

1 **The presence of Type I-F CRISPR/Cas systems is associated**
2 **with antimicrobial susceptibility in *Escherichia coli***

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15

16 **Running title:** Type I-F CRISPR/Cas and antimicrobial susceptibility in *E. coli*

17

18 **Synopsis**

19 **Background** Clustered Regularly Interspaced Short Palindromic Repeats
20 (CRISPR) and their associated *cas* genes are sequence specific DNA
21 nuclease systems found in bacteria and archaea. CRISPR/Cas systems use
22 RNA transcripts of previously acquired DNA (spacers) to target invading
23 genetic elements with the same sequence, including plasmids. In this
24 research we studied the relationship between CRISPR/Cas systems and
25 multi-drug resistance in *Escherichia coli*.

26 **Methods** The presence of Type I-E and Type I-F CRISPR systems were
27 investigated among 82 antimicrobial susceptible and 96 MDR clinical *E. coli*
28 isolates by PCR and DNA sequencing. Phylogrouping and MLST were
29 performed to determine relatedness of isolates. RT-PCR was performed to
30 ascertain the expression of associated *cas* genes.

31 **Results** Type I-F CRISPR was associated with the B2 phylogroup and was
32 significantly overrepresented in the susceptible group (22.0%) compared to
33 the MDR group (2.1%). The majority of CRISPR I-F containing isolates had
34 spacer sequences that matched IncF and IncI plasmids. RT-PCR
35 demonstrated that Type I-F *cas* genes were expressed and therefore
36 potentially functional.

37 **Conclusion** The CRISPR I-F system is more likely to be found in
38 antimicrobial susceptible *E. coli*. Given that the Type I-F system is expressed
39 in wild-type isolates, we suggest that this difference could be due to the

- 40 CRISPR system potentially interfering with the acquisition of antimicrobial
41 resistance plasmids, maintaining susceptibility in these isolates.

42 **Introduction**

43 Clustered Regularly Interspaced Short Palindromic Repeat (CRISPR) loci
44 were first observed over 20 years ago and have since been found in the
45 genomes of many bacteria and archaea.¹ Along with their associated Cas
46 proteins, the system collectively known as CRISPR/Cas, has been described
47 as providing adaptive immunity for bacteria, targeting potentially deleterious or
48 costly invading DNA such as phages or plasmids. CRISPR loci consist of
49 short 21 to 47 base pair (bp) repeats separated by similarly sized non-
50 repeating sequences called spacers.² The repeat arrays are often, but not
51 always, associated with *cas* genes which encode the proteins involved in the
52 function of the CRISPR/Cas system. The CRISPR/Cas system leads to the
53 enzymatic cleavage of double stranded DNA in precise sites determined by
54 the sequence of the spacer.³ The process can be divided into two stages;
55 acquisition and interference. In the acquisition stage, Cas1 and Cas2 proteins
56 scan invading DNA for a short 3-6 bp motif (called the Protospacer Adjacent
57 Motif or PAM). Sequences immediately next to the PAM are processed and
58 integrated into the CRISPR array; these spacers are then transcribed and
59 processed into CRISPR RNA (crRNA), and this RNA is used in the
60 interference stage to guide the Cas nuclease complex to cleave
61 complementary DNA.¹

62 With recent attention focused on the genetic engineering potential of
63 CRISPR/Cas, its natural role has received less attention. The existence of an
64 adaptive immune system that rids bacteria of mobile genetic elements
65 (MGEs) is paradoxical in terms of survival. Indeed, the ubiquitous distribution
66 of mobile genetic elements among bacterial species suggests that CRISPR

67 systems are not always functional, or that they may have other roles such as
68 the regulation of gene expression⁴ and/or as yet undiscovered roles. In some
69 environments, host bacteria clearly benefit from plasmid-encoded traits such
70 as antimicrobial resistance and possession of CRISPR systems to rid the cell
71 of such as plasmids is likely to be rapidly selected against. The assumption
72 that CRISPR functions as an immune system has been called into question in
73 *E. coli*.⁵ We set out to explore this paradox.

