

1 **Fitness effects of CTX-M-15-encoding IncF plasmids on their native**
2 ***Escherichia coli* ST131 H30Rx hosts**

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25 Running title: Fitness effects of IncF plasmids on *E. coli* ST131 H30Rx

26 **Abstract**

27 **Objectives**

28 The objective of this study was to investigate effects of large CTX-M-15-encoding IncF
29 plasmids on the fitness of their native *E. coli* ST131 H30Rx hosts in order to understand
30 possible plasmid-host coevolution.

31 **Methods**

32 We selected five *E. coli* ST131 H30Rx strains of diverse origin, each carrying a multireplicon
33 IncF plasmid encoding the gene *blactx-m-15*. The plasmid was eliminated from each isolate by
34 displacement using an incompatible plasmid vector pMDP5_cureEC958. Whole-genome
35 sequencing (WGS) was performed to obtain complete chromosome and plasmid sequences of
36 wild-type isolates and to detect chromosomal mutations in plasmid-free strains. Competition
37 assays were conducted to determine the relative fitness of plasmid-free clones compared to the
38 corresponding wild-type isolates.

39 **Results**

40 We were able to successfully eliminate the IncF plasmids from all of the wild-type strains using
41 the curing vector pMDP5_cureEC958. The chromosomes of plasmid-free clones contained zero
42 to six point mutations. Plasmid-free strains of three isolates showed no significant difference in
43 relative fitness compared to the corresponding plasmid-free strains. In the two remaining
44 isolates, the plasmids produced a small but significant fitness cost.

45 **Conclusion**

46 We conclude that IncF plasmids produce moderate fitness effects in their *E. coli* ST131 H30Rx
47 hosts. This fitness compatibility is likely to promote the maintenance of antibiotic resistance in
48 this worrisome *E. coli* lineage.

49 Introduction

50 Extraintestinal pathogenic *E. coli* (ExPEC) represent a huge public health burden¹ as these
51 strains are a common source of numerous diseases, from mild to life-threatening infections,
52 such as urinary tract or blood-stream associated infections, bacteraemia and meningitis².
53 Globally emerging multi-drug resistance, especially to antimicrobials of clinical importance,
54 such as fluoroquinolones and cephalosporins, is of special concern in this species in recent
55 years. Due to the resistance, only a few or even no therapeutic options are left for a treatment
56 of infections caused by these bacteria^{2,3}.

57 Resistance to clinically important antimicrobials among ExPEC strains was scarce before 2000.
58 Since then, the number of ExPEC isolates resistant to fluoroquinolones and cephalosporins has
59 been increasing exponentially⁴. Highly virulent and multi-drug resistant *E. coli* ST131 is one
60 of the clinically most important ExPEC strains due to its worldwide predominance. Even though
61 *E. coli* ST131 was mainly associated with human infections⁵, recent findings brought a
62 disturbing evidence of its dissemination among companion animals, poultry, livestock, food
63 products, wildlife and environment, including wastewater treatment plant effluents^{6,7}.

64 A study from 2015⁸ analysed the evolution of *E. coli* ST131 lineage. Based on investigations
65 from the mid-2000s, it is apparent that previous consumption and misuse of antimicrobials is
66 linked with the emergence of resistant pathogens^{8,9}. Antimicrobials, such as fluoroquinolones
67 and cephalosporins, were often used for the treatment of human infections which resulted in the
68 emergence of fluoroquinolone resistant subclone *E. coli* ST131 H30R. Furthermore, by
69 acquisition of an incompatibility F (IncF) plasmid carrying an ESBL gene *bla*_{CTX-M-15}, a distinct
70 fluoroquinolone resistant extended-spectrum beta-lactamase (ESBL) producing *E. coli* ST131
71 subclone H30Rx emerged^{9,10}. Compensatory mutations in combination with virulence
72 determinants and resistance to clinically important antimicrobials allowed the subclone H30Rx

73 to outcompete other sublineages of *E. coli* ST131 and in the late 2000s became a globally
74 disseminated and most prevalent ExPEC subclone^{8,11}.

75 IncF are complex epidemic resistance plasmids composed of more than one plasmid replicon.
76 CTX-M-15-encoding IncF plasmids present in the subclone *H30Rx* usually harbour two
77 plasmid replicons with plasmid multilocus sequence type (pMLST) F2:A1:B-. These plasmids
78 typically harbour other resistance genes apart from *bla*_{CTX-M-15}, such as *bla*_{TEM-1}, *bla*_{OXA-1},
79 *aac(6')-Ib-cr*, *catB4*, *aadA5*, *mph(A)*, *dfrA7*, *tet(A)* and *sull*. Additionally, the narrow-host
80 IncF plasmids encode partitioning and addiction systems to ensure their maintenance^{6,8,12}.
81 Carriage of such large plasmids providing selective advantage for a bacterial host via additional
82 virulence and antibiotic determinants, usually imposes a fitness cost to its host¹³. On the other
83 hand, a previous study suggested that *E. coli* ST131 *H30Rx* is adapted to large IncF plasmids⁸.

