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# Context-dependent Requirements for FimH and Other Canonical Virulence Factors in Gut Colonization by Extraintestinal Pathogenic *Escherichia coli*

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Running Title: ExPEC persistence within the gut

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**ABSTRACT**

1 Extraintestinal pathogenic *Escherichia coli* (ExPEC) act as commensals within the mammalian
2 gut, but can induce pathology upon dissemination to other host environments such as the urinary
3 tract and bloodstream. It is thought that ExPEC genomes are shaped in large part by evolutionary
4 forces encountered within the gut where the bacteria spend much of their time, provoking the
5 question of how their extraintestinal virulence traits arose. The principle of coincidental
6 evolution, in which a gene that evolved in one niche happens to be advantageous in another, has
7 been used to argue that ExPEC virulence factors originated in response to selective pressures
8 within the gut ecosystem. As a test of this hypothesis, the fitness of ExPEC mutants lacking
9 canonical virulence factors was assessed within the intact murine gut in the absence of any
10 antibiotic treatment. We found that most of the tested factors—including CNF1, Usp, colibactin,
11 flagella, and the plasmid pUTI89—were dispensable for gut colonization. Deletion of genes
12 encoding the adhesin PapG or the toxin HlyA caused transient defects, but did not affect longer-
13 term persistence. In contrast, a mutant missing the type 1 pilus-associated adhesin FimH
14 displayed reduced persistence within the gut. However, this phenotype was variable, being
15 dependent on the presence of specific competing strains and partially attributable to aberrant
16 flagellin expression by the *fimH* mutant. These data indicate that FimH and other key ExPEC-
17 associated factors are not strictly required for gut colonization, suggesting that selective
18 pressures within the gut do not drive the development of all extraintestinal virulence traits.

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19 **INTRODUCTION**

20 The bacterium *Escherichia coli* was first described by Theodor Escherich in 1884, and
21 has since become a critical model organism that has been used to understand the fundamentals of
22 molecular biology (1). *E. coli* is able to live in a variety of locations, including the soil, water,
23 and the human gut. Although it is a prominent member of the neonatal microbiota, it is quickly
24 overshadowed by burgeoning anaerobic bacteria as oxygen becomes scarce within the gut
25 following birth (2, 3). In adults, *E. coli* is present at about $10^7$ to $10^9$ Colony Forming Units
26 (CFU)/g feces, a level that is 100-10,000-fold-lower than the resident anaerobes (4). Despite
27 being a minor component of the microbiota, the estimated number of *E. coli* cells that are
28 transmitted via fecal matter from each human being to the environment in a single day is
29 staggering – about $10^{11}$ CFU (5).

30 Understanding the role of *E. coli* within the microbiota is complicated by the fact that *E.*
31 *coli* is a very diverse species with a wide spectrum of phenotypes (1). Some *E. coli* strains live
32 harmlessly in the gut, while others act as pathogens, causing diarrhea and hemorrhaging (6).
33 Others have been linked with the development of Crohn's Disease and colorectal cancer (7-9).
34 One strain, Nissle 1917, acts as a probiotic that assuages inflammation in addition to inhibiting
35 colonization by pathogens such as *Salmonella* (10, 11). A group of strains known as
36 Extraintestinal Pathogenic *Escherichia coli* (ExPEC) generally act as commensals within the gut,
37 but can disseminate to other host environments and subsequently cause disease (12). ExPEC
38 include uropathogenic *E. coli* (UPEC), which cause the overwhelming majority of urinary tract
39 infections (UTIs) (13). These infections are especially prevalent among women, about half of
40 whom will have at least one UTI during their lifetime. ExPEC is also responsible for other, more
41 serious conditions, including sepsis and neonatal meningitis (12, 14).

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42 The gut is thought to be the major ExPEC reservoir that seeds extraintestinal infections.
43 Evidence for this notion is that the same ExPEC strain can often be isolated from both the feces
44 and urine of individual patients suffering from UTIs (15-18). Indeed, ExPEC strains are
45 frequently difficult to clear from the gut with antibiotic treatments, even when the pathogens are
46 effectively eliminated from the urinary tract (19). Furthermore, *E. coli* strains belonging to
47 phylogenetic group B2, which includes many ExPEC isolates, are much more likely to be long-
48 term residents within the gut in comparison with other *E. coli* populations (20-22). The majority
49 of adults carry group B2 *E. coli* strains within the gut, irrespective of extraintestinal infections
50 (22). Cumulatively these observations suggest that ExPEC primarily inhabit the gut, with
51 sporadic departures to extraintestinal sites.

52 Given that ExPEC reside mostly within the gut, and that transmission of ExPEC among
53 individuals likely occurs chiefly through fecal-oral routes (4, 23-25), it is expected that ExPEC
54 genomes have been shaped in large part by the evolutionary pressures present within the gut.
55 How then, did extraintestinal virulence factors come into being? The hypothesis of coincidental
56 evolution has often been evoked to answer this question (26). In general terms, coincidental
57 evolution is when a factor evolves in one context, but happens to be useful in another context as
58 well (27). When the hypothesis of coincidental evolution is applied to the ExPEC life cycle, the
59 implication is that factors that promote virulence in extraintestinal niches evolved in the gut for a
60 function possibly unrelated to virulence.

61 Little concrete evidence has been put forth to support or contradict coincidental evolution
62 in the context of ExPEC infection, other than the fact that known extraintestinal virulence factors
63 are often encoded by gut isolates (21, 26, 28). One prediction of this hypothesis is that
64 extraintestinal virulence factors should play a role in gut colonization. To date, this possibility

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65 has only been addressed by one study in which an ExPEC mutant that lacks multiple
66 pathogenicity-associated islands (PAIs) was found to be defective in its ability to persist within
67 the murine intestinal tract (5). It is clear that more experimental work needs to be done to
68 determine to what extent coincidental evolution applies to single virulence factors within
69 ExPEC. To address this issue, several canonical virulence factors were individually deleted from
70 a reference ExPEC isolate and the resulting mutants were tested for their ability to colonize and
71 persist within the mouse gastrointestinal tract. Among eight virulence factors that were
72 examined, only the type 1 pilus-associated mannose-binding adhesin FimH had a notable
73 persistence defect within the gut. However, this defect was variable and partly contingent upon
74 aberrant flagellin expression by the *fimH* mutant and the presence of specific competing strains.
75 These findings are discussed in the context of both coincidental evolution and the development
76 of anti-ExPEC therapeutics.
77
78 **RESULTS**
79 **ExPEC stably colonizes the murine intestinal tract in the presence of the natural, intact**
80 **microbiota.** To examine ExPEC colonization of the intestinal tract, we employed a model in
81 which adult specific pathogen free (SPF) Balb/c mice were intragastrically gavaged with $\sim10^9$
82 CFUs of the reference cystitis isolate F11. At various time points post-gavage, feces were
83 collected, and the numbers of viable ExPE C present were enumerated. F11 and other bacterial
84 strains used in this study were engineered to express either chloramphenicol ($Clm^R$) or
85 kanamycin ($Kan^R$) resistance cassettes so that they could be easily identified by plating fecal
86 homogenates on selective media. Following inoculation, the fecal titers of F11 remained fairly
87 stable for up to 75 days, with median values ranging between $10^6$ and $10^7$ CFU/g feces after the