74 Two subtypes of CRISPR are known in *E. coli*, Type I-E and Type I-F.⁶
75 In both types, the genes are clustered and closely flanked by two repeat
76 arrays each; CRISPR1 and CRISPR2 for Type I-E, and CRISPR3 and
77 CRISPR4 for I-F. Both systems are similar but the Type I-E system has 8
78 genes whereas Type I-F has 6.⁷ The functionality of the Type I-E system has
79 been brought in to question due to the finding that Type I-E *cas* genes are
80 repressed by the global regulator H-NS under laboratory conditions.⁸
81 Conversely, Type I-F *cas* genes have been shown to be constitutively
82 expressed.⁹ Due to the spacer content of the two systems, it has previously
83 been hypothesized that the Type I-E system may be specialized in targeting
84 bacteriophages whereas the Type I-F system is more associated with
85 plasmids.⁶

86 In this study, we examine the relationship between CRISPR and
87 antimicrobial resistance plasmids in *E. coli* by comparing the prevalence of
88 CRISPR Type I-E and I-F systems in antimicrobial susceptible and resistant
89 isolates. Additionally, by investigating expression of Type I-F *cas* genes, we
90 aim to gain an insight into the activity of these systems and potential
91 interference against natural antimicrobial resistance plasmids.

92 **Methods**

93 ***E. coli* isolates and antimicrobial susceptibility testing**

94 A total of 178 clinical *E. coli* isolates, derived from three sources, were
95 available for the study. Isolates were split into two groups; MDR¹⁰ comprising
96 96 isolates and fully susceptible, comprising 82 isolates, based on known
97 susceptibility testing results to 10 or more antimicrobials. The three sources
98 comprised; 90 (33 susceptible and 57 MDR) recent urine isolates from the
99 Royal Free Hospital (RFH), London collected between 2014 and 2015; 39
100 MDR isolates from Jaroden Hospital and Alexandria University in Egypt
101 between 2009 and 2011, and 49 susceptible community urine isolates from
102 South West of England collected between 2005-2006. Egyptian isolates were
103 chosen on the basis of resistance to 3rd generation cephalosporins and
104 carbapenems. RFH isolates were picked at random from available fully
105 susceptible or MDR *E. coli* isolates from the urine bench. The isolates from
106 South West England represented the first 50 fully susceptible isolates in a
107 larger collection forming part of another study. All isolates were subjected to
108 additional susceptibility testing to antimicrobials commonly associated with
109 plasmid-acquired genes (ampicillin, gentamicin, tetracycline, ciprofloxacin,
110 chloramphenicol and sulfamethoxazole) using the EUCAST disc diffusion
111 method. All isolates are listed in Table S1.

112 **PCR and DNA sequencing**

113 PCR was used to screen for four known CRISPR arrays with primers from
114 Touchon *et al.*⁵ (listed in Table 1). PCR reactions were prepared using
115 HotStar Taq Mastermix (Qiagen) according to manufacturer's instructions

116 (12.5 µl MasterMix, 0.2-1 mM of each primer and 20-100 ng of DNA up to 25
117 µl total volume). PCR products were visualized in agarose/ethidium bromide
118 gels under UV light. The presence of CRISPR3 and CRISPR4 arrays were
119 confirmed with Sanger DNA sequencing (Beckman Coulter Genomics)
120 followed by CRISPR identification using CRISPRfinder.²

121 **Analysis of *E. coli* by phylogrouping, MLST and plasmid replicon typing**

122 Phylogenetic groups were determined using multiplex PCR according to the
123 revised method of Clermont *et al.* 2013¹¹ and isolates that were unclassified
124 according to the method were re-confirmed to be *E. coli* by MALDI-TOF.
125 MLST was also performed on Type I-F CRISPR containing isolates using the
126 7 gene Achtman method.¹² O25b-ST131 clones were detected using PCR.¹³
127 PCR-based replicon typing was used to screen CRISPR I-F containing *E. coli*
128 for the presence of IncF and IncI group plasmids.¹⁴

129 **Spacer analysis**

130 CRISPRfinder² was used to determine the number and sequences of the
131 spacers within CRISPR3 and CRISPR4 repeat arrays. Nucleotide BLAST and
132 CRISPRTarget¹⁵ were used to search for matching sequences for Type I-F
133 spacers and a subset of Type I-E spacers. An identity score of 29 was used
134 as a lower threshold for plasmid matches of interest, excluding matches to
135 CRISPR regions from other isolates.

136 **RT-PCR for expression analysis**

137 RT-PCR for Type I-F *csy1* and *cas1* was performed using One Step RT-PCR
138 kit (Qiagen) using previously described primers (Table 1). The housekeeping
139 gene *rpsL* was used as a control. RNA was extracted from bacteria in the

140 logarithmic phase using an RNeasy minikit (Qiagen) and treated with DNase
141 using Turbo DNA (Ambion) according to manufacturers' instructions. Extracts
142 were confirmed to be devoid of detectable DNA with PCR using the HotStar
143 Taq kit (Qiagen).