84 In this study, we analyse plasmid-host interactions between this intriguing *E. coli* subclone and
85 its plasmids. We aimed to estimate the fitness impact of the large F2:A1:B- IncF plasmids,
86 previously recognised as a foundation of *H30Rx* sublineage emergence, on its native host. Five
87 representatives of the *E. coli* ST131 *H30Rx* were selected for elimination of IncF plasmid using
88 the curing vector pMDP5_cureEC958. Plasmid fitness effects were subsequently calculated
89 using competition assays between the plasmid-carrying and plasmid-free isogenic clones.

90 **Materials and Methods**

91 **Bacterial strains**

92 Five *E. coli* ST131 *H30Rx* strains were selected out of the collection of 169 *E. coli* ST131
93 isolates of diverse origin from several geographic regions⁶. Selected isolates were of human (n
94 = 3), environmental (n = 1) and companion animal (n = 1) origin. Each strain carried a large
95 IncF plasmid harbouring *bla*_{CTX-M-15}. Additional information on isolates obtained during our
96 previous study⁶ is presented in Table S1.

97 Bacterial strains were routinely grown on Luria-Bertani agar (LBA; Sigma-Aldrich, Saint
98 Louis, USA) supplemented with cefotaxime (2 mg/L) at 37 °C overnight if not specified
99 otherwise. Competition assays were performed in Luria-Bertani broth (LBB; Becton Dickinson,
100 MD, USA).

101 **Construction of curing vector**

102 The curing vector pMDP5_cureEC958 was designed based on the pCURE2 plasmid¹⁴. The
103 backbone vector pMDP5 was assembled from three vectors including pUC19 (ori and MCS,
104 126-1480 nt), pKD3 (chloramphenicol resistant gene, 85-980 nt) and pCURE2 (*sacB* gene, 209-
105 2076 nt). Subsequently, a curing fragment (containing RepFIIA, RepFIA, *ccdB*, *sok*, *pemI*, and
106 *vagC*) was synthesised and cloned into pMDP5, creating the curing vector pMDP5_cureEC958
107 (Figure 1). All cloning and DNA synthesis steps were performed by Epoch Life Science (Texas,
108 USA). The sequence of pMDP5_cureEC958 was deposited to GenBank under accession
109 number MZ723317.

110 **Plasmid curing**

111 Plasmid-free variants were created using a curing method described by Hale and colleagues¹⁴
112 with the curing vector pMDP5_cureEC958. The plasmid curing vector was introduced into the
113 wild-type strains by electroporation (1.8 kV, 25 µFar, 200 Ω) using Gene Pulser Xcell™
114 electroporation system (Bio-Rad Laboratories Inc., California, USA) as described before¹⁵.
115 Cultures harbouring both, the wild-type plasmid and the designed construct, were selected on
116 LBA supplemented chloramphenicol (30 mg/L) and the presence of *blactX-M* and *catA1* genes
117 was verified by PCR^{16,17}. Transformants were cultivated on LBA containing chloramphenicol
118 (30 mg/L) in order to eliminate the IncF plasmid. After successful removal of the wild-type
119 plasmid, verified by the same PCR, the pMDP5_cureEC958 was eliminated on a non-selective
120 LBA plates supplemented with 5% sucrose. Three to four plasmid-free clones of each isolate
121 were selected.

122 Selected plasmid-free clones were sequenced on MiSeq platform (Illumina) as described below
123 to investigate possible single nucleotide mutations (SNPs) on chromosomes. Subsequently, one
124 plasmid-free clone of isolates without chromosomal mutations was selected to reintroduce the
125 corresponding wild-type IncF plasmid as a control of experiment. Plasmid DNA was extracted
126 from wild-type strains using Genopure Plasmid Midi Kit (Roche Diagnostics GmbH,
127 Mannheim, Germany). Plasmids were reintroduced by electroporation and their presence was
128 confirmed by PCR assays for gene *blaCTX-M* and for FII and multiplex (FIA, FIB, FIC) IncF
129 replicons^{16,18}.

130 **Whole-genome sequencing**

131 Wild-type isolates were subjected for short- and long-read sequencing. Additionally, plasmid-
132 free strains and plasmid-free strains with reintroduced wild-type IncF plasmid were selected for
133 short-read sequencing. Genomic DNA for short-read sequencing was extracted using
134 NucleoSpin® Tissue kit (Macherey-Nagel, GmbH & Co. KG, Germany), library was prepared
135 by Nextera® XT Library Preparation kit (Illumina, San Diego, CA, USA) and sequenced using
136 2x250 bp paired-end sequencing on MiSeq (Illumina) platform. NucleoSpin® Microbial DNA
137 kit (Macherey-Nagel) was used for the extraction of genomic DNA aimed for long-read
138 sequencing. Libraries were constructed using SMRTbell Express Template Prep Kit 2.0 (Pacific
139 Biosciences, PacBio, USA) followed by single molecule real-time (SMRT) sequencing on
140 Sequel I Platform (PacBio).

