta-lactams is highly frequent in the *E. coli* clone ST131.

Keywords *E. coli* ST131 clone · Quinolones · Beta-lactams · Resistance · IncF plasmids

Introduction

Extra-intestinal pathogenic *E. coli* (ExPEC) is a major human pathogen, the most common cause of urinary tract infections and the most common gram-negative bacterium associated with bloodstream infections in both developed and developing countries [1]. WHO reports state that FQ-resistance (FQ-R) among ExPEC is very widespread across the globe. A single clone which belongs to the O25b-B2 subgroup, ST131 clone, is predominantly responsible for this global multidrug-resistant (MDR) ExPEC pandemic [2].

Population genetics has indicated that ST131 consists of different lineages or clades, e.g., clade A associated mostly with *fimH*41 fimbria, clade B mostly associated with *fimH*22, and clade C mostly associated with *fimH*30 [3]. Global longitudinal studies (going back as far as the 1960s) showed that clade B was presiding among ST131 before the 1990s,

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but since the 2000s, clade C became the most dominant lineage (currently up to 80% of global ST131 belongs to clade C) [2]. Next-generation sequencing (NGS) identified two sub-clades within clade C named C1/H30R (associated with FQ-R) and C2/H30-Rx (associated with the ESBL CTX-M-15), both sub-clades with extensive global distribution [3]. Substitutions such as S83L, D87N, and D87G into GyrA and S80R, S80I, and E84V into ParC have been frequently associated with FQ-R in C1/H30R and C2/H30-Rx subclades. Phylogenetic studies have shed some light regarding the origin and evolution of ST131 clade C [4, 5], showing that clade C evolved from clade B during the mid-late 1980s.

Additionally, ESBL CTX-M-15 present in C2/*H30*-Rx and CTX-M-27 present in C1/*H30*R sub-clades have been linked to IncF plasmids, F2:A1:B and F1:A2:B20 formula, respectively [5–7]. Non-ESBL ST131 producers also carried other IncF plasmids [8], and several virulence factors have been identified in these plasmids [9].

The rapidity of this increase of resistance is somewhat surprising because clinically relevant levels of resistance in *E. coli* require multiple genetic changes [10, 11], some of which are associated with modified bacterial fitness. The role of the interplay of mutations associated with FQ-R and IncF plasmids, as well as its impact on the stability of these multi-resistance plasmids and on the evolution of FQ-R bacteria, is unknown. A full understanding of the factors driving this problem would be useful for developing an effective strategy to restrict the increase in resistance.

Despite the abundant research on *E. coli* to date, the mechanisms driving extra-intestinal evolution, stability, and resistance in ExPEC remain poorly understood. ST131 clade C is one important example: its transmission success was likely driven, in part, by the acquisition of FQ resistance and ESBL production [12].

The main objective of this study was to investigate the relationship and the impact of different topoisomerase mutations frequently found related to FQ-R in *E. coli* ST131 clone on the stability of IncF plasmids and, on the other hand, the impact of IncF plasmids acquisition on topoisomerase modifications emergence using isogenic *E. coli* mutants. These data will help to understand mechanisms implicated in this fast adaptation process in the *E. coli* ST131 clone.

Material and methods

Strains, growth conditions, and antimicrobial agents

Wild-type *E. coli* ATCC 25922 was used as the starting strain for all constructions (Table S1) [13]. Luria broth (LB, Invitrogen, Madrid, Spain), liquid or solid, was routinely used. Strains were grown at 37 °C. Kanamycin (Sigma-Aldrich, Madrid, Spain) was used at 30 µg/mL for pCR-BluntII-TOPO stability. Chloramphenicol (Sigma-Aldrich) and ampicillin (Sigma-Aldrich) were used at 25 and 100 µg/mL for pST76C and pUC19RP12 use (Table S1).

We selected six clinical *E. coli* ST131 isolates from a previous study [14] with different FAB formulas and identical addiction systems except for VagC (Table S2). Plasmid sequences used in this study were obtained for transformants generated in our lab into the *E. coli* Dh10B receptor strain. These IncF plasmids were used as donors for further experiments.

Quinolones used were nalidixic acid (Sigma-Aldrich), ciprofloxacin (Sigma-Aldrich), levofloxacin (Sigma-Aldrich), moxifloxacin (Sigma-Aldrich), norfloxacin (Sigma-Aldrich), and ofloxacin (Sigma-Aldrich). Stock solutions were prepared in accordance with Clinical and Laboratory Standards Institute (CLSI) guidelines (www.clsi.org) [13].