144 **Statistics**

145 Results were analysed using GraphPad Prism 7. A significance level of α :
146 0.05 was used for all statistics. Fisher's exact test was used for comparisons
147 of CRISPR presence between the susceptible and resistant isolates.

148

149 **Results and Discussion**

150 All 178 *E. coli* isolates were screened for four CRISPR arrays (CRISPR 1-4)
151 found in this species. Overall, over half of the *E. coli* isolates had at least one
152 of the screened CRISPR arrays (53.9%) and Type I-E repeat arrays
153 (CRISPR1 and/or 2) were more common (39.9%) than Type I-F repeats
154 (CRISPR3 and/or 4) (15.7%). The overall distribution of CRISPR array types
155 differed significantly between susceptible and MDR groups ($P < 0.0001$);
156 CRISPR1 and 2 arrays were overwhelmingly the most prevalent amongst
157 resistant isolates, whereas in susceptible isolates approximately equal
158 numbers of both array types (CRISPR1/2 or CRISPR3/4) were found (Figure
159 1). Type I-E and Type I-F repeats were largely mutually exclusive among the
160 isolates; only 4 out of the 178 isolates studied had repeats associated with
161 both CRISPR types, in line with previous findings.⁵ None of the isolates had
162 all four repeat arrays.

163 In addition to screening for the individual repeat arrays, isolates that
164 were shown to have Type I-F repeats (CRISPR3 and CRISPR4) were also
165 screened for Type I-F *cas* genes. Out of the 82 susceptible isolates, 18
166 (22.0%) had Type I-F systems, defined here as having CRISPR3 and
167 CRISPR4 as well as the associated genes, and an additional 8 (9.8%) had
168 only CRISPR3 repeat arrays but without the *cas* genes. This differs
169 significantly from the resistant isolates ($P < 0.0001$) where only two isolates
170 out of 97 (2.1%) had Type I-F systems and none had CRISPR3 on their own.
171 Type I-F overrepresentation in susceptible isolates was also demonstrated in
172 only the Royal Free Hospital subset of isolates collected from the same
173 hospital and over the same time period ($p = 0.0108$). 21.2% of the 33
174 susceptible RFH isolates had CRISPR I-F whereas only 3.5% of the 57
175 resistant RFH isolates had the system. None of the highly resistant Egyptian
176 isolates had CRISPR3 or 4.

177 There were a total of 65 distinct CRISPR3 spacers and 39 distinct
178 CRISPR4 spacers with no overlap between the two arrays in terms of spacer
179 content. Some spacers were common and appeared in multiple non-clonal
180 isolates, including both susceptible and resistant isolates (Table 2, Figure 2).
181 Type I-E repeats from 49 susceptible isolates were also sequenced for
182 comparison. Interestingly, there were 152 and 117 distinct spacers for
183 CRISPR1 and CRISPR2 respectively, which is greater than the number of
184 distinct spacers for CRISPR3 and CRISPR4 for the entire group of 178
185 isolates. However, none of the CRISPR1 and CRISPR2 associated spacers
186 corresponded to known plasmids and only one corresponded to a known
187 phage. Most spacers were cryptic with no homology to any known genes. This

188 is in contrast to the work of Diez-Villasenor *et al.* who reported a much larger
189 proportion of spacers with a known origin.¹⁶ On the other hand, Nucleotide
190 BLAST for the Type I-F spacers revealed that five of the spacers matched
191 conserved regions within IncFII, IncFIB and IncI1 type plasmids with a
192 minimum of 97% homology (31/32 nucleotides) (Table 2). One spacer
193 corresponding to *klcA*, encoding a putative anti-restriction protein, appeared in
194 20 isolates in total. The *klcA* gene is conserved among IncI1 and IncFII
195 plasmid scaffolds, including those associated with the epidemic *E. coli* ST131;
196 typified by the CTX-M-15 encoding plasmids pEK516 and pEK499.^{17, 18}
197 Interestingly, three of the spacers identified (2, 3 and 4 in Table 2) are found
198 in the same, largely cryptic, region which is shared between IncI1 and IncFII
199 plasmids. PCR-based screening for plasmid replicons confirmed the absence
200 of plasmids corresponding to the spacer content of susceptible *E. coli* isolates
201 containing Type I-F CRISPR loci. The contrast between the spacer content
202 between Type I-E and I-F systems supports the hypothesis that the systems
203 have different functions within *E. coli* with the Type I-F seemingly being
204 associated more with plasmids.