141 **Data analysis**

142 Raw reads acquired by Illumina sequencing were trimmed using Trimmomatic v0.39¹⁹ to
143 remove adaptor residues and discard low quality read regions ($Q \leq 20$). SPAdes v3.13.1²⁰ with
144 the “--careful” configuration was used to obtain *de novo* assemblies. Center for Genomic
145 Epidemiology tools (PlasmidFinder v2.1, pMLST v2.0, ResFinder v4.0) were used to verify the
146 presence of plasmid replicons and genes intermediating antibiotic resistance

147 (<https://cge.cbs.dtu.dk/services/>). HGAP4 in SMRT Link v.6 (PacBio) was used to obtain
148 polished long reads in fastq format. Hybrid assembly of trimmed short and long reads was
149 performed using Unicycler v0.4.8²¹ and corrected with Pilon v1.23²² in order to reconstruct
150 chromosome and plasmid sequences of wild-type isolates. Complete circular sequences of
151 plasmids were manually annotated using Geneious v7.1.9 (Biomatters, Auckland, New
152 Zealand) in compliance with annotation form of previous studies²³.

153 **Comparative genomics**

154 Phylogenetic relatedness of wild-type isolates was estimated. Prokka v1.14.1²⁴ was used to
155 predict open reading frames of isolates assemblies and their core genome was aligned using
156 Roary v3.12.0²⁵. Subsequently, the alignment was used to generate phylogenetic tree in
157 RAxML v8.2.11²⁶ under GTR+GAMMA model supported by 1,000 bootstraps. A nucleotide
158 similarity between the isolates was estimated using the core genome alignment in snp-dists
159 v0.6.3 (<https://github.com/tseemann/snp-dists>) considering the number of SNPs. The
160 phylogenetic tree was visualized in iTOL v5.7²⁷.

161 BLAST (Basic Local Alignment Search Tool) of NCBI (National Center for Biotechnology
162 Information, MD, USA) was used to find and download a plasmid sequence with the highest
163 coverage and identity from GenBank. Genetic content of the IncF plasmids was compared using
164 BLAST Ring Image Generator (BRIG) v0.95²⁸ and Clinker v0.0.13²⁹. Presence and
165 nomenclature of specific insertion sequences in IncF plasmids were confirmed using ISfinder
166 database³⁰ and toxin-antitoxin systems were verified using Conserved Domain Database³¹.

167 Comparison of the wild-type isolates to the corresponding plasmid-free strains and to the
168 plasmid-free strains with reintroduced plasmids was made to verify the identity of the strains
169 as well as the identity of the wild-type and reintroduced plasmids. Corresponding sequences
170 were aligned using algorithm BWA-MEM v0.7.17³² and manually checked in Geneious v7.1.9.

171 **Single nucleotide polymorphism analysis**

172 Corrected short reads of plasmid-free variants were mapped to the corresponding wild-type *de*
173 *novo* assemblies using Bowtie2 v2.3.5³³. Variant calling was performed by VarScan v2.4.4³⁴
174 based on the coverage of mapped reads. Minimum variant frequency was 80% and called
175 variants were manually checked in Geneious v7.1.9. Corresponding wild-type reads were
176 mapped and analysed as well in order to normalize obtained results.

177 **Transferability of IncF plasmids**

178 Wild-type *E. coli* ST131 H30Rx isolates were used as donors while laboratory strain *E. coli*
179 TOP10 (Invitrogen Life Technologies, Carlsbad, CA, USA) and corresponding plasmid-free
180 variants of studied isolates were used as recipients for the estimation of conjugation ability of
181 IncF plasmids using filter mating assays based on a previous study³⁵. Conjugations were
182 conducted in technical triplicates and biological duplicates.

183 **Relative fitness measurements**

184 Competition assays were performed to compare the relative fitness of the wild-type strains and
185 their plasmid-free clones using flow cytometry as previously described³⁶. Only the plasmid-
186 free strains without chromosomal mutations were selected for fitness experiments. A small non-
187 mobilisable pBGC plasmid (MT702881)³⁷ producing green fluorescent protein (GFP) was
188 transformed to the wild-type strains by electroporation¹⁵. Transformants were selected on LBA
189 plates containing cefotaxime (2 mg/L) and chloramphenicol (30 mg/L) and subsequently
190 verified by PCR assays for genes *blactX-M* and *gfp*^{16,37}.

191 Two competition assays, each consisting of six biological replicates, were performed for each
192 isolate. Competitions were performed between GFP-tagged wild-type strains and their untagged
193 plasmid-free variants while each included a competition between tagged and untagged wild-
194 types for normalisation. Overnight cultures were mixed in ratio 1:1 and diluted 1:400 for the

195 competition. GFP expression in the wild-type strains resulting in fluorescence was induced by
196 incubation in 0.9% sodium chloride solution containing 0.5% L-arabinose for 1.5 hours.
197 Plasmid-free and wild-type populations were competed at 37 °C for 22 hours shaking at 225
198 rpm. Initial and final proportions were measured on NovoCyte (ACEA Biosciences) flow
199 cytometer recording 50,000 events of each mixture. Relative fitness of plasmid-free clones
200 compared to corresponding wild-types was estimated using the formula:

$$201 w = \frac{\ln(\frac{N_{final,GFP-}}{N_{initial,GFP-}})}{\ln(\frac{N_{final,GFP+}}{N_{initial,GFP+}})}$$

202 where w represents relative fitness, $N_{initial, GFP-}$ and $N_{final, GFP-}$ are initial and final values of
203 untagged population and $N_{initial, GFP+}$ and $N_{final, GFP+}$ are proportions of GFP-marked population
204 before and after competition. Relative fitness of plasmid-free clones was statistically processed
205 using Student's T-test where relative fitness with p value < 0.05 was evaluated as statistically
206 significant. Obtained data was normalized using a competition between the tagged and
207 untagged wild-type populations in order to capture relative fitness of plasmid-free clones in
208 comparison to the corresponding (untagged) wild-type isolates. Competitions between wild-
209 type strains and constructed plasmid-free strains with reintroduced wild-type IncF plasmid were
210 performed as a control.

