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1 **Comprehensive analysis of type 1 fimbriae regulation in *fimB*-null strains**
2 **from the multidrug resistant *Escherichia coli* ST131 clone**

3

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22

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29 **Summary**

30 Uropathogenic *Escherichia coli* (UPEC) of sequence type 131 (*E. coli* ST131) are a pandemic
31 multidrug resistant clone associated with urinary tract and bloodstream infections. Type 1
32 fimbriae, a major UPEC virulence factor, are essential for ST131 bladder colonization. The
33 globally dominant sub-lineage of ST131 strains, clade C/H30-R, possess an ISEc55 insertion in
34 the *fimB* gene that controls phase-variable type 1 fimbriae expression via the invertible *fimS*
35 promoter. We report that the inactivation of *fimB* in these strains causes altered regulation of type
36 1 fimbriae expression. Using a novel molecular approach, we demonstrate that 'off' to 'on' *fimS*
37 inversion is reduced in these strains and mediated by the *fimE* and *fimX* genes. Unlike typical
38 UPEC strains, the nucleoid-associated H-NS protein does not strongly repress *fimE* transcription
39 in these strains. Using a genetic screen to identify novel regulators of *fimE* and *fimX* in the clade
40 C/H30-R ST131 strain EC958, we defined a new role for the *guaB* gene as a regulator of type 1
41 fimbriae and a mouse bladder colonization factor. Our results provide a comprehensive analysis
42 of type 1 fimbriae regulation in the globally predominant group of ST131 strains, and highlight
43 important differences in its control compared to non-ST131 UPEC.

44 **Introduction**

45 Uropathogenic *Escherichia coli* (UPEC) are the leading cause of urinary tract infection (UTI),
46 resulting in over 150 million cases worldwide every year (1) and amounting to billions of dollars
47 spent in direct and associated healthcare costs (2). The increased incidence of UTIs caused by
48 multidrug resistant (MDR) strains, including strains that belong to high-risk globally pandemic
49 clones such as *E. coli* sequence type 131 (*E. coli* ST131), presents significant new challenges for
50 the management and treatment of UTI.

51
52 *E. coli* ST131 is a globally disseminated MDR clone originally identified due to its close
53 association with the spread of the *bla*_{CTX-M-15} extended spectrum β -lactamase (ESBL) gene (3-5).
54 Genomic analysis of *E. coli* ST131 has identified a globally dominant fluoroquinolone resistant-
55 *fimH30* subgroup previously defined as *H30-R* (6) or clade C (7), as well as two additional less
56 prevalent clades (referred to as A and B) (7). Type 1 fimbriae represent the best-characterized *E.*
57 *coli* ST131 virulence factor and are required for ST131 colonization of the mouse bladder (8). Its
58 pharmacological inhibition has been shown to prevent the establishment of acute MDR UTI and
59 treat chronic bladder infection in mice (9).

60
61 The *E. coli* type 1 fimbriae (*fim*) gene cluster contains nine genes, which encode the major (FimA)
62 and minor (FimFGH) structural components, the chaperone-usher transport and assembly apparatus
63 (FimCD) and two regulatory proteins (FimB and FimE) (10, 11). Type 1 fimbrial expression is
64 phase variable due to inversion of the *fim* switch (*fimS*), a 314 bp invertible DNA element which
65 contains a promoter that drives transcription of the *fimACDFGH* genes (12). 'On' or 'off'
66 orientation of *fimS* results in a fimbriated or bald phenotype, respectively. Two tyrosine-like

67 recombinases, FimB and FimE, catalyze the inversion of *fimS* (13). The FimB recombinase
68 possesses bidirectional switching activity ('off'-to-'on' and 'on'-to-'off'), while FimE primarily
69 catalyzes 'on'-to-'off' *fimS* inversion (13, 14). FimE can also mediate 'off'-to-'on' inversion of *fimS*
70 under some growth conditions, but at lower efficiency (15, 16). The transcription of the *fimB* and
71 *fimE* genes is driven by individual promoters, both of which are repressed by the histone-like
72 nucleoid associated protein H-NS in *E. coli* K-12 strains (17). The inversion of *fimS* is subject to a
73 complex regulatory network that involves several other global regulators, including integration host
74 factor (IHF) (18) and the leucine-responsive regulatory protein (Lrp) (19), as well as other proteins
75 that directly or indirectly impact on *fimS* orientation (reviewed in (20)). The 'off'-to-'on' switching
76 of *fimS* is enhanced by growth in static liquid culture, which results in the enrichment of type 1
77 fimbriated cells and the formation of a pellicle at the air-liquid interface (21-23).

78
79 In addition to *fimB* and *fimE*, three other genes encoding tyrosine-like recombinases have been
80 identified in UPEC (24). The products of two of these genes, FimX (24-26) and IpuA (24) can
81 mediate *fimS* inversion. It has been shown that FimX possesses specificity for *fimS* 'off' to 'on'
82 inversion, while IpuA can mediate bidirectional inversion (24, 26). In the reference UPEC strain
83 UTI89, FimX mediates slow 'off' to 'on' *fimS* inversion *in vitro*, but rapid *fimS* 'on' switching
84 during infection of the mouse bladder (25). The *fimX* gene is located adjacent to the *hyxR* gene at a
85 site distal to the *fim* genes on the chromosome (25, 27). An invertible DNA switch analogous to
86 *fimS* lies between the *fimX-hyxR* genes (27). FimX-mediated inversion of this *in cis* cognate
87 switch influences the transcription of *hyxR*, which encodes a LuxR-like response regulator that
88 controls tolerance to reactive nitrogen intermediates (27).

89

90 *E. coli* ST131 strains from the globally dominant clade C/H30-R group (hereafter referred to as
91 clade C) possess a 1,895bp insertion element within the *fimB* recombinase gene (*fimB*::*ISEc55*;
92 Fig. 1) (8, 28). Given the role of *fimB* in *fimS* ‘on’ switching, it is therefore likely that the
93 presence of this insertion affects type 1 fimbriae expression in ST131. Indeed, the *fimB*::*ISEc55*
94 insertion in ST131 has been associated with a slower ‘off’-to-‘on’ switching phenotype *in vitro*
95 (8).

96
97 In this study, we investigated the impact of the *fimB*::*ISEc55* insertion on type 1 fimbriae
98 expression by comparing ST131 strains that possessed either an intact or disrupted *fimB* gene.
99 We confirmed that strains containing the *fimB*::*ISEc55* insertion display slow ‘off’ to ‘on’ type 1
100 fimbriae switching phenotype and demonstrated its association with increased transcription of
101 the *fimE* gene. Notably, H-NS repression of *fimE* in these strains was also altered compared to
102 non-ST131 strains, highlighting an important difference in type 1 fimbriae regulation between
103 ST131 strains and other well-characterized UPEC reference strains. We also examined the role
104 of the FimE and FimX recombinases in *fimS* inversion in the ST131 clade C reference strain
105 EC958, and described the use of a novel molecular approach based on Illumina deep sequencing
106 to quantitate *fimS* orientation. Finally, we show that in EC958, guanosine 5' triphosphate (GTP)
107 homeostasis and the bacterial stringent response triggered by the (p)ppGpp alarmone contribute
108 to type 1 fimbriae expression via *fimE* and *fimX* regulation.

109 **Results**

110 *Most E. coli ST131 strains can express type 1 fimbriae regardless of their fimB status*

111 We previously sequenced a large collection of *E. coli* ST131 isolates from different geographical
112 locations (7), including the high-quality reference genome of EC958, a representative clade C
113 strain (8, 29). Despite most strains encoding intact and seemingly functional type 1 fimbriae
114 operons, type 1 fimbriae expression was not a conserved trait among *E. coli* ST131 strains under
115 standard laboratory growth (7, 8). This variability in type 1 fimbriae expression was significantly
116 associated with the presence of an insertion element within the *fimB* recombinase gene that was
117 identified in most clade C ST131 strains studied by us and others (7, 8, 28). Here, we extended
118 these findings in a larger collection of 91 ST131 strains (71 previously published, detailed in
119 Materials and Methods) for which we provide a comprehensive type 1 fimbriae expression
120 profile. In this collection, 57 strains contained the *fimB::ISEc55* insertion (and were from clade
121 C) and 34 contained an intact *fimB* gene (and were from clades A and B). Using yeast cell
122 agglutination, a standard method for monitoring the production of mannose-sensitive fimbriae
123 (30), we determined type 1 fimbriae expression following growth in different conditions (Table
124 1). Following overnight shaking growth in LB broth at 37°C, only 14% of the *fimB::ISEc55*
125 containing strains expressed type 1 fimbriae compared to 70.6% of strains with an intact *fimB*
126 gene, $\chi^2(1, N = 91) = 29.87, P < 0.0001$. This significant difference was also observed following
127 overnight static growth, $\chi^2(1, N = 91) = 9.063, P < 0.01$, but no significant association was seen
128 between *fimB* status and type 1 fimbriae expression when strains were further statically
129 subcultured (Table 1; days 2 and 3). By three successive rounds of static subculture, the majority
130 of ST131 strains were positive for type 1 fimbriae expression irrespective of *fimB* gene status.
131 However, a step-wise increase in the proportion of type 1 positive strains was only seen for those

132 containing the *fimB::ISEc55* insertion, suggesting a slower rate of ‘on’ switching for most of
133 these strains.

134

135 In our analysis, ten strains (five with an intact *fimB* and five with a *fimB::ISEc55* insertion)
136 remained negative for type 1 fimbriae expression and were further subjected to six additional
137 rounds of 24-hour static subculture. This resulted in the expression of type 1 fimbriae by four
138 strains, three out of which had an intact *fimB* gene. Of the six remaining strains (S1, S18, S27,
139 S36, S61, S77), S36 (Supporting Information Fig. S1) and S77 (7) had large deletions within
140 coding regions of the type 1 fimbrial operon, accounting for their negative yeast agglutination
141 phenotype. To test the capacity of *fimS* to invert to an ‘on’ orientation in S1, S18, S27 and S61,
142 each strain was transformed with a plasmid containing the *fimB* gene from CFT073 (pFimB) and
143 subjected to static culture followed by a screening PCR to determine *fimS* orientation (24). Over-
144 expression of FimB in this manner resulted in *fimS* inversion to an ‘on’ orientation in three of the
145 strains (S1, S18 and S61), however functional type 1 fimbriae expression as assessed by yeast
146 cell agglutination could only be detected for S18 (Table 2). Sequence analysis of *fimS* from S27
147 revealed a single cytosine deletion (Supporting Information Fig. S2) 8bp downstream of the left
148 inverted repeat in *fimS* (31), which may account for the ‘locked off’ status of *fimS* in this strain.

149

150 *The transcription of *fimE* is enhanced in ST131 strains with a *fimB::ISEc55* insertion*

151 We hypothesized that the *fimB::ISEc55* insertion may alter the transcription of the downstream
152 *fimE* recombinase gene. In order to test this, we examined the level of *fimE* transcription using
153 qRT-PCR in EC958 as well as four additional clade C ST131 strains with the same *fimB::ISEc55*
154 insertion (S4, S54, S60, S88), and compared this to the level of *fimE* transcription in four ST131

155 strains with an intact *fimB* gene (S17, S31, S55, S90). Strains were grown in M9 minimal
156 glucose medium with aeration to mid-log phase for analysis. In all cases, the level of *fimE*
157 transcription was significantly higher in strains with the *fimB*::*ISEc55* insertion compared to
158 strains with an intact *fimB* gene (overall 6.26-fold increase in *fimE*; Fig. 2A; $P < 0.05$).

159

160 FimX is another accessory tyrosine recombinase that contributes to turning 'on' *fimS* in UPEC
161 (32). Given that all nine ST131 strains used for this analysis also screened positive for the *fimX*
162 gene by PCR, *fimX* transcript levels were also examined. There was no significant difference in
163 *fimX* transcription between the two strain sets (Fig. 2B).