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88 first day (Fig. 1A). These data demonstrate that ExPEC can efficiently initiate and maintain
89 colonization of the SPF mouse gut, in line with recent reports from our group and others (24, 29-
90 31). Consistent with the observation that nonpathogenic *E. coli* mostly reside within the large
91 bowel (32), the cecum and colon carried the largest load of F11 at the 2-week time point,
92 although considerable numbers of F11 were also present within the small intestine (Fig. 1B).
93 It is of note that the SPF mice utilized in these experiments were not treated with
94 antibiotics and therefore each possesses an intact microbiota. This is in contrast to other
95 commonly used mouse models in which mice are treated with streptomycin and/or other
96 antibiotics in order to disrupt the intestinal microbiota and open up niches that can then be
97 occupied by incoming microbes (32). Since antibiotic treatment was not required for consistent
98 colonization of the gut by F11 (or by other ExPEC isolates (24, 29)), we wondered whether
99 ExPEC is simply more adept at gut colonization than nonpathogenic *E. coli* strains. As a test of
100 this idea, we first competed F11 head-to-head with MG1655, an often-used nonpathogenic *E.*
101 *coli* K12 strain. Following oral gavage with equal numbers of F11 and MG1655, the K12
102 bacteria were cleared from all of the mice by day 3, while F11 stably persisted (Fig. 1C). When
103 mice were gavaged with MG1655 alone, it was lost at a slower rate, but was still cleared from
104 80% of the mice by day 14 (Fig. 1D). This is in sharp contrast to the persistent phenotype
105 observed with F11. Similar results were obtained using SPF C57Bl/6 mice (Fig. S1A).
106 To assess whether ExPEC colonization of the colon is marked by inflammation (colitis),
107 the mice were weighed at several time points after colonization, as weight loss frequently
108 accompanies colitis. Mice that were gavaged with F11 consistently experienced transient weight
109 loss at the one-day time point, in comparison to mice that were inoculated with MG1655 (Fig.
110 1E). Since the ExPEC-associated pore forming toxin α-hemolysin (HlyA) was previously shown

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111 to induce inflammation within the gut in other mouse models (33-35), we hypothesized that
112 HlyA contributes to the transient weight loss seen in F11-colonized animals. When mice were
113 colonized with F11Δ*hlyA*, fecal titers of the mutant were notably lower at 1 and 3 days post-
114 inoculation, relative to wild type (WT) F11 (Fig. 1D). However, the Δ*hlyA* mutant persisted
115 much like the WT strain at later time points. These data, coupled with the observation that
116 F11Δ*hlyA* does not elicit transient weight loss by the host (Fig. 1E), suggest that HlyA both
117 enhances initial colonization of the gut by F11 and stimulates short-term inflammation that
118 causes transient weight loss. By the 14-day time point, the colons of mice that were gavaged with
119 WT F11, F11Δ*hlyA*, or MG1655 appear unperturbed, having normal crypt architecture and no
120 evidence of infiltrating immune cells, as assessed by histological analysis. Altogether, these data
121 indicate that F11 colonization induces transient inflammation of the intestinal tract with
122 coordinate weight loss by the host. HlyA can facilitate these processes, but is not required for the
123 longer-term persistence of F11 within the gut.
124
125 **Not all ExPEC-associated toxins promote ExPEC fitness within the gut.** In light of the
126 hypothesis of coincidental evolution and observations showing that HlyA can enhance initial
127 colonization of the gut by F11 (Fig. 1D), we wished to examine possible roles for other canonical
128 ExPEC-associated toxins as mediators of intestinal colonization. The first of these was cytotoxic
129 necrotizing factor type 1 (CNF1), a secreted toxin that catalyzes the deamidation of a specific
130 glutamine residue within Rho family GTPases (36). This causes constitutive activation of Rho
131 GTPases, leading to aberrant host cytoskeletal rearrangements and multinucleation (2, 37, 38).
132 CNF1 has been linked with ExPEC strains in epidemiological studies (38), and can enhance host
133 inflammatory responses and ExPEC virulence in mouse models of UTI (39) and prostatitis (40).

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134 However, the effects of CNF1 on ExPEC fitness within the host remain unclear, clouded
135 somewhat by conflicting reports (39-42). To test if CNF1 plays a role in the gut, mice were
136 gavaged with $10^9$ CFU of either WT F11 or F11Δ*cnf1* and intestinal colonization levels were
137 tracked by homogenizing and plating feces at various time points. Median bacterial levels for
138 both the WT and mutant strains ranged between $10^5$ and $10^6$ CFU per gram of feces over the
139 course of two weeks (Fig. 2A). At no point were the WT and F11Δ*cnf1* titers significantly
140 different, suggesting that CNF1 does not impact the ability of F11 to colonize the mouse gut.
141 Like HlyA and CNF1, Uropathogenic specific protein (Usp) is often encoded by ExPEC
142 isolates (43, 44). Usp is also associated with *E. coli* fecal isolates that are capable of long-term
143 persistence within the human infant intestinal tract (45). Interestingly, Usp has genotoxic
144 nuclease activity as well as homology to colicins, a group of toxins that can be used by bacteria
145 to harm competing microbes (46-48). Given that interbacterial competition within the gut is
146 commonplace, we tested whether Usp is important for ExPEC gut colonization. The F11Δ*usp*
147 mutant did not exhibit any notable defects within the gut, indicating that Usp is not required in
148 this niche (Fig. 2B).
149 Another toxin that has been linked to ExPEC pathogenesis is colibactin, which is
150 produced by a number of factors encoded by the polyketide synthase (*pks*) genomic island. The
151 *pks* island is not typically carried by intestinal pathogenic *E. coli* strains, but is enriched in
152 extraintestinal isolates relative to commensal fecal isolates (49). Colibactin induces DNA
153 damage and cell cycle arrest in host cells (49, 50), and the presence of colibactin-producing
154 bacteria has been linked with the development of colorectal cancer (51). In extraintestinal
155 infections, colibactin-deficient bacterial strains are not as virulent as their WT counterparts.
156 Mutation of the *pks* island reduces ExPEC translocation from the gut in neonatal rats (52) and