211 **Results**

212 **Strain and plasmid features**

213 In order to compare fitness effects of the plasmid on its native host, *E. coli* ST131 H30Rx
214 isolates carrying a single large *bla*_{CTX-M-15} harbouring IncF plasmid were selected. The
215 phylogenetic analysis of the five selected strains (Table S1) was based on the core-genome
216 alignment of 4,803 genes and showed 78-440 SNPs differences (Figure S1).

217 All five plasmids contained two IncF replicons (RepFIA, RepFII) with pMLST formula
218 F2:A1:B-, slightly varied in size and antibiotic resistance genes content (Figure S2). All
219 plasmids provided multi-drug resistance profile, encoding genes for ESBL as well as for other
220 antimicrobials and contained insertion sequences, mostly IS26 (Table 1). The *ccdB* and *pemK*
221 toxin-antitoxin systems were encoded in all plasmids within replicons RepFIA and RepFII,
222 respectively. Each plasmid harboured two copies of the addiction system *vapBC*. All but one
223 plasmid (pM45) harboured *hok/sok* system and plasmids of human isolates encoded *parDE*
224 system.

225 All IncF plasmids in our study have proved to be non-conjugative. Genetic analysis of transfer
226 (*tra*) regions showed diverse defects in all plasmids likely resulting in their non-functionality
227 (Figure 2). The *tra* region of pOV24 was disrupted in two parts by the *bla_{TEM-1}* gene, usually
228 transposed within a composite mobile genetic element, but 3' flanking sequence IS26 was
229 disrupted by *ISEcp1* element. Furthermore, the *traC* gene was truncated by another IS26 and
230 genes *traW* and *traU*, encoding proteins for pilus assembly and DNA transfer, were missing.
231 The *tra* region of pM70 was disrupted by composite mobile element containing *bla_{TEM-1}* gene
232 flanked by *IS15DI* and IS26 similarly as in pOV24. Moreover, part of the second half of the *tra*
233 genes was translocated 29.5 kb from the first part of the *tra* region and truncated by IS26 in
234 *traN* gene. The first part of the *tra* region, including *traJ*, serving as a transcriptional activator
235 of the *tra* region, was completely missing in plasmids pM24 and pM45. Plasmid pDog168
236 lacked most of this part of the region as well with an exception of the *traM* gene.

237 **Plasmid curing**

238 To study plasmid-associated fitness effects on their native host, all five wild-type strains were
239 cured of the naturally occurring IncF plasmids. A curing vector pMDP5_cureEC958 was
240 designed for the generation of plasmid-free variants, harbouring selective genes (*catA1* and

241 *sacB*), replicons RepFIA, RepFII, and antitoxins of the addiction systems encoded by wild-type
242 plasmids (Figure 1).

243 Four plasmid-free clones per each of four isolates (M24, M45, M70 and Dog168) and all three
244 grown plasmid-free clones of the isolate OV24 were selected for further analyses. Sequence
245 comparison of plasmid-free clones to the corresponding wild-type isolates discovered zero to
246 six chromosomal mutations. Mutations occurred in 52.6% of plasmid-free strains (in 10 out of
247 19). Nearly all mutations (92%, 23/25) occurred in protein coding sequences, only two of them
248 were located in intergenic regions. Additionally, most of the mutations in coding sequences
249 (82.6%, 19/23) were non-synonymous. No mutations occurred only in one of the plasmid-free
250 clones of the OV24 (1/3) and M70 (1/4) isolates, in two Dog168 (2/4), two M24 (2/4) and three
251 M45 (3/4) plasmid-free clones. Detailed list of genetic changes in plasmid-free clones is in
252 Table S2.

253 Four of all plasmid-free strains with the reintroduced wild-type plasmid (40%, 4/10) harboured
254 one mutation. Although, only one of them was non-synonymous.

255 **Fitness impact of IncF plasmids on their native host**

256 To maintain isogenic conditions in the competition experiment, only the plasmid-free clones
257 with no mutations in their chromosome were selected for the measurement of the IncF-
258 associated fitness effects on their native host. Relative fitness of plasmid-free clones was
259 estimated in comparison to the corresponding wild-type isolates considering a background
260 fitness of wild-types as 1 (Table 2). Analysis of two competition assays both consisting of six
261 biological replicates for each combination plasmid-free clone and wild-type revealed non-
262 significant fitness effects ($p > 0.05$) of IncF plasmids in three isolates (Dog168, OV24, M45).
263 Moderate increase ($p < 0.05$) of relative fitness was observed in plasmid-free strains of two
264 isolates (M24 and M70) as visualised in Figure 3, revealing a small plasmid fitness cost.