164

165 *The fimB::ISEc55 insertion does not contain an additional fimE promoter*

166 The transcription start site (TSS) of *fimE* has previously been mapped to a T residue 166 bases
167 upstream of the GTG start codon in the *E. coli* K-12 strain VL751 (17). As we observed
168 significantly higher *fimE* transcript levels in ST131 strains with the *fimB*::*ISEc55* insertion, we
169 hypothesized that there may be an additional promoter located within the insertion element that
170 could drive *fimE* transcription. The *fimE* TSS was mapped using 5' RACE to a G residue 164
171 bases upstream of the start codon in all five of the *fimB*::*ISEc55* ST131 strains tested (EC958,
172 S4, S54, S60, S88; Fig. 3). As a control, we also mapped the TSS of *fimE* in one ST131 strain
173 with an intact *fimB* gene (S90), and showed that it was identical. A consensus -35 (TTGTTA)
174 and -10 (AAAATA) promoter sequence, separated by 18 bp, was conserved in all ST131 strains
175 examined.

176

177 Since the TSS was conserved in all ST131 strains tested, consensus sequences of *fimE* promoter
178 and coding regions (1074 bp) from clades A, B and C strains were constructed from previously
179 published genome sequences (7) to ascertain if there were any differences that could explain the
180 higher *fimE* transcription observed in clade C strains. These were compared to corresponding
181 *fimE* sequences from two non-ST131 UPEC strains (CFT073 and UTI89) and *E. coli* K-12 strain
182 MG1655 by multiple sequence alignment (Supporting Information Fig. S3). Sequences from
183 ST131 clade A and C strains were 100% identical and closely related to *fimE* from MG1655. In
184 contrast, the sequence of the *fimE* promoter and coding region from ST131 clade B strains
185 contained several nucleotide changes, and was most similar to the corresponding sequence from
186 CFT073 and UTI89. The most parsimonious explanation for our observation in light of the
187 known phylogeny of ST131 (7) is an independent recombination in the *fimE* region of clade B
188 strains after divergence from clade C. This is distinct from the previously reported *fimD-uxuR*
189 recombination event in clade C that encompassed the *fimH30* allele (28). Nucleotide changes in
190 the coding region were synonymous with only a single amino acid substitution (V198A) found in
191 FimE from clade B ST131 strains (also present in UTI89, Supporting Information Fig. S3).
192 These changes, however, are unlikely to account for the increased *fimE* transcript levels observed
193 in strains with the *fimB::ISEc55* insertion, as this is exclusively found in clade C ST131 strains.

194

195 *FimE and FimX are responsible for inverting fimS to the 'on' orientation in EC958*

196 Despite the lack of an intact *fimB* gene, the majority of clade C ST131 strains still expressed
197 functional type 1 fimbriae after multiple rounds of static growth, indicating that they possess an
198 alternative recombinase capable of switching 'on' *fimS*. Based on genomic analysis, EC958
199 contains two tyrosine-like recombinase genes that have been shown to mediate inversion of *fimS*

200 in other *E. coli* strains; namely *fimE* (15) and *fimX* (24-26). We therefore constructed a series of
201 isogenic mutants in EC958 lacking *fimE* (EC958*fimE*), *fimX* (EC958*fimX*) or both genes
202 (EC958*fimE fimX*) and monitored their capacity to express type 1 fimbriae by yeast agglutination
203 and western blot analysis (using an antibody against the major fimbrial subunit FimA) following
204 static growth with repeated subculture over a period of 5 days (Fig. 4).

205

206 Type 1 fimbriae expression was detected in EC958 following 3 successive rounds of 24-hour
207 static growth. In contrast, type 1 fimbriae expression by EC958*fimE* was delayed and only
208 observed after 4 successive rounds of static growth, demonstrating a direct role for FimE in ‘off’
209 to ‘on’ *fimS* inversion (Fig. 4A). This also implicated a role for FimX in *fimS* inversion, and
210 although the single EC958*fimX* mutant demonstrated a type 1 fimbriae expression profile
211 identical to wild-type, the EC958*fimE fimX* double mutant failed to express type 1 fimbriae after
212 prolonged static subculture for up to 5 days (Fig. 4A). This result was further validated by
213 complementation of the EC958*fimE fimX* double mutant with a plasmid containing either *fimE* or
214 *fimX* (pFimE or pFimX), both of which resulted in rapid type 1 fimbriae production within two
215 days of static subculture (Fig. 4B). Thus, our data demonstrate that FimE is the major
216 recombinase responsible for ‘off’ to ‘on’ *fimS* inversion in EC958, and that FimX can also
217 mediate this switching mechanism, albeit less efficiently than FimE during *in vitro* static growth.

218

219 *Direct quantitation of fim switching in EC958 using Illumina sequencing*

220 In order to quantitate the efficiency of FimE- and FimX-mediated *fimS* inversion in EC958, we
221 utilized a novel read-mapping method based on Illumina sequencing data that we refer to as
222 DNA Invertible Switch Counter (or DISCus). EC958, EC958*fimE* and EC958*fimX* were grown

223 under static conditions as described in the previous section. Aliquots from the air-liquid interface
224 of each subculture were harvested on days 1-5, and genomic DNA was extracted and sequenced
225 using Illumina Technology. Using DISCUS, we determined the percentage of reads that
226 corresponded to either the 'on' or 'off' orientation of *fimS* at each time point by read mapping to
227 *fimS* pseudo-reference sequences representing both orientations. The starting inoculum for these
228 experiments was an overnight shaking culture, which possessed only 1% *fimS* 'on' reads. In the
229 static subculture time course, EC958 produced a steadily increasing *fimS* 'on' switching profile,
230 with a maximum of 71% 'on' reads at day 5 (Fig. 5A). EC958*fimX* produced a similar *fimS*
231 switching profile, with the exception that 'on' switching was slower on day 2 compared to wild-
232 type (Fig. 5A). In contrast, *fimS* 'on' switching in EC958*fimE* occurred at a significantly slower
233 rate. Only 11.6% of 'on' reads were detected at day 4 of static subculture, which then increased
234 to a level comparative to EC958 and EC958*fimX* at day 5. DISCUS analysis of the EC958*fimE*
235 *fimX* double mutant did not identify any significant *fimS* 'on' switching, supporting our
236 observation that FimE and FimX are the only recombinases capable of inverting *fimS* in EC958.
237 Overall, the quantitative *fimS* switching data closely paralleled the type 1 fimbriae expression
238 analysis for each strain based on yeast agglutination and FimA protein production (Fig. 4).

239
240 To examine the specificity of FimE and FimX for *fimS* inversion in greater detail, we
241 complemented the EC958*fimE fimX* double mutant by introduction of a plasmid containing *fimE*
242 (pFimE) or *fimX* (pFimX). The EC958*fimE fimX* (pSU2718, empty vector control), EC958*fimE*
243 *fimX*(pFimE) and EC958*fimE fimX*(pFimX) strains were subcultured by static growth over 3
244 days as described above, and DISCUS was used to analyze the *fimS* orientation from Illumina
245 sequences generated at each time point. Interestingly, data from these experiments demonstrated

246 that when the recombinases were over-expressed in this manner, FimX exhibited greater
247 propensity for *fimS* ‘off’ to ‘on’ inversion than FimE (Fig. 5B).

248

249 *FimE does not invert the fimX switch in EC958*

250 The *fimX* switch, located between the *fimX-hyxR* genes, comprises two 16 bp inverted repeats
251 flanking a 278 bp central region (27). In addition to mediating *in trans* inversion of *fimS* (27),
252 FimX also mediates *in cis* bidirectional inversion of this switch. To assess this in greater detail,
253 we analyzed the *fimX* switch inversion rate from the sequence data generated above. In the
254 experiments that involved repeated static subculture of EC958, EC958*fimE* and EC958*fimX*, the
255 *fimX* switch remained primarily in the ‘off’ orientation (92-97% ‘off’) throughout the 5-day
256 static subculture period (Fig. 6A). However, in the experiments involving subculture of the
257 complemented strains EC958*fimE fimX*(pFimE) and EC958*fimE fimX*(pFimX), and the control
258 strain EC958*fimE fimX*(pSU2718), we observed very rapid FimX-mediated *fimX* switch
259 inversion within a day (Fig. 6B). Taken together, these results demonstrate a rapid and strong
260 interaction of FimX with its cognate switch. Furthermore, these results also demonstrate for the
261 first time that FimE is unable to invert the *fimX* switch in EC958.

262

263 *H-NS is not a major repressor of fimE gene expression in EC958*

264 The histone-like nucleoid structuring protein (H-NS) is a global transcriptional regulator (33). H-
265 NS has been shown to directly regulate type 1 fimbriae expression in *E. coli* K-12 by binding to
266 sequences adjacent to and within *fimS* (34) and also to the promoters of *fimB* and *fimE* (17, 35).
267 At 37°C, H-NS down-regulates *fimE* transcription and *hns* deletion leads to increased *fimE*
268 promoter activity (35). We constructed a *hns* deletion in EC958 and three non-ST131 UPEC

269 strains (UTI89, 536 and IHE3034) and measured *fimE* transcript levels of all wild-type strains
270 and corresponding *hns* mutants by qRT-PCR. At 37°C, deletion of *hns* led to significantly
271 increased *fimE* transcription compared to the wild-type in all non-ST131 UPEC strains (Fig. 7B-
272 D). However, in EC958, there was only a relatively small increase in *fimE* transcription in the
273 *hns* mutant compared to the wild-type (Fig. 7A). Thus, H-NS does not strongly repress *fimE*
274 transcription in EC958.

275

276 *Identification of genes that affect fimE and fimX promoter activity in EC958*

277 In order to investigate the regulation of the *fimE* and *fimX* genes in EC958, two promoter-
278 reporter strains were generated. The *lacIZ* genes of EC958 were initially inactivated to generate
279 strain EC958*lac*, and this strain was subsequently modified by inserting the *lacZ* gene as a
280 chromosomally located transcriptional fusion to the *fimE* (EC958*fimE::lacZ*) or *fimX*
281 (EC958*fimX::lacZ*) promoter. When grown on LB agar containing X-gal, EC958*fimE::lacZ*
282 colonies were pale blue (indicating weak transcription) and EC958*fimX::lacZ* colonies were
283 white (indicating no transcription). The two reporter strains were then subjected to Tn5
284 mutagenesis; in each case 50,000-60,000 transposon mutants were generated and visually
285 screened based on their blue colour intensity following growth on LB X-gal agar. Individual
286 colonies that were dark blue (compared to the control strain) were selected and further analyzed
287 for β -galactosidase activity. Genes identified to contain transposon insertions that altered the β -
288 galactosidase activity of EC958*fimE::lacZ* or EC958*fimX::lacZ* are listed in Tables 3 and 4,
289 respectively. Strikingly, multiple independent mutants containing insertions in the *guaB* gene
290 were identified for both EC958*fimE::lacZ* and EC958*fimX::lacZ*; thus the remainder of this
291 study focused on understanding the regulatory role of *guaB* on *fimE* and *fimX* transcription.

292

293 *Deletion of guaB in EC958 leads to up-regulation of the fimE and fimX recombinase genes but*
294 *slower fimS 'on' switching*

295 In order to study how *guaB* regulates *fimE* and *fimX*, we constructed defined deletion mutants in
296 EC958*fimE::lacZ* and EC958*fimX::lacZ* to generate the following strains: EC958*fimE::lacZ guaB*
297 and EC958*fimX::lacZ guaB*. Activity from the *fimE* and *fimX* promoters was measured by
298 determining the β -galactosidase activity from control and *guaB* mutant strains (Fig. 8A, B).
299 Deletion of *guaB* resulted in significantly increased promoter activity from both
300 EC958*fimE::lacZ guaB* (2-fold, $P < 0.05$, Fig. 8A) and EC958*fimX::lacZ guaB* (1.5-fold, $P < 0.05$,
301 Fig. 8B). Furthermore, complementation of the mutant strains by over-expressing *guaB in trans*
302 (via plasmid pGuaB) resulted in promoter activity comparable to the parental controls. These
303 results strongly suggest that the *guaB* gene product contributes to the regulation of *fimE* and *fimX*
304 in EC958.