Construction of isogenic E. coli strains

Substitutions S83L, D87N, and D87G into GyrA and S80R, S80I, and E84V into ParC and combinations mutations were obtained by gene replacement, as described by Posfai et al. [15] (Table S2). The method is based on the recombination and repair activities of the bacteria. Briefly, an internal fragment of the mutated gene (*gyrA* or *parC*) was cloned into a conventional pCR-BluntII-TOPO (Invitrogen) vector using specific primers, shown in Table S1. Also, strains containing these substitutions are indicated in Table S2. The mutated fragment was subcloned into the BamH1 site of suicide plasmid pST76-C. The construction was introduced into *E. coli* ATCC 25922 and derivates by electroporation. The mutated fragment was inserted into the chromosome by homologous recombination. The resolution of this cointegrate was by intramolecular recombination of the allele pair. The resolution process was mediated by the pUC19RP12 vector and confirmed by PCR.

Whole-genome sequencing

All strains, both donors and transconjugants/transformants derivatives, included in this study were subjected to whole-genome sequencing with a MiSeq (Illumina, San Diego, CA, USA). For this, the libraries were prepared with the Nextera XT DNA library preparation kit (Illumina), and the sequencing was done with a V3 600-cycle reagent cartridge. Sequencing was achieved with an average of at least $\times 30$ coverage. Illumina sequences were assembled de novo (clinical strains) or using a reference (*E. coli* ATCC 25922 derivatives, Genome Accession CP009072) that uses CLC Genomics Workbench (Qiagen, Netherland). The genomes were annotated with Rapid Annotation using Subsystem

Technology (RAST), plasmidFinder, and Resfinder (https://cge.cbs.dtu.dk/services/ResFinder/) [16]. Plasmids sequences are available at BioProject ID PRJNA756876 (https://www.ncbi.nlm.nih.gov/).

MIC determination

MICs were determined in triplicate for each bacterial strain, using two different techniques: the gradient strip test and broth microdilution, following EUCAST reference methods [17].

Conjugation and transformation assays

Conjugation experiments were carried out using LB medium with rifampicin-resistant *E. coli* ATCC 25922 (and all derivatives EC02, EC04, EC06, EC80, EC81, and EC82) as the recipients. These isogenic pairs were obtained under selective pressure (200 mg/L of rifampicin). Transconjugants were selected on LB agar plates containing rifampicin at 100 mg/L and amoxicillin at 100 mg/L (or cefotaxime at 4 mg/L) for selection of plasmid-mediated FQ-R.

When plasmids were not transferable by conjugation, a transformation assay was performed. Natural plasmids were electroporated into *E. coli* and plated onto LB agar containing rifampicin (Sigma-Aldrich) at 100 mg/L and amoxicillin (Sigma-Aldrich) at 100 mg/L.

Mutant frequency assays

To evaluate whether the different genetic combinations might become resistant to ciprofloxacin more easily if they possessed IncF plasmids from ST131 clinical strain donors, the frequency of mutant selection was determined by plating an overnight culture of viable microorganisms on MH agar plates without and with ciprofloxacin (4 × MIC). For each strain used in the study, a 0.5 McFarland standard was obtained, a 10^{-4} dilution was performed (starting inoculum of ~ 10^4 CFU/mL) and incubated at 37 °C overnight. One hundred μL (corresponding to ~10^9 CFU/mL) of overnight culture was inoculated in the presence of 4 × MIC of ciprofloxacin. The colony count was determined after 48 h at 37 °C from at least three independent assays. The frequency of mutants was expressed as the ratio of the number of resistant mutants recovered to the total viable bacteria obtained from the drug-free MH agar plates.

Plasmid stability assays

For each strain used in the study, a 0.5 McFarland standard was obtained, a 10^{-4} dilution was performed (starting inoculum of ~ 10^4 CFU/mL) and incubated at 37 °C overnight. This process was repeated for 7 days. To estimate the number of cells containing these IncF natural plasmids in a given *E. coli* population, sequential dilutions of cultures for testing were seeded onto LB plates containing rifampicin at 100 mg/L (total population) with or without amoxicillin at 100 mg/L or cefotaxime at 4 mg/L (population conserving IncF plasmids). The number of CFU was recorded after culturing the plates for 24 h at 37 °C. Data for each strain–plasmid combination are the results of at least four independent experiments.