265 Relative fitness differences of the two M24 plasmid-free clones were statistically significant
266 (with p -value 1.83×10^{-6} and 4.07×10^{-4} , respectively), however, the increase in relative fitness
267 was moderate (1.04 and 1.02). A similar result was observed for the M70 plasmid-free clone,
268 which showed a moderate but significant increase in relative fitness compared to the wild type
269 clone ($w = 1.03$, $p = 1.5 \times 10^{-3}$).

270 **Discussion**

271 Even though the *E. coli* ST131 has attracted much attention in the last few years due to its
272 predominance in ExPEC infections, its success is still not fully elucidated. Plasmids play a key
273 role in bacterial survival under a selection pressure by providing virulence and antibiotic
274 resistance genes, but it is known that they often impose a fitness burden to their hosts³⁸⁻⁴⁰.
275 However, the fitness cost of a specific plasmid can differ in various hosts⁴¹.

276 In our study, we investigated the fitness effects of strictly clade-specific¹² F2:A1:B- IncF
277 plasmids, previously recognised as a source of *H30Rx* subclone emergence^{8,11}, on these native
278 hosts. Five native *E. coli* ST131 *H30Rx* hosts were cured of the large *blaCTX-M-15*-harbouring
279 IncF plasmids and then competed against their corresponding wild-type strain to calculate the
280 plasmid fitness impact.

281 **Plasmid curing**

282 Plasmid curing was previously recognised as a best way to study effects of a plasmid on a
283 bacterial population³⁸. Traditionally used methods for plasmid curing involved bacterial growth
284 in the presence of a chemical factor⁴². Although these techniques were widely used, efficiency
285 of plasmid curing was low and promoted high accumulation of unwanted mutations⁴³. Recently,
286 the CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) curing is being used
287 more often. The CRISPR curing is based on targeting a specific conserved sequence within
288 plasmids and subsequent plasmid cleavage. This technique is efficient and do not generate new

289 mutations, however, there are still plasmids which avoid targeting⁴⁴. As IncF plasmids are
290 known for their complexity and many combinations of the replicon alleles among the plasmids,
291 the pCURE method¹⁴ with the vector pMDP5_cureEC958 was used in this study. The method
292 is based on incompatibility of the targeted plasmid with an introduced curing vector. Even
293 though many plasmids overcome incompatibility elimination, it is possible to design a construct
294 with more replicons and antitoxin genes to successfully cure a strain of a plasmid. The approach
295 proved efficient in the past^{14,44} as well as in our study. On the other hand, we observed point
296 mutation accumulation in 52.6% (10 out of 19) of the plasmid-free clones. Therefore, WGS
297 after pCURE plasmid curing followed by genomic comparison of plasmid-free clones and their
298 corresponding wild-type isolates is necessary to exclude the clones harbouring mutations and
299 to obtain reliable relative fitness results.

300 **Fitness effects of the F2:A1:B- plasmids**

301 The fitness cost imposed by plasmids is influenced by several factors. It was observed before,
302 that solely the size of plasmids plays no role in their fitness cost, however, maintaining the
303 plasmid-encoded accessory genes can produce an energetic burden. The increasing number of
304 accessory genes, such as antimicrobial resistance genes, correlates with the higher fitness cost⁴⁵.
305 In order to evaluate the fitness impact of the F2:A1:B- plasmids providing their hosts with
306 multi-drug resistance, we estimated relative fitness of the plasmid-free strains in comparison to
307 the corresponding wild-type isolates. Competitions followed by measurement using flow
308 cytometer were used for this purpose. This method is considered much more sensitive than
309 growth curve analysis allowing to detect even subtle differences in relative fitness¹³. We
310 demonstrated that fitness impact of these IncF plasmids on their native hosts in non-selective
311 conditions was moderate to negligible in human as well as in animal and environmental isolates
312 which is in concordance with previous studies on F2:A1:B- plasmids⁴⁶⁻⁴⁸. A study of Shin and
313 Ko⁴⁷ focused on effects of CTX-M-14 and CTX-M-15 IncF plasmids from human clinical

314 isolates and their impact on a laboratory *E. coli* J53 strain. Based on the growth curve analysis
315 of the transconjugants, the authors proposed that strains harbouring *bla*_{CTX-M} on IncF plasmids
316 were as competitive as the naive host. Ranjan and his colleagues⁴⁸ studied competitiveness of
317 *E. coli* ST131 harbouring IncF plasmids and their plasmid-free variants against colicin-
318 producing *E. coli* ST10. The authors observed similar fitness ($p > 0.05$) of the wild-type *E. coli*
319 ST131 and their plasmid-free variants based on growth curves analysis. However, growth
320 curves in this experiment were conducted on selective plates which could affect the fitness of
321 the strains as some antibiotic resistance genes are genetically linked to each other and co-
322 selected. Therefore, the supplementation of plates with antibiotics could create a selection
323 pressure where carriage of plasmids would be more beneficial for the strain survival⁴⁹.
324 Mahéralult and colleagues⁴⁶ provided the investigation on two human clinical *bla*_{CTX-M-15-}
325 harbouring F2:A1:B- plasmids and their impact on an *E. coli* J53-2. Even though the fitness
326 cost of an IncF plasmid occurred initially after conjugation, the authors observed that this fitness
327 cost alleviated and a transconjugant carrying the IncF plasmid proved more competitive than a
328 transconjugant harbouring an IncC plasmid.