305

306 To further assess the effect of *guaB* on *fimS* inversion, we generated an EC958*guaB* mutant and
307 determined its switching profile using DISCUS. Static subculture of EC958 and EC958*guaB* was
308 performed over a 5-day period as described in previous sections. DISCUS analysis of *fimS* was
309 performed on samples at days 1, 3 and 5. Analysis of samples taken at days 3 and 5 revealed a
310 significantly slower 'on' switching profile for EC958*guaB* compared to EC958 (Fig. 8C).
311 Furthermore, this slower *fimS* switching rate was restored to wild-type level following
312 complementation of EC958*guaB* with plasmid pGuaB (Fig. 8C). Taken together, this data
313 strongly supports a role for the *guaB* gene in *fimS* inversion.

314

315 *The effect of guaB deletion on fimE and fimX transcription is partially dependent on GTP*
316 *homeostasis and mimics (p)ppGpp stress response conditions*

317 Guanosine tetra- and pentaphosphate, also known as (p)ppGpp, is a stress response alarmone that
318 has been shown to increase type 1 fimbriae expression by up-regulating *fimB* and to a lesser
319 extent, *fimE* transcription in *E. coli* (36). GuaB catalyzes the rate-limiting step in GMP
320 biosynthesis and its deletion depletes the intracellular GTP pool (37, 38). Production of large
321 amounts of (p)ppGpp can also result in a sharp decline in the intracellular GTP level both by
322 direct GTP consumption and by (p)ppGpp-mediated GuaB inhibition (38, 39). Thus, (p)ppGpp is
323 involved in regulating intracellular GTP homeostasis (38).

324

325 We hypothesized that the effect of *guaB* deletion on *fimE* and *fimX* transcription in EC958 may
326 be mediated through (p)ppGpp and tested this using pooled human urine as a growth medium.
327 We predicted that *guaB* deletion would induce the stringent response as a result of reduced
328 cellular GTP and trigger (p)ppGpp production by the action of purine salvage pathways (40)
329 utilizing guanine and guanosine species present in human urine (41, 42). Elevated levels of
330 (p)ppGpp, in turn, would cause increased transcription of *fimE* and *fimX*. To test this, we first
331 used a sub-inhibitory concentration of serine hydroxamate (SHX, 0.2 mM) to induce (p)ppGpp
332 generation within the cell via depletion of serine-charged t-RNA, leading to activation of the
333 amino acid starvation pathway (43). Stationary phase cultures of EC958*fimE::lacZ* and
334 EC958*fimX::lacZ* grown in the presence or absence of SHX were analyzed for β -galactosidase
335 activity (Fig. 9). The addition of SHX led to a significant de-repression of the *fimE* promoter
336 (Fig. 9A; $P < 0.05$), albeit not to the level seen in EC958*fimE::lacZ guaB*. No significant change
337 was observed in *fimX* promoter activity (Fig. 9B).

338

339 Next, we attempted to replenish the depleted intracellular GTP pool in *guaB* deletion reporter
340 strains by direct supplementation with GTP. The rationale behind this was that restoring
341 intracellular GTP levels would rescue the cell from stress, leading to a decline in (p)ppGpp
342 production and a subsequent decrease in *fimE* and *fimX* transcription. Thus, EC958*fimE::lacZ*
343 *guaB* and EC958*fimX::lacZ guaB* were grown in pooled human urine supplemented with 0.5 mM
344 GTP. For both strains, the addition of GTP led to a significant decrease in *fimE* ($P<0.01$) and
345 *fimX* ($P<0.05$) promoter activities to levels similar to those observed in the parent
346 EC958*fimE::lacZ* (Fig. 9C) and EC958*fimX::lacZ* strains (Fig. 9D). Taken together, these results
347 suggest that GTP depletion in a *guaB* mutant background mimics conditions observed during
348 (p)ppGpp stress and is at least partially responsible for increased transcription of *fimE* and *fimX*.

349

350 *The guaB gene is required for EC958 persistence in urine and bladder colonization in a mouse*
351 *UTI model*

352 In order to assess the contribution of *guaB* to the virulence of EC958, we tested the ability of the
353 EC958*guaB* mutant to survive in the mouse urinary tract using a competitive infection assay. We
354 employed an EC958*lac* strain as the wild-type to enable differentiation of both strains on
355 MacConkey lactose medium; EC958*lac* colonized the mouse bladder in equivalent numbers to
356 wild-type EC958 in a mixed competitive infection (Supporting Information Fig. S4A, B).
357 EC958*lac* and EC958*guaB* inocula were prepared by four successive rounds of static subculture
358 in LB broth (3x48 hours followed by 1x24 hours); under these conditions both strains expressed
359 similar levels of type 1 fimbriae (Supporting Information Fig. S4C). Female C57BL/6 mice were
360 co-inoculated with 1:1 ratio of EC958*lac* and EC958*guaB*, and the colonization of each strain

361 was assessed at 24 hours post infection. In these experiments, EC958*guaB* was significantly
362 outcompeted by EC958*lac* in the bladder ($P<0.01$) and urine ($P<0.05$) of infected mice (Fig. 10).

363

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364 Discussion

365 The emergence and rapid spread of the UPEC ST131 clone has been well documented since
366 2008 with epidemiological reports from all over the world. Like other UPEC, *E. coli* ST131
367 strains utilize type 1 fimbriae for colonization of the urinary tract. However, most ST131 isolates
368 from the globally predominant clade C sub-lineage possess an *ISEc55* insertion element within
369 the tyrosine-like recombinase *fimB* gene that is associated with reduced type 1 fimbriae
370 expression (8, 28). Here, we define the molecular basis of *fimS* inversion in this clinically-
371 relevant fluoroquinolone-resistant group of *E. coli* ST131 strains.

372
373 The high prevalence of the *fimB::ISEc55* insertion in ST131 strains from clade C led us to assess
374 the impact of this insertion on type 1 fimbriae expression. We have previously reported a delayed
375 ‘on’ switching phenotype of type 1 fimbriae expression in a subset of the strains used in the
376 current study (8). We therefore compared the type 1 fimbriae expression profile of a large
377 collection of ST131 strains that possessed an intact or disrupted *fimB* gene over multiple rounds
378 of static subculture. Overall, in accordance with previous observations (8), most ST131 strains
379 containing the *fimB::ISEc55* insertion were able to express type 1 fimbriae, but exhibited a
380 slower ‘off’ to ‘on’ switching profile during static growth. Interestingly, in the present analysis,
381 six strains were unable to express type 1 fimbriae after prolonged subculture, suggesting they
382 lack the capacity to make functional type 1 fimbriae. Two of these strains had large deletions in
383 the *fim* operon and transformation of the remaining four strains with a plasmid containing *fimB*
384 induced type 1 fimbriae expression in only one strain. All three remaining strains harboured the
385 *fimX* recombinase gene previously reported to switch *fimS* ‘on’ (24), indicating that they may
386 contain other mutations that disrupt this phenotype.

387

388 The presence of the *ISEc55* insertion element in *fimB* was associated with increased transcription
389 of the downstream *fimE* gene in the clade C representative EC958 strain and four other clade C
390 ST131 strains. Mapping of the *fimE* promoter in these strains revealed that *ISEc55* did not
391 contain an independent promoter driving *fimE* transcription. However, we did observe that
392 deletion of H-NS, a global transcriptional regulator and a known repressor of *fimE* (35), did not
393 have a major effect on *fimE* transcription in EC958. H-NS is known to bind preferentially to
394 intrinsically-curved DNA and AT-rich sequences, usually found in promoter regions, to exert
395 regulatory effects on its many target genes (44, 45). Our observations indicate an *in trans* effect
396 of *ISEc55* that could alter the local DNA structure and inhibit H-NS binding. Indeed, analogous
397 alterations in the transcription of genes adjacent to other insertion elements have been
398 documented in *E. coli* (46-48).

399

400 Analysis of *E. coli* ST131 genomes has revealed that the *fim* region is associated with
401 recombination (7, 28). Here, we provide a comprehensive analysis of type 1 fimbriae regulation
402 in fluoroquinolone-resistant clade C ST131 strains that contain the *fimB::ISEc55* insertion. The
403 expression of type 1 fimbriae by the majority of ST131 strains containing the *fimB::ISEc55*
404 insertion suggested that they possess an alternative recombinase/s capable of *fimS* inversion.
405 Using a combined mutagenesis and complementation strategy, we showed that both FimE and
406 FimX could mediate *fimS* inversion in EC958, with FimE demonstrating the greatest *fimS*
407 inversion activity under the static growth conditions employed in this study. We did not observe
408 any significant difference in *fimX* transcript levels in ST131 strains with an intact *fimB* and those
409 with the *fimB::ISEc55* insertion. We also developed DISCus, a novel read-mapping approach

410 based on Illumina sequencing, to quantitatively determine the percentage of *fimS* ‘on’ or ‘off’
411 populations. Our data suggest that *fimE* plays a more important role than *fimX* in regulating the
412 orientation of *fimS* during *in vitro* culture. This is in agreement with a previous report that
413 detected relatively low levels of FimX activity *in vitro*, but observed rapid expression of type 1
414 fimbriae upon experimental infection in strains with FimX as the only active recombinase (25).
415 The role of FimX in the inversion of *fimS* in EC958 during *in vivo* infection remains to be
416 elucidated. In this respect, DISCUS could be used to monitor *fimS* orientation in such populations.
417 We also note that DISCUS could be applied more broadly to analyze DNA invertible elements in
418 other bacteria.

419

420 To identify potential regulators of the *fimE* and *fimX* genes in EC958, we employed random
421 transposon mutagenesis of the *fimE* and *fimX* reporter strains and identified several genes that
422 when mutated caused increased activity of the *fimE* and *fimX* promoters. The *guaB* gene, which
423 encodes an enzyme that catalyzes the first rate limiting step in *de novo* GTP/GDP biosynthesis
424 and is responsible for maintaining GTP homeostasis (49), was identified in screens of both
425 reporter strains and was the focus of subsequent experimental analysis (see below). Mutation of
426 several other genes was also linked with increased *fimE* promoter activity (*yubO*, *pdxH*, *lrhA*,
427 *dprA*) or increased *fimX* promoter activity (*betA*, *yjjA*). The LysR family transcriptional regulator
428 *lrhA* has previously been shown to repress both *fimB* and *fimE* (50), and our identification of
429 *lrhA* in this study as a repressor of *fimE* is consistent with these results. The *yubO* gene is located
430 on a 135.6 kb IncF plasmid in EC958 and encodes a protein of unknown function (51). Three
431 independent Tn5 mutants were identified in *yubO*, however specific mutagenesis of *yubO* failed
432 to reproduce a significant increase in *fimE* transcription (data not shown). Thus, a definite role

433 for *yubO* in type 1 fimbriae regulation remains to be demonstrated. The *dprA* gene encodes a
434 putative DNA processing protein implicated in natural bacterial transformation (52) and has been
435 previously reported to be expressed *in vivo* during UTI (53). However, the molecular mechanism
436 by which *dprA* might affect *fimE* transcription, as well as the role of *pdxH* (encodes an enzyme
437 required for vitamin B6 biosynthesis (54)), *betA* (encodes a choline dehydrogenase (55)) and *yjjA*
438 (encodes a putative metal chaperone) in type 1 fimbriae expression, remains to be determined.