205 Phylogrouping was performed for all 178 isolates and their phylogroup
206 composition breaks down as follows: A 10.7%, B1 5.6%, B2 55.1%, C 2.3%,
207 D 14.6%, E 2.8%, F 7.9%, unclassified 1.1%. The results confirmed a
208 previously reported¹⁶ association between CRISPR subtype and phylogenetic
209 groups. CRISPR I-F systems were only found in B2 group isolates whereas I-
210 E systems never appeared in B2 isolates. However, lone CRISPR3 arrays
211 without *cas* genes or CRISPR4 were detected in phylogroups A, D, E and F
212 (Table S1). Our results show that B2 isolates are more common in the

213 susceptible group (65.4%) than in the resistant group (49.0%). Since Type I-F
214 systems only appear in the B2 group, we analysed Type I-F presence within
215 the B2 group only and found a significant difference between susceptible and
216 MDR resistant isolates within B2 ($p = 0.0001$) (Figure 3). Further, MLST
217 analysis was performed on all isolates with Type I-F systems as well as 10
218 randomly selected susceptible isolates without Type I-F CRISPR. All isolates
219 underwent PCR screening for the O25b-ST131 epidemic clone.¹³ The MLST
220 types of Type I-F positive isolates suggested clustering within particular STs,
221 with nine of the 20 isolates with Type I-F systems belonging to the ST95
222 clonal complex, including one of the resistant isolates with Type I-F systems.
223 The second most common sequence type among Type I-F isolates was
224 ST141 (3/20). Plasmid-corresponding spacers were largely limited to these
225 two sequence types (Figure 2). The MLST types of isolates without I-F
226 CRISPR systems were representative of urinary *E. coli* found in other studies
227 (Table S1).¹⁹

228 A previous study found that unlike the Type I-E system, Type I-F genes
229 can be expressed under laboratory conditions.⁹ We therefore used RT-PCR to
230 investigate the expression of the *csy1* and *cas1* genes at the log phase of
231 growth in 7 Type I-F isolates (6 from the susceptible group and 1 from the
232 MDR group). These two genes are the first in the two putative transcriptional
233 units of the Type I-F *cas* genes.⁹ In all seven of the Type I-F strains tested,
234 both transcriptional units were expressed in the log phase of growth.

235 In this work we show that the presence of the Type I-F CRISPR
236 systems is strongly associated with antimicrobial susceptibility in *E. coli*.
237 Reinforcing previous research,^{5, 16} we also demonstrate that Type I-F systems

238 are only typically associated with the B2 phylogenetic group. However, our
239 results are in contrast to a previous study, which did not show an association
240 between the distribution of CRISPR and antimicrobial resistance plasmids in
241 *E. coli*.²⁰ When only considering the B2 group, which is the only group that
242 can contain Type I-F genes, the presence of Type I-F system is still strongly
243 associated with antimicrobial susceptibility. We also show that Type I-F genes
244 are expressed in a number of clinical isolates of *E. coli* and therefore
245 theoretically capable of interfering with antimicrobial resistance plasmids.
246 Indeed, a study by Almendros *et al.* demonstrated that an isolate with an
247 expressed Type I-F system was also capable of interfering with plasmid
248 constructs containing matching spacers.⁹

249 Previous work has shown that some B2 lineages such as ST131 are
250 associated with antimicrobial resistance.^{21, 22} None of the ST131 isolates
251 included within our study contained Type I-F systems. What was more striking
252 was the finding that CRISPR3 and CRISPR4 arrays incorporated spacer
253 sequences derived from IncFII and IncI1 plasmid scaffolds commonly linked
254 to resistant *E. coli* clones such as ST131. ST95 strains are often
255 underrepresented in resistant groups of *E. coli*^{23, 24} and our data suggest that
256 CRISPR may be a contributing factor, given that 9/20 of the Type I-F positive
257 isolates reported here belonged to the ST95 clonal complex. We suggest that
258 B2 strains with active Type I-F CRISPR systems may be interfering with the
259 uptake or survival of antimicrobial resistance plasmids within the isolate,
260 hence helping to keep them susceptible to antimicrobials.