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157 reduces lymphopenia in septic mice (53). Whether the *pks* island also plays a role in bacterial
158 fitness during gut colonization is not clear. Whereas one study observed decreased colonization
159 of the small intestines of neonatal rats by colibactin mutants (52), another found no colonization
160 differences between a *pks* mutant and WT bacteria (54). To test whether colibactin production is
161 important in our adult mouse model of gut colonization, we orally inoculated mice with either
162 WT F11 or F11Δ*clbCDEFG*, which lacks a key operon within the *pks* island (55). There was no
163 difference in the colonization ability of the WT and mutant strains (Fig. 2C), suggesting that
164 colibactin biosynthesis does not contribute to bacterial fitness in this model of gut colonization.
165
166 **Flagella are not required for ExPEC gut colonization.** Flagella are filamentous organelles
167 comprised of polymers of the flagellin protein FliC (56). Though best known for their role in
168 motility, flagella can also promote bacterial attachment and biofilm formation, and can potently
169 stimulate host inflammatory responses (57, 58). Several studies have provided evidence that
170 flagella enhance ExPEC colonization of the mouse urinary tract (59-62). In contrast, flagella are
171 not critical for gut colonization by nonpathogenic *E. coli* in streptomycin-treated mice (63). To
172 determine if flagella are required for gut colonization by ExPEC in the face of an intact
173 microbiota, we inoculated adult Balb/c mice with WT F11 or an isogenic mutant lacking *fliC*. In
174 these assays, no clear differences between the WT strain and F11Δ*fliC* were observed (Fig. 2D),
175 suggesting that flagella do not promote ExPEC fitness in the gut.
176
177 **The pUTI89 plasmid is not important for gut colonization.** Many ExPEC isolates, including
178 F11, carry plasmids that are identical or closely related to the pUTI89 plasmid that was first
179 identified in the cystitis isolate UTI89 (64, 65). This plasmid, which is roughly 114 kb in length,

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180 encodes conjugation machinery, several virulence factors, and numerous hypothetical genes.
181 Loss of pUTI89 impairs the fitness and intracellular growth of UTI89 during the early stages of a
182 UTI in adult mice (66). Likewise, deletion of a closely related plasmid from the neonatal
183 meningitis *E. coli* isolate RS218 attenuates bacterial virulence during systemic infections in rat
184 pups (65). In addition, a number of pUTI89-associated genes have been linked with ExPEC
185 mucus and glucose metabolism *in vitro* (30). To determine if pUTI89 facilitates gut colonization
186 by F11, an F11 derivative that was cured of the plasmid was tested in our mouse model. The
187 cured strain exhibited no defect when compared to the WT control strain (Fig. 2E), suggesting
188 that pUTI89 is dispensable for ExPEC fitness within the gut.
189

190 **Disruption of *fimH* reduces gut colonization fitness, whereas the lack of *papG* has no effect.**
191 ExPEC strains encode many types of hair-like adhesive organelles known as pili, or fimbriae.
192 Two of the most often-studied ExPEC-associated adhesins are P and type 1 pili (T1P) (67). P pili
193 terminate with the PapG adhesin, which binds host globoseries glycosphingolipids and can
194 facilitate bacterial infection of the kidneys. T1P are capped by the mannose-binding adhesin
195 FimH, which promotes biofilm formation as well as bacterial attachment to and invasion of
196 bladder epithelial cells. In our non-competitive gut colonization assays in which the WT and
197 mutant bacterial strains are kept separate, the deletion of either *papG* or *fimH* did not
198 significantly impair the ability of F11 to colonize the intestinal tract (Fig. 3A and B). However,
199 in these assays we noted that the Δ*fimH* mutant was cleared in 3 out of 10 mice–more than
200 observed with any of the other tested mutants, though not statistically significant (Fig. 3B).
201 These results prompted us to examine the *fimH* mutant further using competitive assays in which
202 the WT and mutant strains are inoculated as a 1:1 mixture into mice via oral gavage. In these and

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203 other competitive assays, total ExPEC levels remain fairly steady over time at around $10^6$ CFU/g
204 feces, even when ratios of the individual competing ExPEC strains are variable.
205 As a control, we first competed $Kan^R$- and $Clm^R$-tagged F11 strains against one another
206 to assess if the resistance cassettes alone compromised bacterial fitness within the intestinal tract.
207 In these control assays, F11-$Kan^R$ exhibited a modest defect, amounting to about a 3-fold
208 decrease relative to F11-$Clm^R$ on day 14, with a median competitive index (CI; see Methods) of
209 -0.49 (Fig. 3C). Likewise, no striking differences between F11-$Clm^R$ and F11Δ*papG* ($Kan^R$)
210 were observed in competitive assays, with the exception of a transient 6.8-fold decrease (median
211 CI of -0.83) in prevalence of the *papG* mutant on day 5 (Fig. 3D). In contrast, when F11Δ*fimH*
212 ($Kan^R$) was competed against F11-$Clm^R$, the Δ*fimH* mutant became progressively worse than the
213 control strain starting at day 7 post-inoculation (Fig. 3E). By day 10, there was about a 360-fold
214 reduction in the relative levels of F11Δ*fimH* recovered in the feces, reflecting a median CI of
215 -2.55. In addition, the Δ*fimH* mutant began to be cleared as early as day 3, and was not detected
216 in the feces of nearly half of the mice by day 10 (green, Fig. 3E). In comparison, F11Δ*papG*
217 dropped below levels of detection in only one mouse during the 14-d time course of these
218 competitive assays (red, Fig. 3D). Of note, the differences between strains indicated in Figs. 3C-
219 E are not statistically significant when *p* values are corrected for multiple comparisons.
220 However, the amplitude (effect size) of the phenotypes observed with the Δ*fimH* mutant suggests
221 that disruption of this gene can markedly impair ExPEC persistence within the gut in competitive
222 assays.
223
224 **T1P expression by F11 is modest following excretion from the gut.** T1P are phase variable,
225 being turned ON or OFF through recombinase-mediated flipping of an invertible promoter