439
440 We focused our molecular investigation on the role of the *guaB* gene in *fimE* and *fimX*
441 regulation. While there is no direct link between *guaB* and expression of type 1 fimbriae in the
442 literature, *guaB* has been shown to be upregulated in women with UTI (53) and is important for
443 UPEC fitness in an experimental model of systemic infection (56). In other studies, the (p)ppGpp
444 alarmone, synthesized from GTP, has been shown to increase type 1 fimbriae expression in
445 *E. coli* (36). Strains lacking any (p)ppGpp exhibit reduced transcription from the *fimB* and *fimE*
446 promoters, although the effect on the latter gene is approximately half of that reported for *fimB*
447 (36). Under conditions of nutritional stress, (p)ppGpp controls global metabolic changes within
448 the cell (57-59) via direct interaction with RNA polymerase (60, 61) and activation of stress-
449 associated sigma factors (57). Synthesis of (p)ppGpp results in a reduction of the intracellular
450 GTP pool (62-64). Recently, it has been reported that (p)ppGpp directly inhibits various GTP
451 biosynthesis enzymes, thus further lowering the level of cellular GTP (38). We hypothesized that
452 deletion of *guaB* would produce a GTP deficient state mimicking that observed during the
453 activation of the stringent response pathway, and that this in turn would increase *fimE* and *fimX*
454 promoter activity. Chemical induction of this pathway in EC958 increased *fimE* promoter
455 activity and the addition of GTP to the culture media of *guaB* mutant strains significantly

456 reduced both *fimE* and *fimX* promoter activity. Taken together, these data support the hypothesis
457 that the cellular GTP concentration influences type 1 fimbriae expression by controlling the
458 transcription of *fimE* and *fimX*. We suggest that this effect is partly mediated by the action of the
459 (p)ppGpp alarmone on *fimE* promoter activity in strains that lack a functional copy of the *fimB*
460 gene. Indeed, DISCUS analysis of EC958*guaB* revealed an overall slower *fimS* ‘on’ switching
461 phenotype compared to the wild-type EC958, possibly due to the strong natural bias for FimE to
462 mediate ‘on’ to ‘off’ switching (14, 65). These findings are also consistent with our mouse
463 colonization data, which showed that an EC958*guaB* mutant was significantly outcompeted by
464 wild-type EC958 with respect to bladder colonization and persistence in urine.

465
466 In conclusion, this work has identified and characterized several novel molecular mechanisms
467 associated with the regulation of type 1 fimbriae in *E. coli* ST131 strains that harbour an *ISEc55*
468 insertion in the *fimB* recombinase gene. Our data demonstrate that type 1 fimbriae regulation in
469 these strains is different to that described for non-ST131 UPEC, highlighting the importance of
470 understanding the control of this essential virulence factor in the context of a clinically dominant
471 MDR resistant clone. Overall, the impact of this mutation on the fitness and global dissemination
472 of ST131 remains to be determined. However, given that the only non-ST131 strain shown to
473 harbour the *fimB::ISEc55* insertion to date is the probiotic Nissle 1917 strain (8), it is tempting to
474 speculate that such modulation of type 1 fimbriae expression may be associated with enhanced
475 colonization of the gut.

476

477 **Experimental Procedures**

478 *Ethics statement*

479 Approval for mouse infection studies was obtained from the University of Queensland Animal
480 Ethics Committee (SCMB/471/09/NHMRC (NF)). Experiments were carried out in strict
481 accordance with the recommendations in the Animal Care and Protection Act (Queensland,
482 2002) and the Australian Code of Practice for the Care and Use of Animals for Scientific
483 Purposes (8th edition, 2013). Approval for the collection of human urine was obtained from the
484 University of Queensland Institutional Human Research Ethics Committee (2015000347) and the
485 Griffith University Human Ethics Research Committee (MSC/06/08/HREC). Participation was
486 voluntary and all individuals provided informed consent prior to participation in the study.

487

488 *Bacterial strains and growth conditions*

489 The *E. coli* strains and plasmids used in this study are listed in Table S1 in the Supporting
490 Information. *E. coli* EC958 is a completely sequenced fluoroquinolone-resistant Clade C ST131
491 strain originally isolated in the United Kingdom in 2005 (7, 8, 29). Other *E. coli* ST131 strains
492 were isolated from urine samples collected at hospitals in Manchester and Preston, Northwest
493 England (n=54) and Brisbane, Australia (n=37). Most of the isolates (n=71) used in the present
494 study have been described previously (7, 8, 29, 66-68). The additional 21 strains were collected
495 from Brisbane, Australia as part of routine methods for UTI diagnosis. Strains were routinely
496 cultured at 37°C on solid or in liquid lysogeny broth (LB) unless otherwise specified. Culture
497 media was supplemented with appropriate antibiotics as required: gentamicin (Gent, 20 µg ml⁻¹),
498 chloramphenicol (Cm, 30 µg ml⁻¹). Human urine, when used as culture media, was pooled from
499 at least three healthy donors with no recent history of antibiotic use.

500

501 *DNA manipulations and genetic techniques*

502 Chromosomal DNA purification, PCR and DNA sequencing was performed as previously
503 described (69). PCR screening for the presence of *fimH* (70) and the insertion element within
504 *fimB* was performed as previously reported (8). The sequence type of UTI isolates used in this
505 study was determined by multilocus sequence typing as previously described (4, 66, 67). DNA
506 extraction for Illumina sequencing was performed using the Ultraclean® Microbial DNA
507 Isolation Kit (MO BIO Laboratories). Restriction enzymes were purchased from New England
508 Biolabs (Genesearch, Australia). All mutants, tagged strains and plasmid constructs were
509 confirmed by PCR followed by Sanger sequencing performed at the Australian Equine Genetics
510 Research Centre (Queensland, Australia). Illumina sequencing was performed at the Australian
511 Genome Research Facility (Melbourne, Australia).

512

513 *Construction of plasmids*

514 The plasmids used for *in trans* complementation were created by PCR amplification of the
515 respective gene from EC958 (*fimB* was amplified from CFT073) using primers listed in
516 Supporting Information Table S1. Relevant PCR products were digested with SacI-HindIII and
517 ligated into SacI-HindIII-digested pSU2718 (71). The cloned genes were under the control of a
518 *lac* promoter; cloned genes were induced by the addition of 1 mM IPTG (Sigma Aldrich) to the
519 culture medium.

520

521 *Construction of deletion mutants and complemented strains*

522 EC958 mutants were constructed using λ -Red mediated homologous recombination as
523 previously described (8) using primers listed in Table S1. A three-step PCR procedure was
524 employed for the generation of products with 500 bp homology regions used in recombination
525 (72). Plasmid pCP20 (73) was modified by the introduction of a gentamicin resistance cassette at
526 the NcoI site to yield pCP20-Gent. For complementation of mutants, removal of the FRT-site-
527 flanked chloramphenicol cassette was mediated by the Flp recombinase expressed by pCP20-
528 Gent. Mutant strains were then complemented by introduction of the respective genes cloned into
529 the low-copy number pSU2718 plasmid. All *lacZ* reporter-tagged transcriptional fusions were
530 generated in an *mKate2*-tagged EC958*lac* background using PCR products with a *lacZ-Cm*
531 cassette flanked by 500 bp homology regions specific to *fimE* or *fimX*.

532

533 *Type 1 fimbriae expression profile*

534 A type 1 fimbriae expression profile was established for all 91 *E. coli* ST131 strains examined in
535 this study. Strains were grown overnight on LB agar, and single colonies were picked and used
536 to inoculate a 5 ml LB for overnight aerobic (shaking) growth. A volume of 1 μ l of each
537 overnight culture was inoculated into a new tube containing 5 ml LB and cultured for 24 hours
538 under static conditions. Strains were sub-cultured from the air-liquid interface in a similar
539 manner for further growth. Type 1 fimbriae expression was determined at each stage of
540 subculture using a yeast cell agglutination assay as previously described (8). Type 1 fimbriae
541 expression of EC958 wild-type and mutant derivatives was monitored similarly. The orientation
542 of *fimS* was evaluated from single colonies by PCR as previously described with primers 2848,
543 2859 and 2850 listed in Table S1 (24). Cells from colonies with *fimS* in the 'off' orientation were
544 used to inoculate 5 ml LB broth and cultured for 24 hours under static conditions; a volume of 10

545 μ l was taken from the air-liquid interface and used to generate a new subculture every 24 hours
546 over a 5-day period. For each subculture, type 1 fimbriae expression was monitored by yeast cell
547 agglutination and western blot analysis of whole cell lysates using a FimA-specific antibody. A
548 volume of 1 ml was also collected from the air-liquid interface at each 24-hour time point; cells
549 were pelleted by centrifugation, genomic DNA was extracted and sequenced using Illumina
550 technology for DISCUS analysis.

551

552 *RNA isolation, cDNA synthesis and quantitative RT-PCR (qRT-PCR) analysis*

553 RNA was extracted from bacteria grown aerobically at 37°C in M9 minimal medium
554 supplemented with 0.2% glucose (w/v) (74). Mid-log phase cultures were standardized to OD₆₀₀
555 = 0.6 prior to RNA extraction using the RNeasy Protect Bacteria Mini Kit (Qiagen) following the
556 manufacturer's guidelines. RNA was treated with RNase-free DNase I to remove contaminating
557 DNA and re-purified using Qiagen RNeasy columns. RNA samples were quantified
558 spectrophotometrically at 260nm. cDNA synthesis was performed with 1 μ g total RNA and
559 random hexamer primers using the SuperScript®III First Strand Synthesis System (Life
560 Technologies) according to the manufacturer's instructions. All cDNA samples were diluted 10-
561 fold prior to semi-quantitative real time PCR (qRT-PCR) analysis. qRT-PCR reactions were
562 performed using SYBR® Green Master Mix (Applied Biosystems) and 500 nM gene specific
563 primers for *fimE*, *fimX* and *gapA* (Supporting Information Table S1) on a ViiA™ 7 instrument
564 (Life technologies) with the following cycling parameters: initial denaturation at 95°C for 10 min
565 followed by 40 cycles of denaturation at 95°C for 15 s, annealing at 60°C for 15 s and elongation
566 at 72°C for 30 s. Each reaction was performed in quadruplicate. Amplicon specificity was
567 confirmed via gel and melting curve analysis. The ViiA™ 7 software determined the threshold

568 cycle (C_t) for each reaction. The expression of *fimE* and *fimX* was normalized to *gapA* expression
569 for each sample and the $2^{-\Delta\Delta C_t}$ method (75) was employed to calculate the difference in
570 expression of the *fim* recombinase genes between strains with an intact *fimB* and those with a
571 *fimB::ISEc55* insertion.

572

573 *Rapid amplification of cDNA ends (5' RACE)*

574 The transcription start site (TSS) of *fimE* was mapped using the 5' RACE system (Life
575 Technologies) according to manufacturer's guidelines. Briefly, total RNA was isolated as
576 described above and first strand cDNA synthesis was performed using SuperScript™ II and a
577 gene-specific primer (Supporting Information Table S1, primer 4342). The resulting cDNA was
578 purified and a homopolymeric tail was added to its 3'-end using TdT and dCTP. The cDNA was
579 then amplified by PCR using an anchor primer (supplied) and a gene-specific primer (Supporting
580 Information Table S1, primer 4343) followed by another round of nested PCR (primer 4344).
581 The purified PCR product was then sequenced to determine the TSS.

582

583 *Sequence analysis of fimE*

584 Nucleotide sequences of ST131 *fimE* promoter and coding regions (1074 bp) were obtained from
585 previously published genomes of strains used in this study (n=26) (7). The corresponding
586 sequences from two reference non-ST131 UPEC strains, UTI89 (GI: 91209055) and CFT073
587 (GI: 26111730) and the *E. coli* K-12 strain MG1655 (GI: 556503834) were included for
588 comparison. Multiple sequence alignment was performed using Clustal X v2.0 (76) to construct
589 a neighbour-joining phylogenetic tree which was then visualised with FigTree v1.4.2

590 [<http://tree.bio.ed.ac.uk/software/figtree/>]. Amino acid sequences of the FimE protein (198 AA)
591 were also aligned using Clustal X2.

592

593 *Quantification of the fimS promoter switching frequency using DISCUS*

594 To accurately measure invertible DNA switching, a read mapping approach was developed to
595 determine the frequency of Illumina sequence reads that mapped uniquely to the ‘on’ or ‘off’
596 orientation of the *fimS* invertible switch. To determine the ratio of ‘off’ to ‘on’ mapped reads, a
597 pseudo-reference containing both *fimS* orientations and 1 kbp of flanking sequence was
598 constructed using the relevant sequence from EC958 (GI:641682562). An analogous approach
599 was taken to construct a pseudo-reference for the *hyxR* promoter region. Illumina 100 bp paired-
600 end reads from ST131 strains were then mapped to the pseudo-reference sequences using
601 Burrows-Wheeler Aligner (77), and inter-converted to SAM (Sequence Alignment/Map) and
602 BAM (Binary Alignment/Map) files using SAMtools where necessary (78). To ascertain the
603 number of reads overlapping the unique bordering regions of the ‘off’ and ‘on’ orientations, and
604 thereby their relative switching frequencies, Bedmaps was used to count reads overlapping 10 bp
605 pseudo-exons, which were created at both edges of the ‘off’ and ‘on’ orientations (79). The
606 number of paired-reads, which traversed these bordering regions, was also counted. This was
607 accomplished by assigning six regions in the pseudo-reference, corresponding to ‘left flank’,
608 ‘right flank’ or ‘switch region’ for both the ‘off’ and ‘on’ orientation. Paired end read locations
609 were determined from the BAM file and counted if they were found to traverse from a ‘switch
610 region’ to either ‘flank’ for the ‘off’ or ‘on’ orientation respectively. Reads were not counted if
611 they were unpaired or traversed from an ‘on’ region to an ‘off’ region. Final read counts were
612 normalised as a percentage of total input reads to allow comparison between independent

613 samples. Python and Bash scripts for generating pseudo-reference sequences and automating
614 DISCUS, respectively, are available in a public repository at
615 <https://github.com/LeahRoberts/DISCUS>.