Statistical analysis

For statistical evaluation, a two-tailed unpaired Student's *t*-test was used when two groups of quantitative variables were compared. Differences were considered significant when p values were ≤ 0.05. All data represent the mean ± SE of at least three independent experiments.

Results and discussion

We focus our investigation on the relationship and the impact of different topoisomerase mutations related to FQ-R in *E. coli* ST131 clone on the stability of IncF plasmids and, on the other hand, the impact of IncF plasmids acquisition on topoisomerase modifications emergence using an isogenic *E. coli* mutants collection. The first step was to characterize six different IncF plasmids to be included in this study [14], five IncFIA plasmids, and one IncFIB plasmid (42 plasmid-strain combinations were assayed in our study, Table S2). IncFIA plasmid characterization determined that all of them bore $bla_{(CTX\text{-}M\text{-}15)}$, $bla_{(OXA\text{-}1)}$, and *aac-(6')-Ib-cr* genes as well as 5 plasmid addiction systems (Pemk, CcdA, ParD, VagC, HoK). The IncFIB plasmid bore $bla_{(CTX\text{-}M\text{-}32)}$ and $bla_{(TEM\text{-}1)}$ genes and the same factors of addiction as the IncFIA except for VagC. The data revealed a conserved profile for resistance and plasmid addiction systems for the selected plasmids.

For unknown reasons, it was not possible to obtain transconjugants or transformants of the plasmid IncF from the 1997 strain (Table S2), and therefore, it could not be evaluated in the rest of the assays.

By comparison of all (IncFIA and IncFIB) plasmids results, moderate or minor differences in the IncF plasmids stability were observed among *E. coli* ATCC 25922 wild-type strain and isogenic mutants encoding substitutions such as S83L, D87N, and D87G into GyrA and/or S80R, S80I, and E84V into ParC and its combinations obtained by gene replacement (Fig. 1). Under our experimental conditions, in general, the fluctuation of bacteria harboring plasmids was less than 0.5-$Log_{(10)}$ in most of all cases. A slight increase in stability for EC04 (S83L + S80R) and a slight decrease for EC80 (S83L + D87G) and EC81 (S83L + S80I) were