329 Previous studies also indicated that a functional conjugation system could have a negative
330 impact on a bacterial fitness and plasmids use several different ways to suppress the conjugative
331 transfer³⁹. Even though the silencing of the conjugative transfer results in a decrease of
332 horizontal spread of the plasmids, the vertical spread is supported by a fitness cost reduction⁵⁰.
333 The *tra* region responsible for conjugative transfer investigated during our study was
334 incomplete, the missing genes and length of the missing sequences differed among studied
335 plasmids. The rearrangements resulted in the non-functionality of the *tra* region of each
336 plasmid. Conjugation malfunction together with plasmid addiction systems ensure the vertical
337 transmission of the IncF plasmids. Additionally, it was pointed out that the initial fitness cost
338 is reduced over time. This phenomenon was observed in long-term evolution experiments, even

339 though the fitness of the plasmid-bearing strains was lower than of those without plasmids in
340 many cases³⁹.

341 We demonstrated that fitness impact of these IncF plasmids on their native hosts in non-
342 selective conditions was moderate to negligible among phylogenetically unrelated isolates of
343 diverse origin. Our results, combined with previous findings, strongly suggest that *E. coli*
344 *H30Rx* and IncF plasmids form successful associations promoting the world-wide
345 dissemination of this ExPEC lineage.

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361 **Transparency declarations**

362 None to declare.

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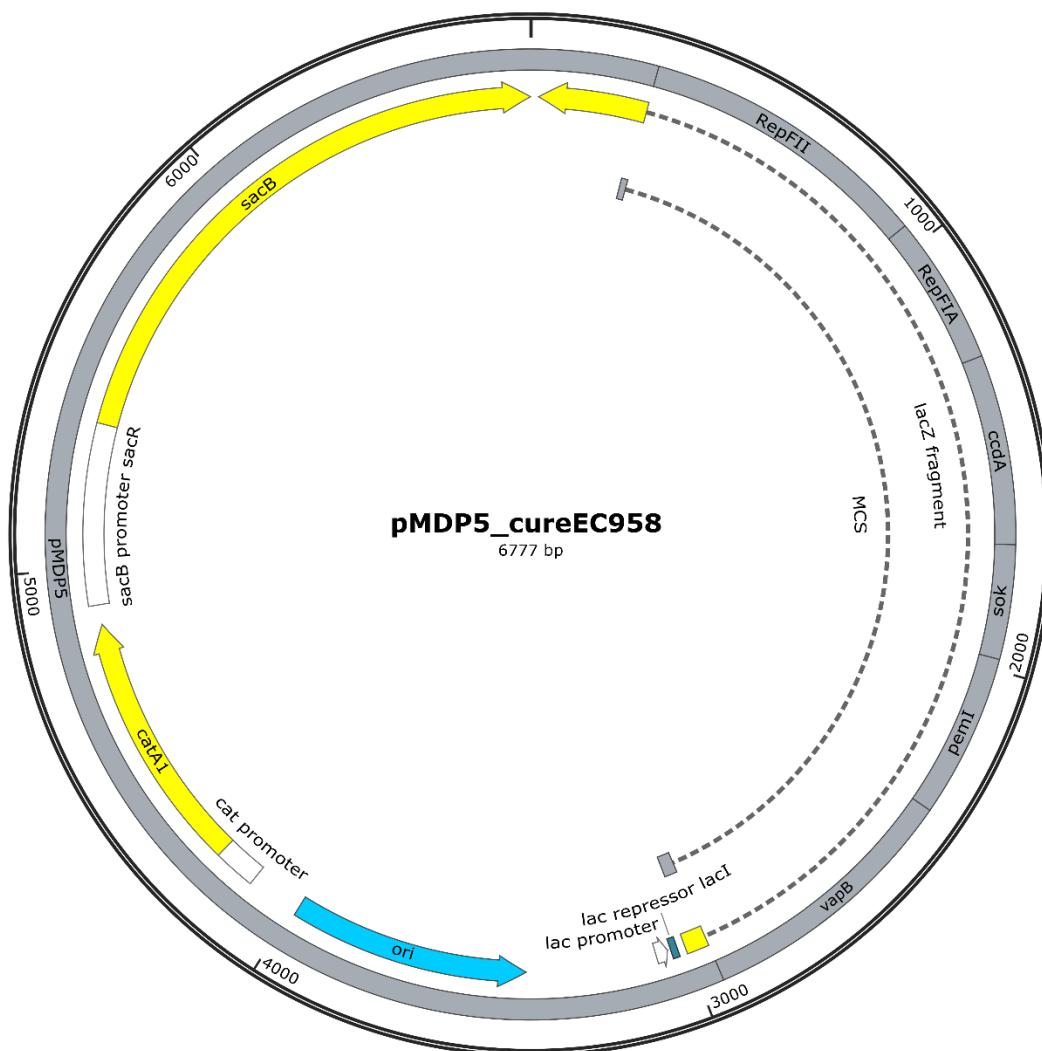
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487

488 **Figure 1** Genetic map of a plasmid vector pMDP5_cureEC958 designed for plasmid curing. It
489 contains chloramphenicol resistance encoding gene *catA1* for selective cultivation of strains
490 harbouring the vector and sucrose sensitivity gene *sacB* for selection of plasmid-free isolates
491 disposed of the vector. For the purpose of plasmid curing, it harbours genes encoding antitoxins
492 VapB, PemI, Sok and CcdA and IncF plasmid replicons RepFIA and RepFII.