616

617 *Transposon mutagenesis analysis*

618 A mini Tn5-Cm transposon containing a chloramphenicol (*Cm*) cassette flanked by Tn5 mosaic
619 ends was PCR amplified from NotI-digested pKD3 plasmid with primers 2279 and 2280
620 (Supporting Information Table S1). The amplicon was phosphorylated using T4 polynucleotide
621 kinase (New England Biolabs) and mixed with EZ-Tn5™ transposase (Epicenter
622 Biotechnologies) to create transposomes according to the manufacturer's guidelines.
623 Electrocompetent cells of EC958 *fimE* and *fimX* promoter-*lacZ* transcriptional fusion strains
624 were prepared as previously described (80). Cells were electroporated with transposomes and
625 after a 2 h recovery period in SOC, were spread on LB plates supplemented with Cm and 5-
626 bromo-4-chloro-3-indolyl-D-galactoside (X-Gal). Approximately 50,000 to 60,000 Cm resistant
627 mutants were obtained for each strain. Mutant colonies were visually screened for increased blue
628 color intensity on X-Gal plates using the parental strains as controls. Selected mutants were
629 further screened for *lacZ* expression by measurement of β -galactosidase activity. The transposon
630 insertion site of mutants with altered β -galactosidase activity was determined using an arbitrary
631 PCR (using primers 2209 and 1799; Supporting Information Table S1) followed by a nested PCR
632 (using primers 2240 and 1801) and sequencing of the final amplicon. Mutation of the *guaB* gene
633 in EC958*fimE::lacZ-cm* and EC958*fimE::lacZ-cm* was performed using λ -Red homologous
634 recombination as described above.

635

636 *β-Galactosidase assays*

637 β -Galactosidase assays were performed essentially as previously described (81). Briefly, strains
638 carrying *fimE* and *fimX* promoter-*lacZ* transcriptional fusions were grown overnight in pooled
639 human urine alone or supplemented with 0.2 mM serine hydroxamate (SHX) or 0.5 mM GTP as
640 indicated. Cultures were diluted in Z-buffer (60 mM Na₂HPO₄, 40 mM NaH₂PO₄, 50 mM β -
641 mercaptoethanol, 10 mM KCl, 1 mM MgSO₄ at pH 7.0) followed by the addition of 0.004% SDS
642 (w/v) and chloroform to permeabilize the cells. The samples were incubated at 28°C, and
643 reactions were initiated by the addition of *ortho*-Nitrophenyl- β -D-galactopyranoside (ONPG).
644 The reactions were stopped with the addition of Na₂CO₃ (300 mM). The enzymatic activity
645 (expressed in Miller units) was assayed in quadruplicate for each strain by measuring the
646 absorbance at 420 nm.

647

648 *Mouse model of UTI*

649 The C57BL/6 mouse model of ascending UTI was employed as previously described (69). All
650 strains used in this experiment were enriched for type 1 fimbriae expression by three successive
651 rounds of static growth in LB for 48 hours followed by one round of static growth for 24 hours
652 for inoculum preparation. All strains were positive for type 1 fimbriae expression as determined
653 by yeast cell agglutination and western blot analysis. Infections were performed as competitive
654 assays; the inoculum contained 1:1 strain mixture of EC958 WT (*lac*⁻ strain) and EC958*guaB*.
655 Bacteria (5x10⁸ CFU in 30 μ l PBS) were injected transurethrally into female C57BL/6 mice (8-
656 10 weeks). Urine was collected from mice 24 h post challenge, after which they were euthanized
657 by cervical dislocation. Bacterial loads in urine and bladder were enumerated as colony forming

658 units (CFU) counts. The two strains were differentiated by colony colour on MacConkey plates:
659 white (*lac*⁻ WT) or pink (EC958*guaB*).

660

661 *Statistical Analysis*

662 All statistical analyses were performed using the GraphPad Prism 6 software package.
663 Significant associations between *fimB* status and type 1 fimbriae expression were investigated
664 with the χ^2 test. Statistical significance of fold-changes in *fimE* transcript levels between ST131
665 strains was determined using a two-tailed Mann Whitney test. The Wilcoxon matched pairs
666 signed-rank test and repeated-measures one-way ANOVA with Dunnett's multiple comparisons
667 test were used to analyse results from β -galactosidase assays. A Pearson's χ^2 test with Yates'
668 continuity correction was used to compare raw read counts generated by DISCUS using RStudio
669 (v0.98.501). The significance level was adjusted to 0.0167 (alpha = 0.05, Bonferroni adjusted for
670 three pairwise comparisons). Data from mouse UTI experiments was analyzed using a two-tailed
671 Wilcoxon matched pairs signed-rank test. In all instances, statistical significance was considered
672 at $P < 0.05$.

673

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682

683

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For Peer Review

966 **Tables**967 **Table 1.** Type 1 fimbriae expression profile of *E. coli* ST131 strains

<i>fimB</i> Status	Number of yeast agglutination positive ST131 strains following overnight growth in LB broth at 37°C (%)			
	Shaking			
	culture	Static subculture		
	Day 1	Day 1	Day 2	Day 3
<i>fimB</i> ::ISEc55 (n=57)	8 (14)	27 (47.4)	34 (59.6)	52 (91.2)
Intact <i>fimB</i> (n=34)	24 (70.6)***	27 (79.4)*	27 (79.4)	29 (85.3)

968 * $P < 0.01$, *** $P < 0.0001$; χ^2 test.

969

970

971 **Table 2.** Effect of FimB over-expression in type 1 fimbriae negative strains

Strain	<i>fimB</i> status	Genotype	<i>fimS</i>	Yeast
			orientation	Agglutination*
S1	<i>fimB::ISEc55</i>	Wild-type	OFF	-
		+ pFimB	ON	-
S18	<i>fimB::ISEc55</i>	Wild-type	OFF	-
		+ pFimB	ON	+
S27	Intact	Wild-type	OFF	-
		+ pFimB	OFF	-
S61	<i>fimB::ISEc55</i>	Wild-type	OFF	-
		+ pFimB	ON	-

972 *Yeast agglutination negative (-), positive (+)

973

974

975 **Table 3.** Mutated genes associated with increased *fimE* promoter activity

EC958 locus tag	Gene	Product	No. independent Tn5 insertions	β -gal activity as Miller units ^a (mean \pm SD)	Fold-change relative to control ^b (mean \pm SD)
EC958_2815	<i>guaB</i>	inosine 5' monophosphate dehydrogenase	3	2102 \pm 56, 1321 \pm 28, 792 \pm 36	4.95 \pm 0.94 3.11 \pm 0.59 1.86 \pm 0.36
EC958_A0030	<i>yubO</i>	Unknown function, plasmid located	3	1175 \pm 139, 830 \pm 70, 702 \pm 40	2.76 \pm 0.61 1.95 \pm 0.40 1.65 \pm 0.32
EC958_1861	<i>pdxH</i>	pyridoxamine 5' phosphate oxidase	2	819 \pm 30, 1936 \pm 131	1.93 \pm 0.36 4.56 \pm 0.91
EC958_2624	<i>lrhA</i>	LysR homologue A	1	2348 \pm 365	5.52 \pm 1.35
EC958_3829	<i>dprA</i>	putative DNA processing protein	1	1358 \pm 59	3.20 \pm 0.62

976 SD, Standard deviation.

977 ^aValues represent data from each independently isolated Tn5 mutant.978 ^b β -gal activity of control strain EC958*fimE::lacZ* = 425 \pm 80.

979

980

981 **Table 4.** Mutated genes associated with increased *fimX* promoter activity

EC958 locus tag	Gene	Product	No. independent Tn5 insertions	β -gal activity as Miller units (mean \pm SD)	Fold-change relative to control ^a (mean \pm SD)
EC958_2815	<i>guaB</i>	inosine 5' monophosphate dehydrogenase	1	94 \pm 7	1.52 \pm 0.15
EC958_0463	<i>betA</i>	choline dehydrogenase	1	108 \pm 9	1.74 \pm 0.18
EC958_0090	<i>yjjA</i>	putative metal chaperone	1	94 \pm 7	1.52 \pm 0.15

982 SD, Standard deviation.

983 ^a β -gal activity of control strain EC958*fimX::lacZ* = 62 \pm 4.

984

985 **Table 5.** Strains and plasmids used in this study

Strain ^a	Description	Reference
CFT073	Wild-type UPEC isolate	(82)
536	Wild-type UPEC isolate	(83)
UTI89	Wild-type UPEC isolate	(84)
IHE3034	Wild-type UPEC isolate	(85)
EC958	Wild-type UPEC isolate; <i>E. coli</i> ST131 ESBL	(8)
S1 pFimB	<i>E. coli</i> ST131 strain S1 pFimB, ESBL, Cm ^r	This study
S18 pFimB	<i>E. coli</i> ST131 strain S18 pFimB, ESBL, Cm ^r	This study
S27 pFimB	<i>E. coli</i> ST131 strain S27 pFimB, ESBL, Cm ^r	This study
S61 pFimB	<i>E. coli</i> ST131 strain S61 pFimB, ESBL, Cm ^r	This study
CFT973 <i>hns</i>	CFT073 <i>hns::kan</i> , Kan ^r	(86)
536 <i>hns</i>	536 <i>hns::kan</i> , Kan ^r	This study
UTI89 <i>hns</i>	UTI89 <i>hns::kan</i> , Kan ^r	This study
IHE3034 <i>hns</i>	IHE3034 <i>hns::kan</i> , Kan ^r	This study
EC958 <i>hns</i>	EC958 <i>hns::cm</i> , ESBL, Cm ^r	(87)
MS3198	CFT073 <i>lacIZ upaH:lacZ-zeo</i>	(88)
EC958 pKOBEG-Gent	EC958 pKOBEG-Gent, ESBL Gent ^r	(8)
EC958 <i>fimE</i>	EC958 <i>fimE::cm</i> , ESBL Cm ^r	This study
EC958 <i>fimX</i>	EC958 <i>fimX::cm</i> , ESBL Cm ^r	This study
EC958 <i>fimE fimX</i>	EC958 <i>fimE fimX::cm</i> , ESBL Cm ^r	This study
EC958 <i>fimE fimX</i> pSU2718	EC958 <i>fimE fimX</i> , ESBL Cm ^r	This study
EC958 <i>fimE fimX</i> pFimE	EC958 <i>fimE fimX</i> pFimE, ESBL Cm ^r	This study
EC958 <i>fimE fimX</i> pFimX	EC958 <i>fimE fimX</i> pFimX, ESBL Cm ^r	This study
EC958 <i>lac::mKate2</i>	EC958 <i>lac::mKate2::cm</i> , ESBL Cm ^r	(87)
EC958 <i>fimE::lacZ-cm</i>	EC958 <i>lac::mKate2 fimE::lacZ-cm</i> , ESBL Cm ^r	This study
EC958 <i>fimX::lacZ-cm</i>	EC958 <i>lac::mKate2 fimX::lacZ-cm</i> , ESBL Cm ^r	This study
EC958 <i>fimE::lacZ</i> <i>guaB</i>	EC958 <i>lac::mKate2 fimE::lacZ guaB::cm</i> , ESBL Cm ^r	This study

EC958 <i>fimX::lacZ</i> <i>guaB</i>	EC958 <i>lac::mKate2 fimX::lacZ guaB::cm</i> , ESBL Cm ^r	This study
EC958 <i>fimE::lacZ</i> <i>guaB</i> (pGuaB)	EC958 <i>lac::mKate2 fimE::lacZ guaB::cm</i> pGuaB, ESBL Cm ^r	This study
EC958 <i>fimX::lacZ</i> <i>guaB</i> (pGuaB)	EC958 <i>lac::mKate2 fimX::lacZ guaB::Cm</i> pGuaB, ESBL Cm ^r	This study

Plasmid

pKOBEG-Gent	λ -Red plasmid, replicates at 37°C, <i>araC</i> arabinose inducible promoter, Gent ^r	(8, 89)
pCP20-Gent	Replicates at 30°C, encodes Flp recombinase, Gent ^r	This study, (73)
pSU2718	Cloning vector, <i>Plac</i> promoter, replicates at 37°C, Cm ^r	(71)
pFimB	pSU2718 <i>fimB</i> ; <i>fimB</i> coding sequence from CFT073, Cm ^r	This study
pFimE	pSU2718 <i>fimE</i> ; <i>fimE</i> coding sequence from EC958, Cm ^r	This study
pFimX	pSU2718 <i>fimX</i> ; <i>fimX</i> coding sequence from EC958, Cm ^r	This study
pGuaB	pSU2718 <i>guaB</i> ; <i>guaB</i> coding sequence from EC958, Cm ^r	This study

986 ^aIn addition to the strains listed above, our *E. coli* ST131 strain collection comprised of 54 strains
987 from the United Kingdom (S1-S54, including EC958; (7, 8, 29, 66, 68) and 37 strains from
988 Australia (S55-S91; (7, 8, 67); Cm^r-chloramphenicol, Gent^r-gentamicin resistant, Kan^r-
989 kanamycin resistant.