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226 element within the *fim* gene cluster (67). The orientation of this *fim* switch can be monitored and
227 quantified by PCR as a means to assess levels of T1P expression (68, 69). Within the feces of
228 mice that are colonized by F11, we found that the *fim* switch is in the ON position in about 25 to
229 35% of the excreted bacteria recovered on days 5, 9, and 14 post-inoculation (Fig. 3F). This is on
230 par with results from shaking broth cultures, in which the *fim* switch is skewed towards the OFF
231 position. These data indicate that T1P expression by feces-associated ExPEC is limited, which
232 may enable the pathogen to better disseminate either within the intestinal tract or after being
233 discharged from the host.
234
235 **Increased flagellin expression partially explains the colonization defect observed with**
236 **F11Δ*fimH*.** T1P, and FimH in particular, may enhance ExPEC persistence within the gut via
237 multiple mechanisms, such as aiding the formation of protective biofilm-like communities or
238 facilitating bacterial attachment to intestinal tissues (58, 70, 71). However, it is conceivable that
239 disruption of *fimH* could also reduce ExPEC fitness within the gut via effects on other bacterial
240 or host processes. Specifically, previous work showed that deletion of the entire *fim* operon in
241 the ExPEC isolate UTI89 causes the upregulation of FliC with a coordinate increase in
242 swimming motility (72). This could be problematic for *fim* mutants since it is known that
243 aberrant overexpression of FliC can impair ExPEC colonization of the gut (5), possibly as a
244 consequence of FliC-mediated stimulation of host inflammatory responses (57). Furthermore,
245 within the intestinal tracts of germ-free or streptomycin-treated mice, mutations that reduce
246 bacterial motility and FliC expression are selected and can promote the persistence of the K12
247 strain MG1655 (73-75). Together, these observations led us to question if defects in the ability of
248 F11Δ*fimH* to survive within the gut in competitive assays are associated with altered FliC

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249 expression.

250 In assessing this possibility, we first noted that deletion of *fimH* does increase the motility
251 of F11 in swim agar plates (Fig. 4A). This phenotype correlates with augmented FliC expression
252 (Fig. 4B), as measured by use of the low-copy reporter construct p*fliC-lux* in which the
253 *luxCDABE* gene cluster encoding bacterial luciferase is transcriptionally fused with the
254 conserved *fliC* promoter (62). These results mirror those reported for a UTI89 mutant lacking the
255 entire *fim* operon (72). Interestingly, in our assays the deletion of *papG* in F11 had nearly the
256 opposite effect of the *fimH* deletion, greatly reducing the motility of F11 and ablating FliC
257 expression (Fig. 4A-B).

258 To test if FliC contributes to the defects in gut colonization observed with F11Δ*fimH*, we
259 generated a double knockout (DKO) mutant lacking both *fimH* and *fliC*. This mutant
260 (F11Δ*fimH*Δ*fliC*) is non-motile in swim plates, similar to the single Δ*fliC* mutant strain (Fig.
261 4A). In competitive gut colonization assays with WT F11, the Δ*fimH*Δ*fliC* mutant exhibited less
262 pronounced deficiencies than F11Δ*fimH* (Fig. 4C; compare with Fig. 3E). The greatest defect
263 was observed at day 14 post-inoculation, at which point F11Δ*fimH*Δ*fliC* was not detected in the
264 feces of 5 of the 13 mice. Relative to the WT strain, F11Δ*fimH*Δ*fliC* titers were reduced by about
265 21-fold on day 14, corresponding with a median CI of -1.33. This defect was substantially
266 smaller than the maximal ~360-fold reduction seen with the single Δ*fimH* mutant in competition
267 with WT F11 on day 10 post-inoculation (see Fig. 3E). In addition, clearance of F11Δ*fimH*Δ*fliC*
268 was not observed until day 7 post-inoculation (Fig 4C), whereas loss of F11Δ*fimH* was evident
269 starting at day 3 (Fig 3C). In light of the delayed defects seen with F11Δ*fimH*Δ*fliC*, we extended
270 the assays to day 21. At this point, the median CI value returned close to 0, though
271 F11Δ*fimH*Δ*fliC* was still undetectable in feces from nearly half of the mice while the WT strain

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272 was present in all but one sample (Fig. 4C). Differences between F11Δ*fimH*Δ*fliC* and the WT
273 strain were not statistically significant, either with or without corrections for multiple
274 comparisons. The less conspicuous defects seen with F11Δ*fimH*Δ*fliC* in these assays suggest that
275 aberrant FliC expression does contribute to the compromised persistence of F11Δ*fimH* within the
276 gut.
277
278 **F11Δ*fimH* outcompetes F11Δ*fimH*Δ*fliC* within the gut.** To better understand the effects that
279 the loss of *fimH* and *fliC* have on bacterial fitness during gut colonization, the Δ*fimH*Δ*fliC* and
280 Δ*fimH* mutants were directly competed. We hypothesized that the Δ*fimH* strain, with heightened
281 FliC expression, would be outcompeted by the Δ*fimH*Δ*fliC* DKO mutant. However,
282 F11Δ*fimH*Δ*fliC* exhibited clear and statistically significant defects in comparison with F11Δ*fimH*
283 (Fig 4D). Fecal titers of the DKO mutant were below levels of detection in one out of 15 mice on
284 day 3 post-inoculation, and by day 14 the DKO mutant was undetectable in the feces of a third of
285 the animals. At this point, F11Δ*fimH* was about 270-fold more abundant than the DKO mutant,
286 corresponding with a median CI of 2.43. These data, as well results from non-competitive assays
287 (see Fig. 3B), indicate that the importance of FimH to ExPEC survival within the intestinal tract
288 is dependent upon the nature of the competing microbes that are present.
289
290 **WT F11 and F11Δ*fimH* can outcompete one other in reciprocal serial colonization assays.**
291 To further evaluate FimH requirements within the intestinal tract, we carried out serial
292 colonization assays in which Balb/c mice were first orally gavaged with WT F11 before the
293 introduction of F11Δ*fimH* 7 d later. Though fecal titers of the Δ*fimH* mutant were initially high
294 and on par with the WT strain, levels of the mutant dropped precipitously and were below the

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295 limits of detection in most mice by day 3 post-inoculation (Fig. 5A). In reciprocal experiments,
296 in which F11Δ*fimH* was allowed to colonize the mice prior to instillation of the WT strain,
297 F11Δ*fimH* persisted while WT F11 was cleared from most of the mice by day 10 (Fig. 5B).
298 These data show that FimH is not strictly required for ExPEC to prevent colonization of the gut
299 by a new competing strain, though the adhesin does seem to aid this process. In addition, these
300 results indicate that WT F11 and F11Δ*fimH* likely vie for the same intestinal niches, with the
301 first strain established having the upper hand irrespective of FimH expression.
302

303 **DISCUSSION**

304 The concept of coincidental evolution, coupled with phylogenetic analyses, suggests that
305 the extraintestinal success of ExPEC strains is a by-product of their ability to colonize the gut
306 (26). A corollary of this hypothesis is that extraintestinal virulence and fitness factors promote
307 ExPEC colonization of the gut. In support of this possibility, researchers previously showed that
308 deletion of the seven major pathogenicity islands (PAIs) of the ExPEC isolate 536 not only
309 reduced the virulence of this pathogen in a murine sepsis model, but also attenuated pathogen
310 persistence within the gut (5). Here, we set out to determine if the principal of coincidental
311 evolution could be applied to individual virulence and fitness determinants encoded by the
312 reference ExPEC strain F11. In non-competitive assays using adult SPF mice, we observed no
313 defects in intestinal colonization or the persistence of ExPEC mutants lacking CNF1, Usp,
314 colibactin, flagellin, or the plasmid pUTI89 (see Fig. 2). Mutants that are missing either *papG* or
315 *hlyA* exhibited transient colonization defects, but these did not affect longer-term ExPEC
316 survival within the gut (Figs. 1D, 3A, and 3D ). These results indicate that at least some ExPEC-
317 linked genes can influence bacterial fitness within the gut, though the effects may be modest.