261 The observation that some of these spacers still persist in multiple
262 isolates and different sequence types may be an indication that they are

263 advantageous, particularly since the spacers can correspond to more than
264 one plasmid. In environments where antimicrobials are scarce or absent,
265 plasmids may confer a fitness cost,²⁵ and in these conditions, B2 strains with
266 Type I-F systems may have an advantage. While we did find two MDR
267 isolates with Type I-F spacers that correspond to antimicrobial resistant
268 plasmids, this could be explained by the fact that CRISPR systems have been
269 shown to have leakage and are not functionally perfect even with exact
270 spacer matches and optimal PAMs.^{9, 26} There is also the possibility that the
271 system has been deactivated as reported in *Staphylococcus epidermis*.²⁵

272 Taken together our findings suggest a role for Type I-F CRISPR in the
273 distribution of antimicrobial resistance among *E. coli* B2 lineages.

274

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287 **Transparency declarations**

288 None to declare.

289

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365

366 **Tables**

367

Name	Sequence (5' to 3')	Purpose	Source
C1 fw	GTTATGCGGATAATGCTACC	CRISPR screening	5
C1 rev	CGTAYYCCGGTRGATTTGAA		
C2 fw	AAATCGTATGAAGTGATGCAT	CRISPR screening	5
C2 g rev	TCGATAATTGTGAACYTMTC		
C3 fw	GCGCTGGATAAAGAGAAAAAT	CRISPR screening	5
C3 rev	GCCCACCATTACCTGTA		
C4 fw	CTGAACAGCGGACTGATTTA	CRISPR screening	5
C4 rev	GTACGACCTGAGCAAAG		
Csy1 fw	TCAGTCATGGTGATTCT	cas gene screening & RT-PCR	9
Csy1 rev	GCAACAGGGAAATAGA		
Cas1 fw	CGGGGTGATGGTAGGCTTTT	cas gene screening & RT-PCR	This study
Cas1 rev	TGGTTTTCTGCCGCGTCTAT		
RPSL fw	CTCGCAAAGTTGCGAAAAGC	RT-PCR control	17
RPSL rev	TTCACGCCATACTTGGAACG		

368

369 **Table 1.** Oligonucleotides used for CRISPR screening and gene expression

370 studies. Oligonucleotides used for phylogenetic grouping, MLST and plasmid

371 replicon typing are primers not included.

372

Name of spacer	CRISPR array	Sequence (5' to 3')	No. of isolates	Protospacer match
Spacer1	CRISPR3	AGCATCTGCATGGTGC CCGTGGTCTTAACAAG	1	IncFII/FIB plasmids
Spacer2	CRISPR3	TGATGGCGCAGCAGTC CTCCCTCCTGCCGCCA	13	Non-coding region of IncI1 and IncFII plasmids
Spacer3	CRISPR3	CTGAACGTTGAAGAGT GCGACCGTCTCTCCTT	20	Putative anti-restriction protein KlcA on IncI1 and IncFII plasmids
Spacer4	CRISPR3	GGAAGAGACGGATGTT GACCAGCGAAATCCGA	1	Hypothetical protein found on IncFII and IncI1 plasmids
Spacer5	CRISPR4	TGTGGCGCTGATGCGT CTGGGCGTCTTTGTAC	8	<i>repA</i> gene of IncFIB plasmids