990

991

992 **Figure Legends**

993 **Fig. 1.** Schematic representation of the type 1 fimbrial operon. The 1895bp *fimB::ISEc55*
994 insertion sequence element is depicted in its genomic context. CFT073, reference UPEC strain;
995 EC958, representative *E. coli* ST131 clade C strain; adapted from Totsika *et al.*(8).

996

997 **Fig. 2.** Quantification of *fimE* and *fimX* transcription in ST131 strains by qRT-PCR.

998 The level of *fimE* (A) and *fimX* (B) transcription relative to housekeeping gene *gapA* was
999 determined by qRT-PCR in mid-log phase ST131 strains with the *fimB::ISEc55* insertion (n=5,
1000 including EC958) and compared to those with an intact *fimB* (n=4) to determine fold-difference
1001 in gene expression between the two groups using the $2^{-\Delta\Delta C_t}$ method. Bars represent group means
1002 \pm standard deviation (SD). Asterisk indicates a statistically significant difference between the two
1003 groups ($P < 0.05$) as determined by the two-tailed Mann Whitney test.

1004

1005 **Fig. 3.** Analysis of *fimE* promoter sequence.

1006 The transcription start site (TSS) of *fimE* was mapped by 5'RACE in EC958 and five other
1007 *E. coli* ST131 strains (S4, S54, S69, S88 and S90). Shown here is the consensus nucleotide
1008 sequence. The -35 and -10 promoter elements are underlined and the TSS mapped to a G residue
1009 164 bases upstream of the GTG start codon (indicated by the curved arrow).

1010

1011 **Fig. 4.** Contribution of FimE and FimX to *fimS* inversion and type 1 fimbriae expression in
1012 EC958.

1013 Western blots demonstrating expression of the FimA major subunit protein of type 1 fimbriae
1014 using α -FimA antibody and yeast cell agglutination assays to monitor type 1 fimbriae expression
1015 (- negative, + positive fimbriae expression) in static cultures.

1016 A. Type 1 fimbriae expression was measured in EC958 (wild-type), EC958*fimE*, EC958*fimX*,
1017 and EC958*fimE fimX* over 5 days of static subculture.

1018 B. Type 1 fimbriae expression in EC958*fimE fimX* strains over 3 days of static subculture.
1019 EC958*fimE fimX* (pSU2718) was used as an empty vector control. Over-expression of FimE
1020 (pFimE) and FimX (pFimX) was induced by the addition of 1mM IPTG to the culture media.

1021

1022 **Fig. 5.** Quantification of *fimS* switch orientation bias in EC958 wild-type, mutants and
1023 complemented strains using DISCUS.

1024 A. *fimS* orientation in EC958, EC958*fimE*, EC958*fimX* and EC958*fimE fimX* over 5 days of
1025 static subculture quantified using DISCUS. Samples from the air-liquid interface of each culture
1026 were taken on each day and used for genomic DNA extraction and Illumina sequencing. Bars
1027 represent *fimS* percentage 'on' population within each culture.

1028 B. DISCUS analysis of *fimS* orientation following *in trans* complementation with FimE (pFimE)
1029 or FimX (pFimX) in the EC958*fimE fimX* double mutant over 3 days of static subculture.

1030

1031 **Fig. 6.** Quantification of *fimX* switch orientation bias in EC958 wild-type, mutants and
1032 complemented strains using DISCUS.

1033 A. DISCUS analysis of *fimX* switch orientation in EC958, EC958*fimE* and EC958*fimX* strains
1034 over 5 days of static subculture. Bars represent percentage *fimX* switch 'on' reads within each
1035 culture population.

1036 B. DISCUS analysis of *fimX* switch orientation in EC958*fimE fimX* and in EC958*fimE fimX*
1037 following *in trans* complementation with FimE (pFimE) or FimX (pFimE) over 3 days of static
1038 subculture.

1039

1040 **Fig. 7.** Effect of *hns* deletion on *fimE* transcript level in different UPEC strains.

1041 A. Fold-change in *fimE* transcription in EC958*hns* compared to wild-type EC958 as determined
1042 by qRT-PCR.

1043 B-D. Fold-change in *fimE* transcription in non-ST131 wild-type UPEC strains and their
1044 corresponding *hns* deletion mutants, namely UTI89 (B), 536 (C) and IHE3034 (D) as determined
1045 by qRT-PCR. Vertical bars represent means \pm SD of three independent replicates.

1046

1047 **Fig. 8.** Effect of *guaB* deletion on *fimE/fimX* promoter activity and *fimS* switching in EC958.

1048 A-B. β -galactosidase activity of EC958 *fimE* (A) and *fimX* (B) promoter-*lacZ* reporter strains
1049 following overnight aerated growth in pooled human urine. All *guaB* mutant strains and
1050 complemented derivatives were constructed in their respective promoter-*lacZ* reporter
1051 backgrounds. Bars represent means \pm SD of at least three independent biological experiments.
1052 Asterisk indicates a statistically significant difference ($P<0.05$) as determined by repeated-
1053 measures one-way ANOVA with Dunnett's multiple comparisons test.

1054 C. DISCUS analysis of the effect of *guaB* deletion on *fimS* switching. EC958
1055 (wild-type), EC958*guaB* and EC958*guaB*(pGuaB) were grown statically in LB (37°C) over a
1056 period of 5 days. Over-expression of GuaB in EC958*guaB*(pGuaB) was induced by the addition
1057 of 1mM IPTG. Genomic DNA was extracted after static subculture on days 1, 3 and 5, and
1058 Illumina sequencing data was analyzed by DISCUS. Bars represent percentage 'on' reads of *fimS*

1059 in each culture population. Asterisks indicate a statistically significant difference (* $P = 0.01$ -
1060 0.001 ; *** $P < 0.0001$) as determined by a Pearson's χ^2 test with Yates' continuity correction.

1061

1062 **Fig. 9.** Regulation of *fimE* and *fimX* promoter activity by (p)ppGpp.

1063 A-B. Effect of serine hydroxamate (SHX, 0.2 mM) induced amino acid starvation on the
1064 promoter activity of *fimE* (A) and *fimX* (B) in EC958*fimE::lacZ* and EC958*fimX::lacZ* strains,
1065 respectively. All *guaB* mutant strains and complemented derivatives were constructed in their
1066 respective promoter-*lacZ* reporter backgrounds. Strains were incubated overnight under static
1067 conditions in pooled human urine and promoter activity was determined by the β -galactosidase
1068 assay. Bars represent means \pm SD of three independent biological experiments with four
1069 technical replicates. Asterisk indicates a statistically significant difference ($P < 0.05$) as
1070 determined by the Wilcoxon matched pairs signed-rank test.

1071 C-D. Effect of GTP (0.5 mM) on the promoter activity of *fimE* (C) and *fimX* (D) in
1072 EC958*fimE::lacZ guaB* and EC958*fimX::lacZ guaB* strains, respectively. Asterisks indicate
1073 statistically significant differences (** $P < 0.01$, * $P < 0.05$) in *guaB* reporter strains upon addition of
1074 GTP, as determined by a repeated-measures one-way ANOVA with Dunnett's multiple
1075 comparisons test.

1076

1077

1078 **Fig. 10.** Mouse urinary tract colonization by EC958 WT and EC958*guaB*. Female C57B/L6 mice
1079 were transurethrally inoculated with a 1:1 mixture of type 1 fimbriae enriched EC958 WT and
1080 EC958*guaB* strains in a competitive infection assay. Each marker represents total colony
1081 forming units (CFU) recovered from each mouse per 1 ml of urine or per 0.1 g of bladder tissue

1082 (as labelled) on selective medium. Lines connect data points for the same mouse and horizontal
1083 bars represent median values. Asterisk indicates statistically significant difference between the
1084 strains for persistence in urine ($P<0.05$) as well as bladder colonization ($P<0.01$) as determined
1085 by the Wilcoxon matched pairs signed-rank test.

1086

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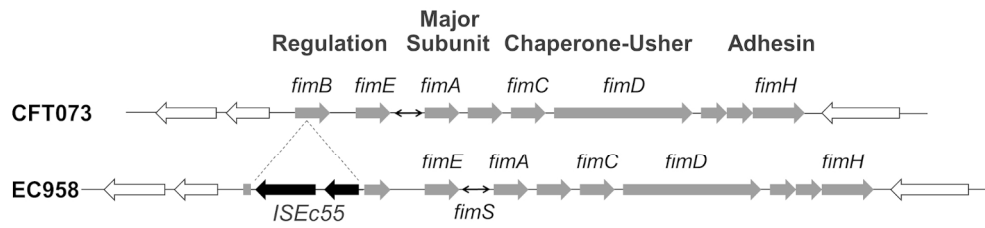


Fig. 1. Schematic representation of the type 1 fimbrial operon. The 1895bp *fimB*::*ISEc55* insertion sequence element is depicted in its genomic context. CFT073, reference UPEC strain; EC958, representative E. coli ST131 clade C strain; adapted from Totsika et al.(8).
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For Peer Review