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318 Discerning more unequivocal phenotypes for individual ExPEC-associated loci within the gut
319 can be complicated and context-dependent, as exemplified by the analysis of Δ*fimH* mutants.
320 In non-competitive assays, we observed no significant differences between WT F11 and
321 F11Δ*fimH*, although the mutant was cleared from a few mice over the course of the 14-d
322 experiments (Fig. 3B). This prompted us to test F11Δ*fimH* further using competitive assays with
323 the WT strain. In these assays, differences between the Δ*fimH* mutant and WT strains were more
324 distinct, but not significantly so if the data are corrected for multiple comparisons. Still, the fact
325 that F11Δ*fimH* titers within the feces from nearly half the mice fell below detectable levels by
326 day 10 post-inoculation suggests that FimH can promote ExPEC persistence within the gut (Fig.
327 3E). These findings are in line with recently published work showing that intestinal persistence
328 of the ExPEC isolate UTI89 within streptomycin-treated mice is facilitated by FimH expression
329 (76). This study, as well as other work with enteric *E. coli* pathogens (71, 77, 78), suggests that
330 FimH can promote ExPEC interactions with intestinal epithelial cells. In our mouse models, we
331 observed that F11 is localized primarily within the lumen of the colon, though substantial
332 numbers of the pathogen are also associated with the colonic tissue (see Fig. S1B). While FimH
333 may mediate interactions between ExPEC and the intestinal epithelium (76), the adhesin could
334 also influence pathogen persistence within the gut via effects on other processes, including
335 biofilm development and the modulation of innate host defenses (58, 70, 79, 80).
336 Defining the contributions of FimH to ExPEC fitness within the gut is further
337 complicated by the observation that the deletion of *fimH* enhances FliC expression by F11 and
338 increases motility (Fig. 4). Analysis of a *fliC* and *fimH* DKO mutant in competition with WT F11
339 indicated that aberrant FliC expression is at least partially responsible for the colonization
340 defects observed with the Δ*fimH* mutant (Figs. 3E and 4C). This situation mirrors results

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341 reported for the ExPEC strain 536, in which reduced intestinal persistence of the mutant lacking
342 seven PAIs was attributed to FliC overexpression (see above, (5)). The expression of flagella is
343 generally thought to have mostly detrimental effects on *E. coli* fitness within the gut, possibly
344 due to an increased burden on bacterial metabolism and the ability of FliC to stimulate host
345 inflammatory pathways (57, 73-75). However, in competitive assays F11Δ*fimH* outperformed
346 F11Δ*fimH*Δ*fliC*, even though the single mutant is hypermotile (Fig. 4D). One potential
347 explanation for this finding is that flagella expression might at times be an advantage for *E. coli*
348 within one or more intestinal niche, as previously suggested (75). This possibility is difficult to
349 reconcile with the observation that F11Δ*fliC* has no substantial defects in our noncompetitive
350 assays (Fig. 2D). It is plausible that crosstalk among bacterial regulators of motility, T1P
351 expression, and other adhesins also contribute to the phenotypes observed in our assays with the
352 *fimH* mutants and other F11 derivatives (81-85), but this will require additional studies to tease
353 apart.

354 The ability of distinct types of bacteria to utilize different spatial and nutritional niches
355 within the gut allows for the coexistence of the diverse organisms that comprise the intestinal
356 microbiota, and helps provide a barrier against colonization by newly arriving microbes (86).
357 This latter effect, known as colonization resistance, is one reason that it is generally necessary to
358 treat mice with an antibiotic like streptomycin to open up niches for incoming bacteria that are
359 delivered into the gastrointestinal tract by oral gavage (32). A striking feature of our
360 experimental system is that F11 is able to colonize and persist indefinitely within the intestinal
361 tract of conventional SPF mice without the need to first administer antibiotics (Fig. 1A). Our
362 group and others have recently reported similar results with distinct ExPEC isolates in different
363 mouse strain backgrounds (24, 29, 31, 87). ExPEC may be able to effectively colonize our

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364 untreated mice because they have very low numbers of endogenous *E. coli* and other
365 Enterobacteriaceae based on 16S rRNA gene sequencing and selective plating assays (CR
366 Russell and MA Mulvey, unpublished data). Nevertheless, these animals are still resistant to
367 colonization by the K12 strain MG1655, indicating that MG1655 lacks one or more genes that
368 ExPEC employs to persist within the gut. When in competition with F11, MG1655 is cleared
369 from the gut at a notably faster rate than in noncompetitive assays (see Fig. 1), suggesting that
370 these two strains compete for the same intestinal niches.

371 A better understanding of the survival mechanisms used by ExPEC within the intestinal
372 tract may aid the development of more efficacious probiotics, while also elucidating new
373 therapeutic strategies to combat ExPEC before it is able to disseminate and cause disease at sites
374 beyond the intestinal tract. The effectiveness of such approaches may be dependent on multiple
375 variables, including timing, the makeup of the microbiota, and the presence of specific
376 competing strains that can alter ExPEC requirements for individual fitness determinants. For
377 example, in contrast to the situation in competitive assays, F11Δ*fimH* has no trouble colonizing
378 the gut in noncompetitive experiments and, once established, this mutant can even persist when
379 challenged with the WT strain (see Figs. 3B and 5B). Thus, while FimH can facilitate ExPEC
380 persistence within the gut in some settings, it is not always an absolute requirement. This
381 conclusion may help reconcile results from older studies in which the expression of T1P was
382 found to be unnecessary for *E. coli* persistence within the intestinal tracts of rodents and human
383 infants (88-90).