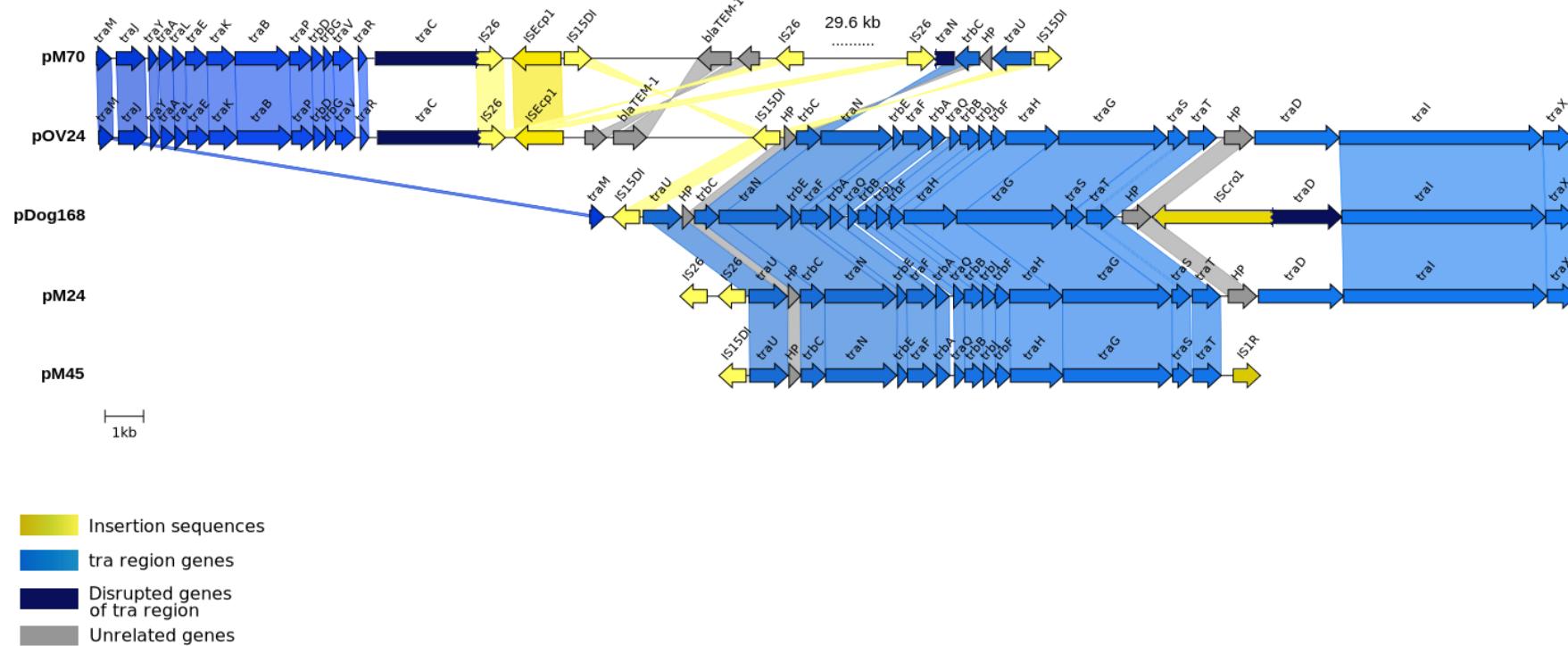
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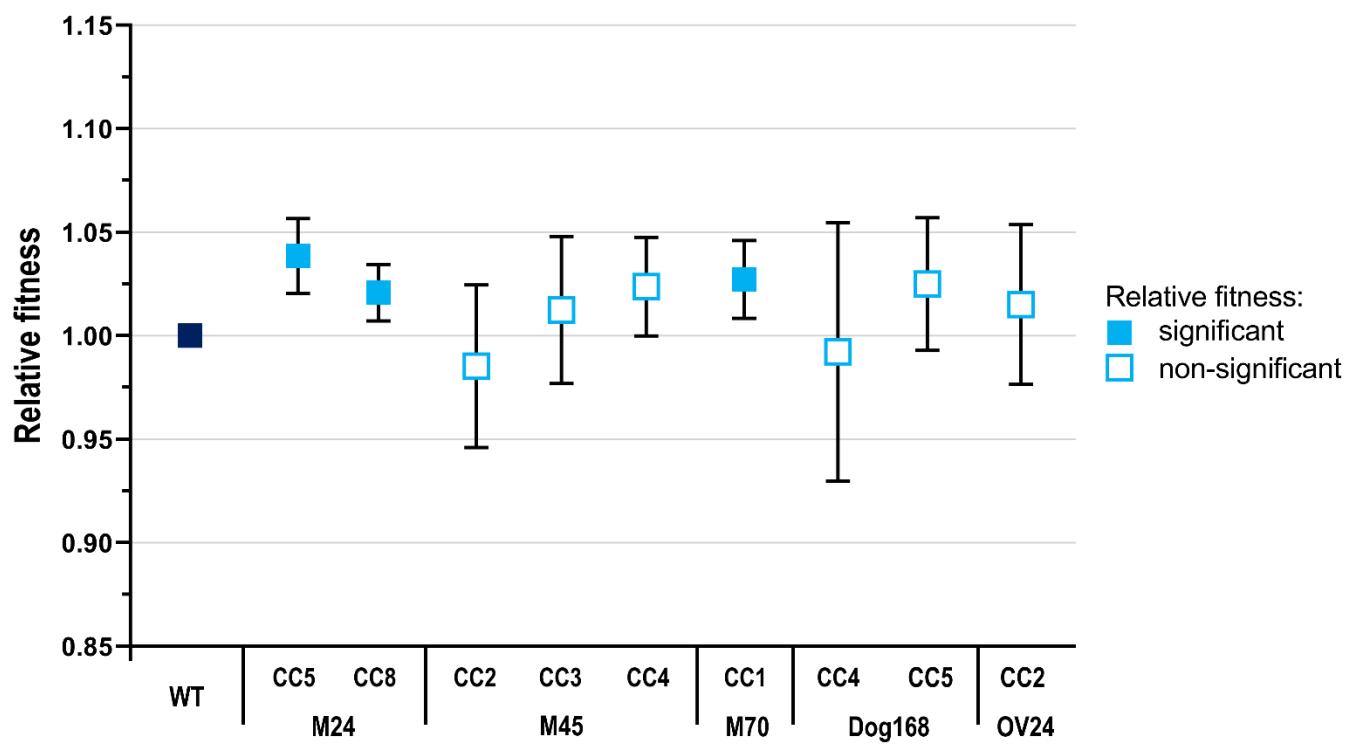
Figure 2 Transfer region of studied plasmids. The shading shows the similarity above 99.9%.