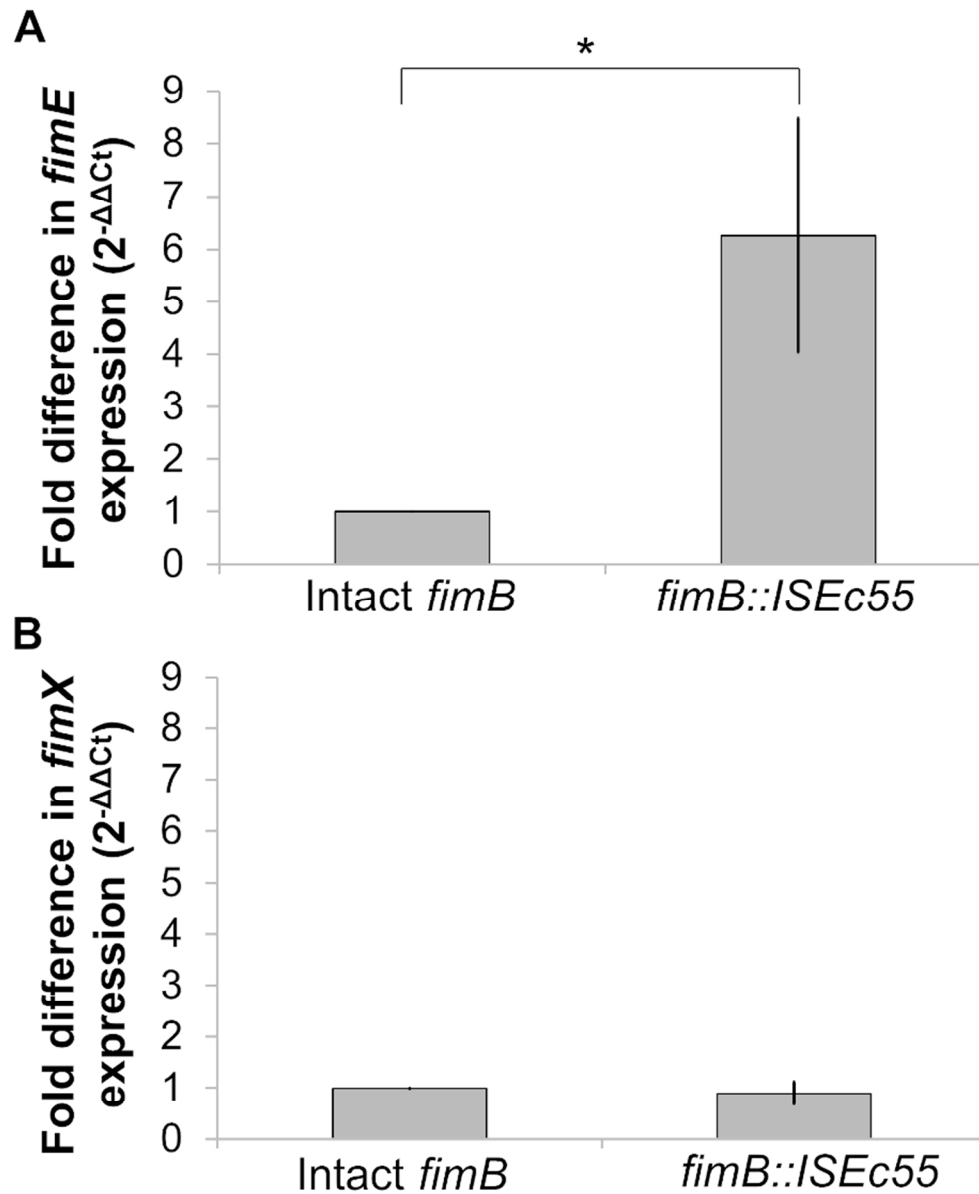


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78x95mm (300 x 300 DPI)

TSS
↓

TCTATTGTTAATTGAATCAAATCAATGAAAATAGATGTTGTCACATCAGTGAT
 -35 -10

ATTTTATTTTTGTATGATATTTAATGTAATTGACTGATAGCCACATCACTCCGT

GTGTGGTTATCTTTTTATCTATTGGGCTAATTTTGACCGATTGAGGTTTCCTAT

AGGTATTCATTCAAATATATCTCAGTTAGGAGTACTACTATTGTGA

Fig. 3. Analysis of *fimE* promoter sequence. The transcription start site (TSS) of *fimE* was mapped by 5'RACE in EC958 and five other *E. coli* ST131 strains (S4, S54, S69, S88 and S90). Shown here is the consensus nucleotide sequence. The -35 and -10 promoter elements are underlined and the TSS mapped to a G residue 164 bases upstream of the GTG start codon (indicated by the curved arrow).
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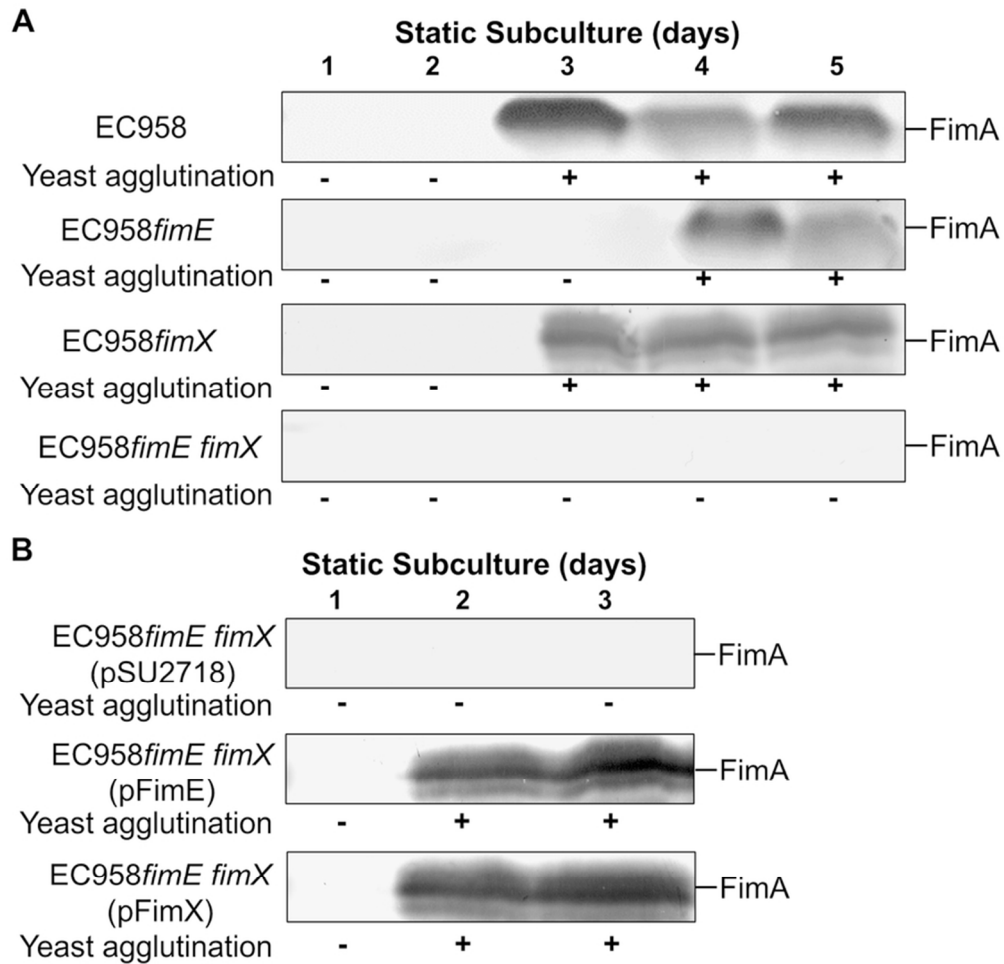


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Western blots demonstrating expression of the FimA major subunit protein of type 1 fimbriae using α -FimA antibody and yeast cell agglutination assays to monitor type 1 fimbriae expression (- negative, + positive fimbriae expression) in static cultures.

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B. Type 1 fimbriae expression in EC958*fimE fimX* strains over 3 days of static subculture. EC958*fimE fimX* (pSU2718) was used as an empty vector control. Over-expression of FimE (pFimE) and FimX (pFimX) was induced by the addition of 1mM IPTG to the culture media.

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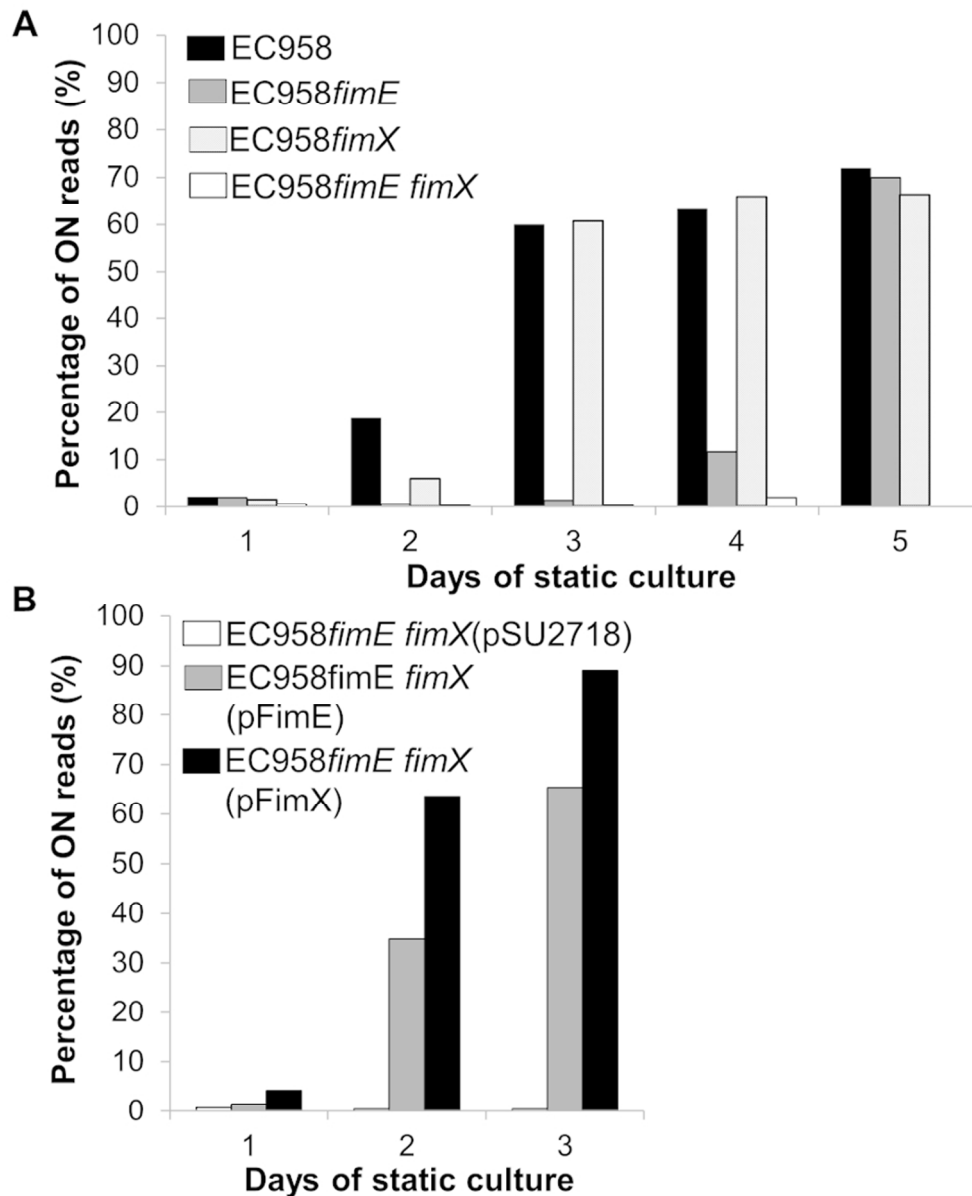


Fig. 5. Quantification of *fimS* switch orientation bias in EC958 wild-type, mutants and complemented strains using DISCUS.

A. *fimS* orientation in EC958, EC958*fimE*, EC958*fimX* and EC958*fimE fimX* over 5 days of static subculture quantified using DISCUS. Samples from the air-liquid interface of each culture were taken on each day and used for genomic DNA extraction and Illumina sequencing. Bars represent *fimS* percentage 'on' population within each culture.