384 Context-dependent variability in the phenotypic effects of fitness determinants like FimH
385 may complicate treatment approaches, as well as our ability to discern how life within the gut
386 affects the evolution of ExPEC virulence traits. Nevertheless, data presented here indicate that it

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387 is reasonable to apply the principle of coincidental evolution to some ExPEC-associated genes,
388 though the phenotypic effects of these genes may be modest and variable dependent on the
389 experimental system that is used. The lack of easily discernable phenotypes for ExPEC-
390 associated loci within the gut suggests that selective forces encountered within other niches have
391 also helped shape ExPEC genomes. For example, extraintestinal virulence has been correlated
392 with the ability of ExPEC strains to resist killing by amoebae (91), while T1P expression has
393 been linked with the transmission of ExPEC between individuals by promoting transient
394 colonization of the oropharynx (88). In total, the results presented here show that piecing
395 together the evolutionary history of ExPEC virulence and fitness traits is a complicated task.
396 However, continuing efforts to resolve this problem will provide a more detailed picture of
397 ExPEC ecology and may help identify niche-specific fitness determinants that could be attractive
398 targets for therapeutic intervention.
399

## 400 MATERIALS AND METHODS

401 **Bacterial strains.** The cystitis isolate F11 and the K-12 strain MG1655 were genetically
402 modified using lambda-red recombination that was facilitated by the pKM208 plasmid (92).
403 Most of the constructs used for recombination were created by PCR using either pKD4 or pKD3
404 as a template to amplify a kanamycin or chloramphenicol resistance cassette, respectively,
405 flanked by ~40 base pairs of DNA with homology to the target insertion site. In some cases,
406 longer homology regions were required, and three-part PCR was performed. This was done by
407 PCR amplification of an antibiotic resistance cassette and regions that are upstream and
408 downstream of the target gene. Primers used were designed to contain sections of homology to
409 allow the three PCR products to be stitched together in a single ligation reaction.

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410 The pCP20 plasmid was used to remove the resistance cassette as necessary (93). To cure
411 F11 of the pUTI89 plasmid, the *ccdAB* toxin-antitoxin system was replaced with a *tetA-sacB*
412 construct and spontaneous loss of the plasmid was selected for on LB agar plates containing
413 fusaric acid and sucrose, as explained previously (94). The strains used in this study are listed in
414 Table S1 along with the primers used to create them. Prior to lambda-red recombination, bacteria
415 were grown shaking in LB broth at 37°C. All growth in petri dishes was done using LB agar
416 supplemented with chloramphenicol (20 μg/ml), kanamycin (50 μg/ml), or ampicillin (50
417 μg/ml), as appropriate.

418 **Mouse gut colonization.** Mice were handled in accordance with protocols approved by
419 the Institutional Animal Care and Use Committee at the University of Utah (Protocol number 15-
420 12015), following US federal guidelines indicated by the Office of Laboratory Animal Welfare
421 (OLAW) and described in the Guide for the Care and Use of Laboratory Animals, 8th Edition.

422 Prior to inoculation into mice, bacteria were grown statically from frozen stocks for 24 h
423 at 37°C in 250 ml flasks containing 20 ml of modified M9 media [$MgSO_4 \bullet 7H_2O$ (1 mM),
424 $CaCl_2 \bullet 2H_2O$ (0.1 mM), D-(+)-glucose (0.1%), nicotinic acid (0.00125%), thiamine HCl
425 (0.00165%), casamino acids (0.2%), $Na_2HPO_4$ (6g/L), $KH_2PO_4$ (3 g/L), $NH_4Cl$ (1 g/L), and NaCl
426 (0.5 g/L) in water]. A total of 12 ml of culture (6 ml of each culture for competitive experiments)
427 was centrifuged at 8,000 X g for 10 min. Bacterial pellets were then washed once and
428 resuspended in 0.5 ml of PBS. To inoculate the mouse gastrointestinal tract, 7-8 week old
429 female, specific pathogen free (SPF) Balb/c or C57Bl/6 mice (The Jackson Laboratory) were
430 orally gavaged with 50 μl PBS containing ~$10^9$ CFU of bacteria. At various time points post-
431 inoculation, individual mice were briefly placed into unused takeout boxes for weighing and
432 feces collection. Feces were homogenized in 1 ml of 0.7% NaCl and then samples were briefly

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433 centrifuged to pellet insoluble debris. Supernatants were serially diluted and spread onto LB agar
434 plates containing either chloramphenicol (20 μg/ml) or kanamycin (50 μg/ml) to select for
435 growth of the relevant bacterial strains. Prior to gavage, fecal samples were analyzed to ensure
436 that there were no endogenous bacteria present that were resistant to chloramphenicol or
437 kanamycin. Mice were housed 3-5 per cage, and were allowed to eat (irradiated Teklad Global
438 Soy Protein-Free Extruded chow) and drink antibiotic-free water *ad libitum*. Competitive indices
439 (CIs) were calculated as the ratio of mutant over WT bacteria recovered in the feces divided by
440 the ratio of mutant over WT bacteria in the inoculum, as noted previously (30).

441 To determine F11 (specifically F11-Clm$^R$) titers within the various regions of the
442 gastrointestinal tract at 14 d post-inoculation, mice were anaesthetized via isofluorane,
443 euthanized by cervical dislocation, and the cecum, colon, small intestine, and stomach were
444 removed. The small intestine was divided into thirds, with the portion closest to the stomach
445 labeled "proximal" and the portion closest to the cecum labeled "distal". A part of each organ
446 was weighed and placed into a Safe-Lock tube (Eppendorf) with three 3.2 mm stainless steel
447 beads, and homogenized using the Bullet Blender (Next Advance). The homogenates were then
448 serially diluted and plated onto LB agar plates containing chloramphenicol (20 μg/mL) to
449 quantify the levels of F11-*clmR* present.

450 For histology, colon tissues were fixed in 10% neutral buffered formalin and submitted to
451 the University of Utah Research Histology core for processing and staining with hematoxylin
452 and eosin (H&E). Uninfected intestinal tissues and tissues recovered from mice at 14 d post-
453 inoculation with either MG1655 or F11Δ*hlyA* were used for comparison. Random colon tissue
454 sections from 5 or more mice in each experimental group were analyzed in a blinded fashion by
455 a trained pathologist (M.P. Bronner).