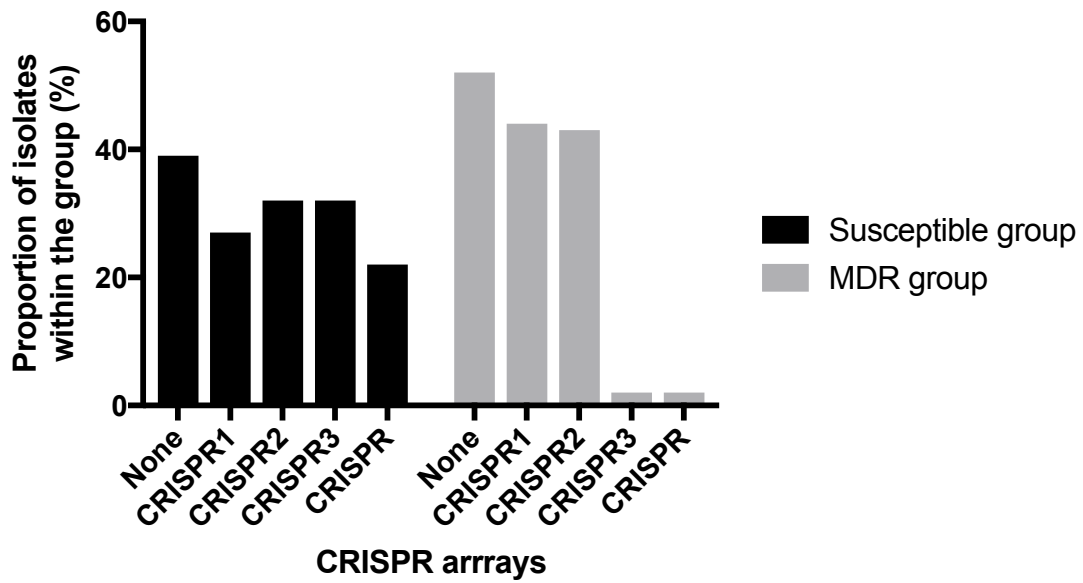
373

374 **Table 2.** Spacer sequences matching antimicrobial resistance plasmids. Five
375 spacers that correspond to plasmid sequences were found using nucleotide
376 BLAST in CRISPR3 and CRISPR4 containing strains, including isolates which
377 had repeat arrays but no I-F genes.

378

379

380 **Figures**



381

382 **Figure 1.** Proportion of *E. coli* isolates with CRISPR 1, 2, 3 and 4 repeat
383 arrays within the susceptible and MDR groups. Overall the two groups had a
384 significantly different distribution of CRISPR arrays ($P < 0.0001$). CRISPR 1 &
385 2 and CRISPR 3 & 4 are often, but not always, found in pairs.

386

387

CRISPR3 arrays	CRISPR4 arrays	No. of isolates	Sequence types
		3	ST80, ST5351
		1	ST141
		1	ST141
		1	ST141
		3	ST95, ST5168
		2	ST95
		1	ST95
		1	ST95
		1	ST95
		1	ST421 (ST95 complex)
		1	ST2015
		1	ST420
		1	ST2582
		1	ST4075
		1	ST4075

388

389 **Figure 2.** CRISPR3 and CRISPR4 array profiles found in isolates containing

390 Type I-F genes. Each box represents a spacer sequence. Isolates of the

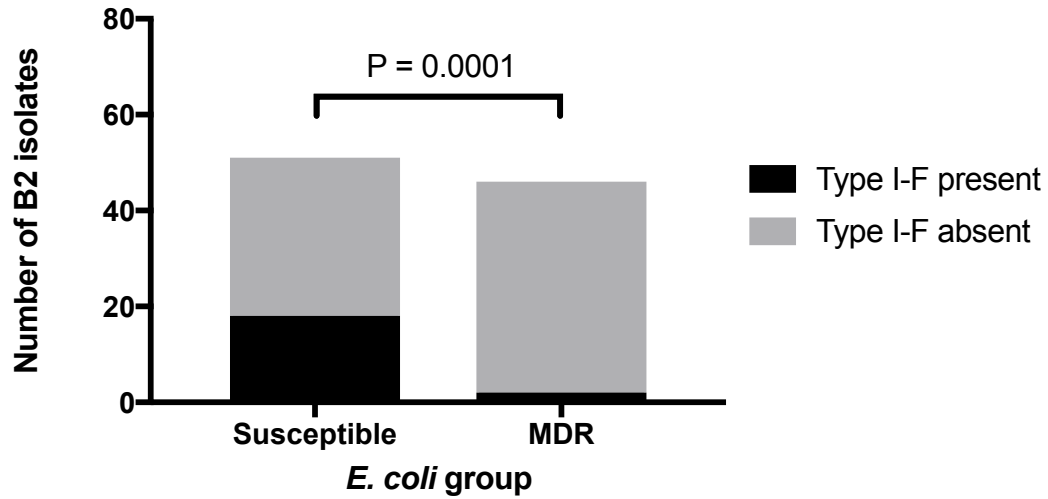
391 same sequence type have similar spacer profiles but often with missing or

392 additional spacers. Shaded spacers correspond to known antimicrobial

393 resistance plasmids listed in Table 2. MLST types of all isolates with complete

394 Type I-F systems are listed

395



396

397 **Figure 3.** Presence of Type I-F systems in susceptible and resistant B2

398 isolates. 'Type I-F systems' are defined here as presence of both Type I-F

399 repeat arrays and cas genes.

400

401