B. DISCUS analysis of *fimS* orientation following in trans complementation with FimE (pFimE) or FimX (pFimX) in the EC958*fimE fimX* double mutant over 3 days of static subculture.
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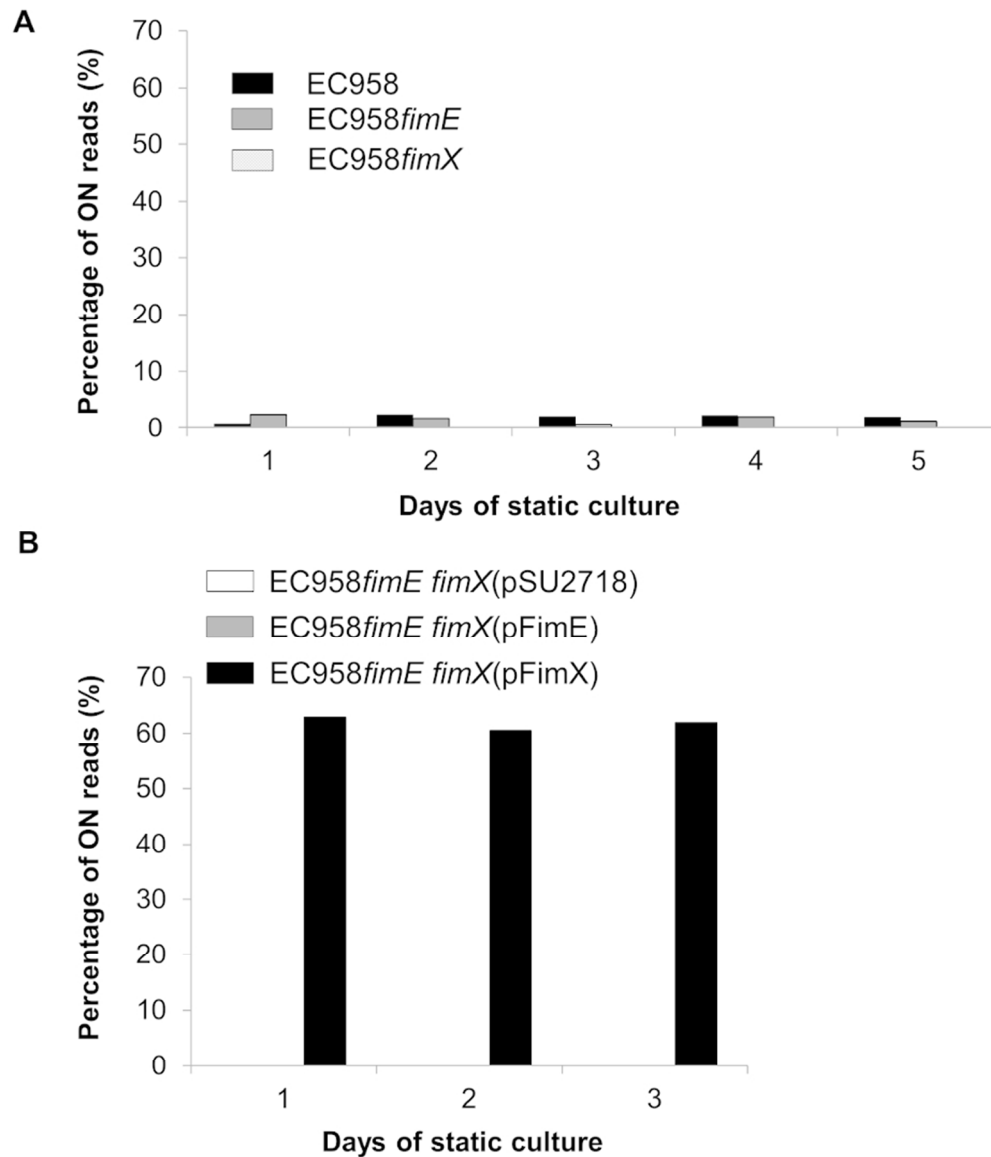


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B. DISCUS analysis of *fimX* switch orientation in EC958*fimE* *fimX* and in EC958*fimE* *fimX* following in trans complementation with *FimE* (pFimE) or *FimX* (pFimE) over 3 days of static subculture.

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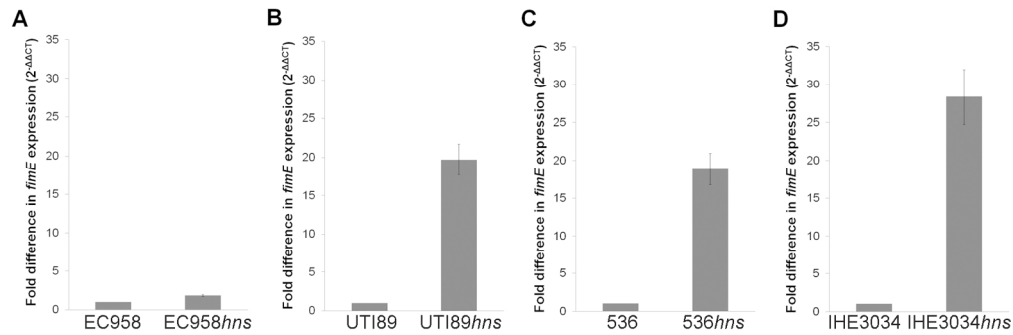


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B-D. Fold-change in *fimE* transcription in non-ST131 wild-type UPEC strains and their corresponding hns deletion mutants, namely UTI89 (B), 536 (C) and IHE3034 (D) as determined by qRT-PCR. Vertical bars represent means \pm SD of three independent replicates.

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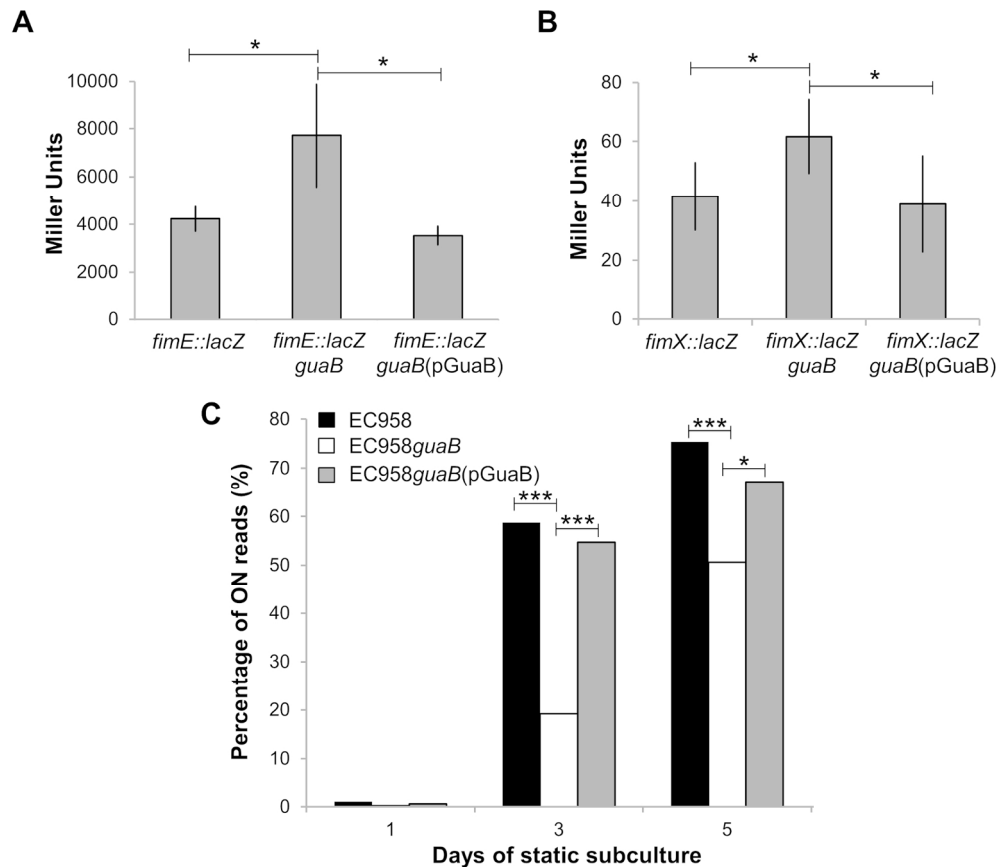


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A-B. β -galactosidase activity of EC958 *fimE* (A) and *fimX* (B) promoter-*lacZ* reporter strains following overnight aerated growth in pooled human urine. All *guaB* mutant strains and complemented derivatives were constructed in their respective promoter-*lacZ* reporter backgrounds. Bars represent means \pm SD of at least three independent biological experiments. Asterisk indicates a statistically significant difference ($P < 0.05$) as determined by repeated-measures one-way ANOVA with Dunnett's multiple comparisons test.

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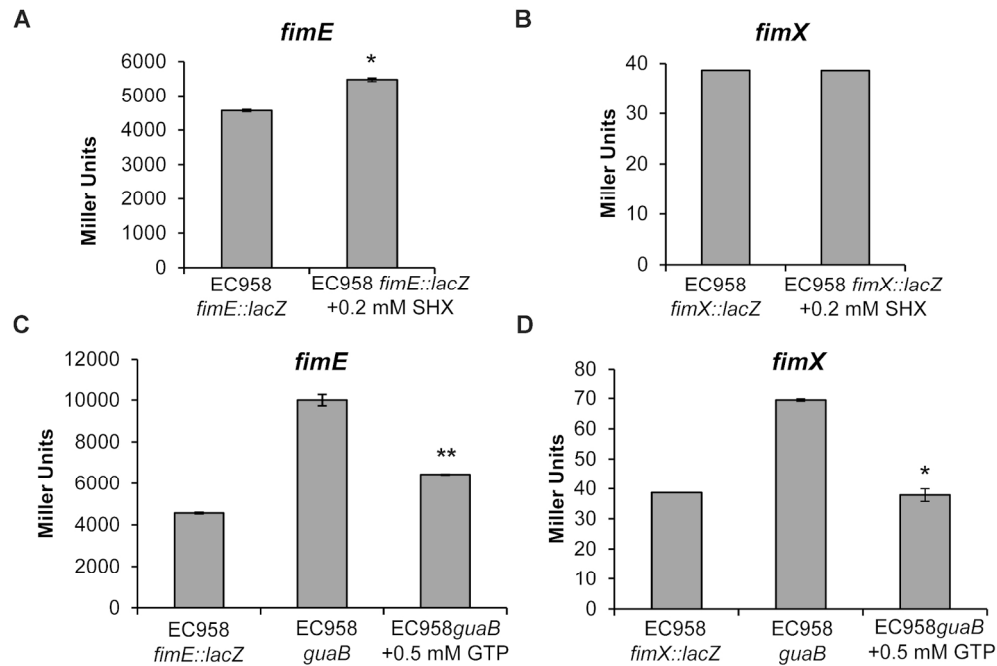


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C-D. Effect of GTP (0.5 mM) on the promoter activity of *fimE* (C) and *fimX* (D) in EC958*fimE::lacZ* *guaB* and EC958*fimX::lacZ* *guaB* strains, respectively. Asterisks indicate statistically significant differences (** $P < 0.01$, * $P < 0.05$) in *guaB* reporter strains upon addition of GTP, as determined by a repeated-measures one-way ANOVA with Dunnett's multiple comparisons test.

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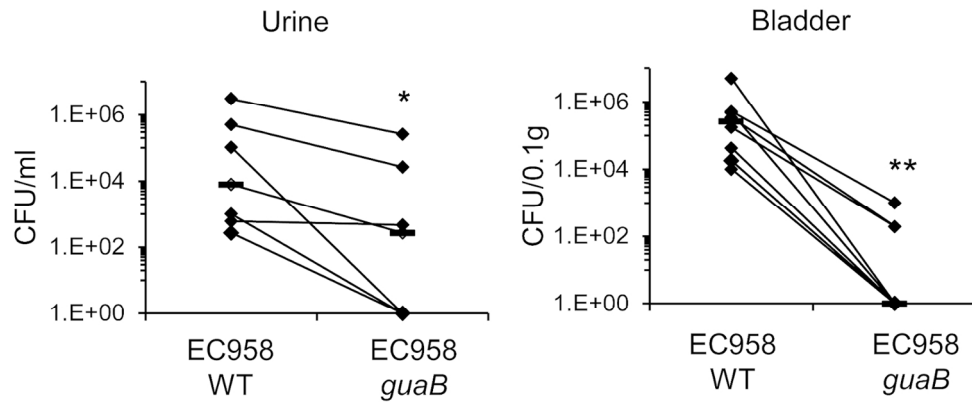


Fig. 10. Mouse urinary tract colonization by EC958 WT and EC958*guaB*. Female C57B/L6 mice were transurethraly inoculated with a 1:1 mixture of type 1 fimbriae enriched EC958 WT and EC958*guaB* strains in a competitive infection assay. Each marker represents total colony forming units (CFU) recovered from each mouse per 1 ml of urine or per 0.1 g of bladder tissue (as labelled) on selective medium. Lines connect data points for the same mouse and horizontal bars represent median values. Asterisk indicates statistically significant difference between the strains for persistence in urine ($P < 0.05$) as well as bladder colonization ($P < 0.01$) as determined by the Wilcoxon matched pairs signed-rank test.

150x61mm (300 x 300 DPI)

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