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456 **Motility Assays.** To test the swimming ability of particular strains, motility agar plates
457 were made by pouring 25 mL of LB soft agar (0.1% agar) into a petri dish. Bacteria (1.5 µl from
458 an overnight culture) were dispensed just below the surface of the plate, which was then
459 incubated at 37° C for 8-10 h prior to imaging with a Stratagene Eagle Eye II System.
460 **Luciferase assays.** The p*fliC-lux* construct was kindly provided by the Mobley lab (62).
461 Overnight shaking cultures of F11, F11Δ*papG*, and F11Δ*fimH* carrying p*fliC-lux* were diluted
462 1:100 into 1 ml of fresh tryptone broth (Fisher Scientific) containing ampicillin (50 µg/ml).
463 Three 100-ul-aliquots of each culture were then transferred to a white 96-well polystyrene plate
464 (Dynex Technologies) and grown statically at 37˚C. Luminescent emission spectra were
465 collected every 30 minutes for 4.5 hours using Gen5 Software with a BioTek Synergy H1 plate
466 reader. The instrument was set to integrate readings over 10 seconds using a gain of 135 and
467 height of 1 mm. Before each reading, the plates were shaken for 1 second. Corresponding growth
468 curves, which were obtained by taking $OD_{600}$ measurements of cultures grown statically in clear
469 96-well polystyrene plates (CytoOne), showed that the strains used in these assays grew
470 similarly.
471 **Analysis of the *fim* switch.** Quantification of the *fim* switch in the ON and OFF positions
472 was DNA was carried out essentially as previously described (68, 69). DNA was purified from
473 feces using the ZR Fecal DNA MiniPrep kit, whereas DNA was purified from broth cultures
474 using the Promega Wizard Genomic DNA Purification kit. To skew the *fim* switch towards the
475 ON position, F11 was grown statically at 37˚C in 20 mL LB broth in 250 mL Erlenmeyer flasks
476 for 24 h, sub-cultured 1:1000 into fresh LB broth, and then incubated for another 24 h. To drive
477 the *fim* switch towards the OFF position, bacteria were grown shaking in LB broth to exponential
478 phase. The primers F11_fimS_F (TACCGCCAGTAATGCTGCTC) and

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479 F11_fimS_R (GTCCCACCATTAACCGTCGT) were used to amplify the *fim* switch region by
480 PCR using the following thermocycler conditions: 95°C for 5 minutes, 10 cycles (95°C for 30
481 sec, 60°C for 20 sec, 72°C for 40 sec), followed by 30 cycles (95°C for 30 sec, 56°C for 20 sec,
482 72°C for 40 sec), and ending with a 5 min incubation at 72°C. The reaction products were
483 column purified, digested with HinfI for 1 h at 37°C, resolved in 1% agarose gels, and imaged.
484 Bands representing the *fim* switch in the ON and OFF positions were quantified using ImageJ.
485 **Statistical Analysis.** All statistical tests were carried out using GraphPad Prism or
486 Stata/IC-14 software. Where indicated, corrections for multiple comparisons were made using
487 the Hochberg procedure (95). $P$ values $\leq 0.05$ were considered significant.
488

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493

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790 **FIGURE LEGENDS**

791 **Figure 1. ExPEC colonizes and persists within the gut of SPF mice without causing serious**
792 **pathology.** Adult female SPF Balb/c mice were inoculated via oral gavage with ~$10^9$ CFU of
793 bacteria. (A) Titers of F11 recovered from the feces of mice at various time points post-gavage.
794 n=5 mice. (B) F11 titers within intestinal tissues at 14 d post-gavage. (C) Mice were gavaged
795 with a 1:1 mixture of F11 and MG1655, and fecal titers were determined for both populations at
796 the indicated time points. *, $p \leq 0.05$ by Wilcoxon signed-rank tests with corrections for multiple
797 comparisons. (D) Fecal titers from non-competitive assays in which mice were orally inoculated
798 with F11, F11Δ*hlyA*, or MG1655. ****, $p \leq 0.0001$ by Mann-Whitney U Tests with corrections
799 for multiple comparisons. #, $p \leq 0.05$ by Mann-Whitney U Tests without corrections. In A-D,
800 bars indicate median values. (E) Relative weights (mean values ± SD) of mice following oral
801 inoculation with F11, F11Δ*hlyA*, or MG1655. Data were normalized to the mass of each mouse
802 prior to gavage. ***, $p \leq 0.001$ with corrections when F11 is compared to either F11Δ*hlyA* or
803 MG1655 by unpaired Student's *t* tests. In B-E, n=10 mice from two independent experiments.
804

805 **Figure 2. Key ExPEC-associated factors are not required for gut colonization.** Mice were
806 orally gavaged with ~$10^9$ CFU of WT F11 or isogenic mutant strains lacking (A) *cnf1*, (B) *usp*,
807 (C) *clbCDEFG*, (D) *fliC*, or (E) plasmid p*UTI89*. At the indicated time points, feces were
808 collected, homogenized, and plated on selective media to determine bacterial titers. Bars
809 represent median values. n=9-11 mice from two independent experiments. No statistically
810 significant differences between WT and mutant titers were discerned using Mann-Whitney U
811 tests.
812

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813 **Figure 3. The persistence of F11Δ*fimH*, but not F11Δ*papG*, within the gut is impaired in**
814 **competitive assays.** Mice were gavaged with WT F11, (A) F11Δ*papG*, or (B) F11Δ*fimH* and
815 fecal titers were determined at the indicated time points. In these non-competitive assays, no
816 significant differences were identified by Mann-Whitney U tests. For competitive assays, mice
817 were gavaged with a 1:1 mixture of (C) F11-Kan$^R$ and F11-Clm$^R$, (D) WT F11 and F11Δ*papG*,
818 (E) or WT F11 and F11Δ*fimH*. *, $p \leq 0.05$ by one sample $t$ tests without correction for multiple
819 comparisons. Pie charts in (E) indicate fraction of mice in which F11Δ*fimH* (green) was not
820 detected. #, $p \leq 0.05$ by Fisher's exact tests without corrections. In A-E, n = 11-13 mice from
821 two independent assays. (F) Graph shows fractions of the *fim* switch in the ON and OFF
822 positions from fecal samples recovered from mice following oral inoculation with F11. Bars
823 indicate mean values ± SEM; n=5-10 mice. Results from F11 grown in static or shaking LB
824 broth are shown for comparison.
825
826 **Figure 4. Flagellin expression impacts the efficacy of gut colonization by F11Δ*fimH*.** (A)
827 WT F11 and the indicated mutant derivatives were inoculated into motility agar to assess
828 swimming. Images of swim plates were taken at 8-10 h post-inoculation and are representative of
829 results from three independent assays. (B) Plot shows results from *fliC* expression reporter assays
830 with WT F11, F11Δ*fimH*, and F11Δ*papG* carrying p*fliC-lux*. Lines indicate mean luminescence
831 values ± SEM from three independent assays performed in triplicate. $p \leq 0.05$ by multiple $t$ tests
832 with (*) or without (#) corrections for multiple comparisons. (C-D) Graphs show results from
833 competitive assays in which mice were orally gavaged with a 1:1 mixture of (C) WT F11 and
834 F11Δ*fimH*Δ*fliC* (DKO) bacteria or (D) F11Δ*fimH* and F11Δ*fimH*Δ*fliC* (DKO). Fecal titers of
835 each strain were determined at the indicated time points by plating on selective media. *, $p \leq$