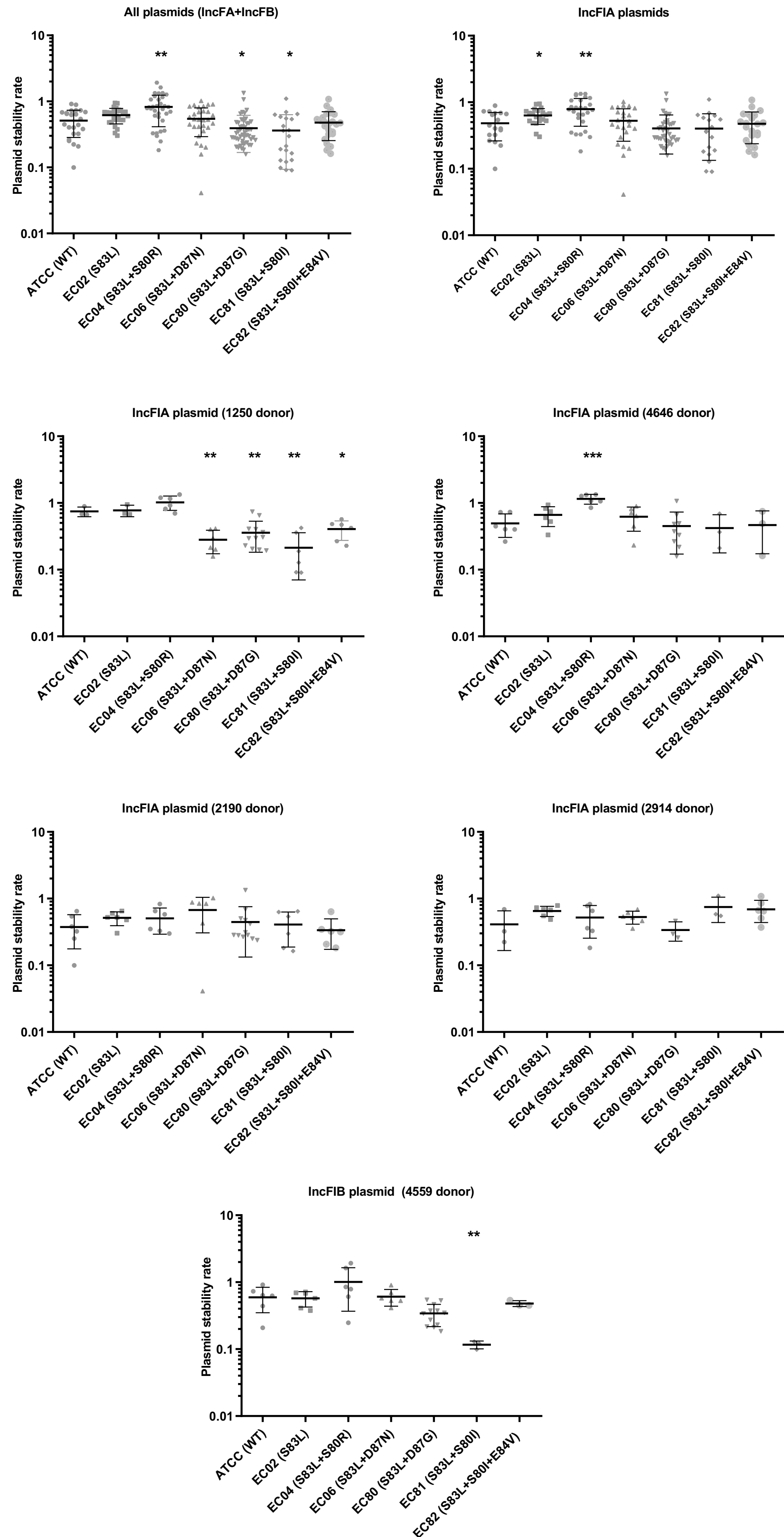

Fig. 1 IncF plasmid stability related to QRDR modifications usually found in ST131 *E. coli* clone. IncFIA plasmids from donors 1250, 2190, 2914, and 4646 and IncFIB plasmids from donor 4559 were included. Impact on plasmid stability was evaluated into multiple genetic backgrounds such as *E. coli* ATCC 25922 (wild type), EC02 (S83L), EC04 (S83L + S80R), EC06 (S83L + D87N), EC80 (S83L + D87G), EC81 (S83L + S80I), and EC82 (S83L + S80I + E84V). Data are represented as the means of at least three independent measurements. Error bars represent standard deviations. Significant *p* values, always compared to *E. coli* ATCC 25922 (wild type), are noted (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$)

significantly observed ($p=0.001$, $p=0.03$, and $p=0.04$, respectively). For IncFIA plasmids, a slight increase in stability for EC02 (S83L) and EC04 (S83L + S80R) was significantly noted ($p=0.02$ and $p=0.002$, respectively). Taking into account results for individual plasmids, for 1250 plasmid, a slight decrease for EC06 (S83L + D87N), EC80 (S83L + D87G), EC81 (S83L + S80I), and EC82 (S83L + S80I + E84V) was significantly observed ($p=0.006$, $p=0.009$, $p=0.002$, and $p=0.01$, respectively); however, this tendency was not reproduced for the rest of IncFIA plasmids (Fig. 1). Neither significant differences in the IncFIA and IncFIB plasmid stability, separately, were observed. Altogether, minor but significant in vitro differences were observed related to the stability of these plasmids with respect to different genetic backgrounds according to QRDR modifications frequently found in ST131 *E. coli* clone. The biological impact of these fluctuations could be trivial, and its in vivo impact at the molecular epidemiology level is unknown.

With respect to mutant frequency tests, it was observed that the presence of these IncF plasmids increased this value significantly compared to the control (up to 1000-fold, $p=0.0002$), being this increase greater in the IncFIA plasmids (Fig. 2). As mentioned earlier, the IncFIA plasmids harbored the low-level FQ-R gene *aac(6′)-Ib-cr*, which could explain the increase of the mutant frequency observed, as this gene is a factor for FQ-R selection. A similar effect has been observed for other low-level FQ-R genes [11, 18–21]. Preincubations at subinhibitory concentrations of ciprofloxacin did not modify this result (data not shown). Interestingly, IncFIB 4559 plasmid, which does not contain any known low-level FQ-R plasmid gene, also increased the mutant frequency level in a similar way (Fig. 2). This data could be indicative that IncF plasmid could facilitate the selection of FQ-R.