497

498

499 **Figure 3** Relative fitness of plasmid-free clones in comparison to the corresponding wild-type isolates. Background fitness of wild-types was estimated
500 as 1. CC stands for cured clone. Bars indicate standard deviation.



501

502 **Table 1** Selected genetic characteristics of CTX-M-15-encoding IncF plasmids in our study.

Plasmid ID	pMLST ^a	Size (bp)	Toxin-antitoxins systems					ARGs ^b						GenBank accession number				
			<i>ccdB</i>	<i>pemIK</i>	<i>vapCB</i>	<i>parED</i>	<i>hok/sok</i>	<i>blaCTX-M-15</i>	<i>blaTEM-1</i>	<i>blaOXA-1</i>	<i>aac(6')-Ib-cr</i>	<i>sulI</i>	<i>aadA5</i>	<i>mph(A)</i>	<i>dfrA17</i>	<i>tet(A)</i>	<i>catB3</i>	
pDog168	F2:A1:B-	131,080	■	■	■	■	■	■	■	■	■	■	■	■	■	■	*	MZ634324
pOV24	F2:A1:B-	144,582	■	■	■	■	■	■	■	■	■	■	■	■	■	■	*	MZ634325
pM45	F2:A1:B-	106,909	■	■	■	■	■	■	■	■	■	■	■	■	■	■	*	MZ634322
pM24	F2:A1:B-	116,543	■	■	■	■	■	■	■	■	■	■	■	■	■	■	*	MZ634326
pM70	F2:A1:B-	126,514	■	■	■	■	■	■	■	■	■	■	■	■	■	■	*	MZ634323

503 ^apMLST – plasmid multilocus sequence type, ^bARGs – antibiotic resistance genes504 Coloured squares represent the presence of the genes, for antibiotic resistance genes with coverage 95% and identity 100%. *Gene *catB3* was disrupted
505 by IS26 leaving 70.3% coverage resulting in gene malfunction.

506 **Table 2** Relative fitness of the plasmid-free clones in comparison to their wild-type isolates

Isolate ID	Plasmid-free clone ID ^a	Relative fitness ^b (\pm SD)	p value
Dog168	CC4	0.992 ± 0.06	0.764
	CC5	1.025 ± 0.03	0.216
OV24	CC2	1.015 ± 0.04	0.284
M45	CC2	0.985 ± 0.04	0.386
	CC3	1.012 ± 0.03	0.444
	CC4	1.024 ± 0.03	0.108
M24	CC5	1.038 ± 0.02	1.8×10^{-6}
	CC8	1.021 ± 0.01	4×10^{-4}
M70	CC1	1.027 ± 0.02	1.5×10^{-3}

507 ^a ID of constructed plasmid-free strains; CC stands for cured clone, ^b Relative fitness of plasmid-
508 free strain compared to the corresponding wild-type isolate which background fitness was
509 estimated as 1. Isolates highlighted in bold showed significant ($p < 0.05$) relative fitness
510 changes, however, the increase was moderate.