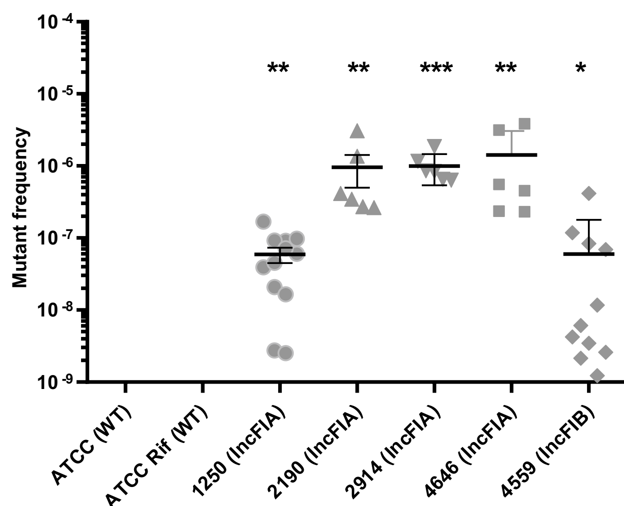

Fig. 2 Mutant frequency against ciprofloxacin for *E. coli* ATCC 25922 harboring IncF plasmids usually found in ST131 *E. coli* clone. IncFIA plasmids from donors 1250, 2190, and 2914 and IncFIB plasmid from donor 4559 were transferred into *E. coli* ATCC 25922 (wild type). The selection concentration was 4 × MIC of the *E. coli* ATCC 25922 strain. Data are represented as the means of at least three independent measurements. Error bars represent standard deviations. Significant *p* values, always compared to *E. coli* ATCC 25922 (wild type), are noted (*$p<0.05$; **$p<0.01$; ***$p<0.001$)

It is important to remark that all the experiment was conducted using the lab strain *E. coli* ATCC 25922 (belonging to ST-73 clone) rather than an *E. coli* ST131 clinical strain. This allowed using an isogenic laboratory model. Hence, the results obtained for the current study should be taken with caution and validated in an ST131 model.

The fitness of an organism comprises the sum total of all contributing phenotypes in the cell, with each variable likely contributing to the organism's success. Overall, ST131 is an excellent real-world model to study the emergence of a superbug. ST131 clade C adapted to environmental changes more rapidly than other ExPEC clones. Without a better mechanistic understanding of the unique adaptations of this important clade, the medical community is unlikely to stop its continuing spread or to anticipate the next clonal wave of MDR ExPEC [12]. Hence, more needs to be done to understand the evolution of these MDR organisms to guide rational drug design, inform antibiotic stewardship programs, and control the evolution of other nascent emerging pathogens [6]. We know the combination of the ST131 genetic background, armed with an IncF plasmid containing $bla_{(CTX\text{-}M\text{-}15)}$, in a sub-clone that could develop compensatory mutations mediating resistance to fluoroquinolones (QRDR chromosomal quinolone resistance) is a recipe for success [6]. Here, the question was whether this process could be enhanced in a genetic background that includes IncF plasmids (with or without low-level FQ-R genes), partially explaining the conjunction of both resistance phenotypes and the evolutionary success of this clone.

Interestingly, toxin–antitoxin systems have been identified on conjugative plasmids. They are implicated to stabilize plasmids or increase plasmid competition via post-segregational killing, but recently, a role in also controlling plasmid copy number was defined [22]. Also, some of these toxin–antitoxin systems interact with topoisomerase type II (DNA gyrase or topoisomerase IV) with potential implications when challenged with anti-topoisomerase antibiotics such as quinolones [23]. Whether plasmid toxin–antitoxin systems could modulate the evolution of *E. coli* ST131 clone related to IncF plasmids and topoisomerase mutations conferring FQ-R is unknown.

In conclusion, FQ-R substitutions in *gyrA* or *parC* genes, frequently found associated with *E. coli* clone ST131, do not modify the stability of ST131-associated IncFIA and IncFIB plasmids under in vitro conditions. IncF-type plasmids present in *E. coli* clone ST131 facilitate the selection of

resistance to quinolones, even in the absence of *aac(6′)-Ib-cr* gene. These results are consistent with the clinical scenario in which the combination of resistance to quinolones and beta-lactams is highly frequent in the *E. coli* clone ST131.

Code availability (software application or custom code)

Not applicable.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s10096-021-04358-4.

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Allowed.

Declarations

Ethics approval (include appropriate approvals or waivers) Not applicable.

Consent to participate and publication On behalf of all authors, we consent to the participation and publication of this work.

Conflict of interest The authors declare no competing interests.

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