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836 0.05 by one sample *t* tests with corrections for multiple comparisons. Pie charts in (C-D) denote
837 the fraction of mice in which the DKO mutant (yellow), WT F11 (blue), or F11Δ*fimH* (green)
838 were not detected. No significant differences were discerned by Fisher's exact tests. n=13-15
839 mice from at least two independent assays.
840
841 **Figure 5. Pre-colonization of mice with F11Δ*fimH* effectively limits colonization by the WT**
842 **strain, and vice versa.** Balb/c mice were inoculated via oral gavage with (A) WT F11
843 (specifically F11-$Clm^R$) and then with F11Δ*fimH* ($Kan^R$) 7 d later. (B) Alternatively, mice were
844 inoculated with F11Δ*fimH* followed 7 d later by the WT strain. Solid lines connect median fecal
845 titers of each strain over time. The zero time point (dotted line) indicates when the second strain
846 (WT F11 or F11Δ*fimH*) was introduced. *, $p<0.05$ by Mann-Whitney U tests with corrections for
847 multiple measurements; n=5 mice.
848
849 **Supplemental Figure S1. F11, but not MG1655, effectively colonizes the intestinal tract of**
850 **SPF C57Bl/6 mice.** Adult female SPF C57Bl/6 mice were inoculated via oral gavage with $\sim 10^9$
851 CFU of either F11 or MG1655. (A) Graph shows titers of F11 and MG1655 recovered from the
852 feces at various time points post-gavage. n=10 mice from two independent non-competitive
853 assays. **, $p<0.01$ by Wilcoxon signed-rank tests, with corrections for multiple comparisons. (B)
854 F11 titers recovered in association with the colonic tissue or within the lumen of the colon at 14
855 d post-gavage. *P* value determined by Mann-Whitney test; n=10 mice.
856

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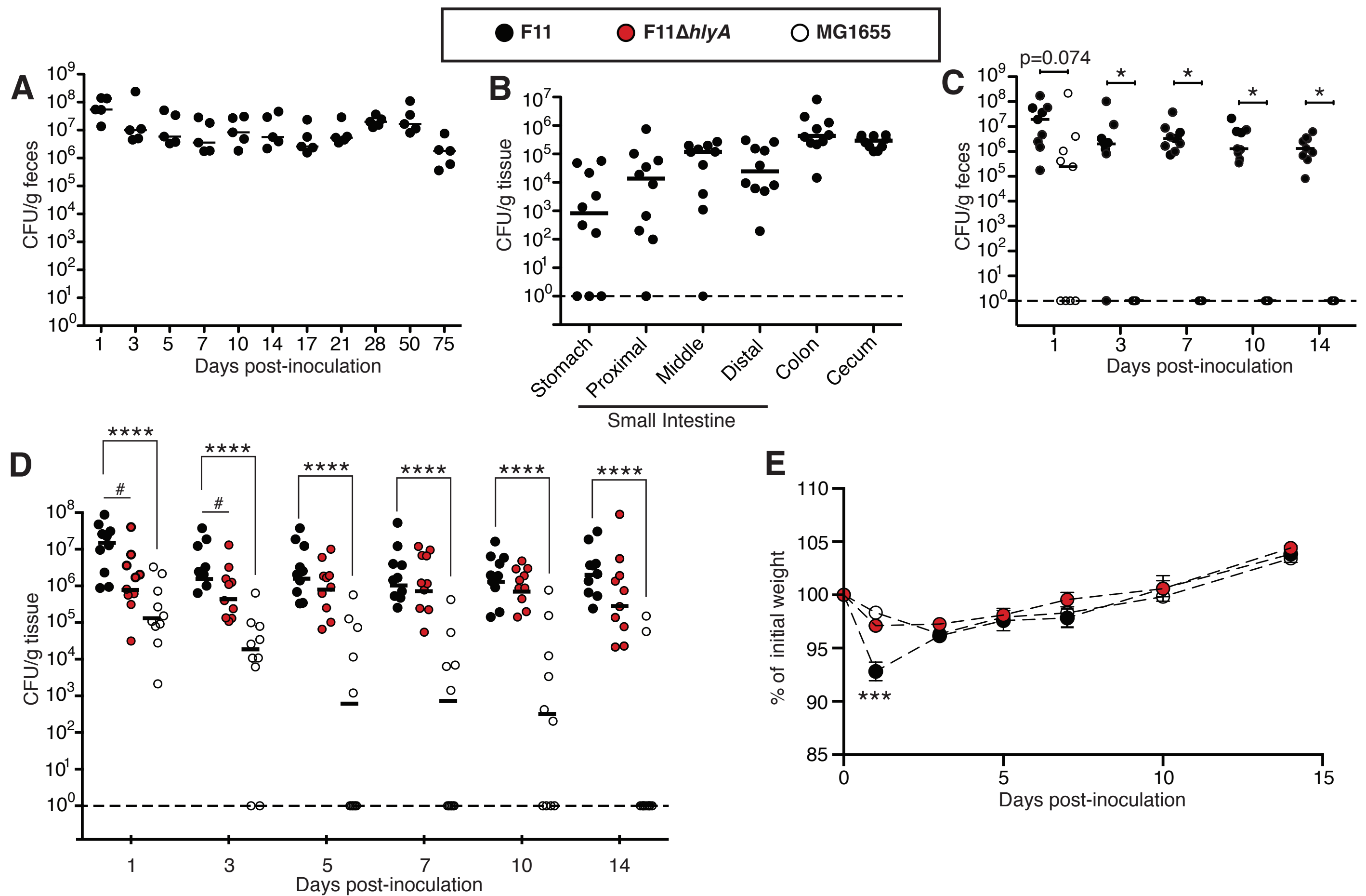


**Figure 1. ExPEC colonizes and persists within the gut of SPF mice without causing serious pathology.** Adult female SPF Balb/c mice were inoculated via oral gavage with ~$10^9$ CFU of bacteria. (A) Titers of F11 recovered from the feces of mice at various time points post-gavage. n=5 mice. (B) F11 titers within intestinal tissues at 14 d post-gavage. (C) Mice were gavaged with a 1:1 mixture of F11 and MG1655, and fecal titers were determined for both populations at the indicated time points. *, $p \leq 0.05$ by Wilcoxon signed-rank tests with corrections for multiple comparisons. (D) Fecal titers from non-competitive assays in which mice were orally inoculated with F11, F11Δ*hlyA*, or MG1655. ****, $p \leq 0.0001$ by Mann-Whitney U Tests with corrections for multiple comparisons. #, $p \leq 0.05$ by Mann-Whitney U Tests without corrections. In A-D, bars indicate median values. (E) Relative weights (mean values ± SD) of mice following oral inoculation with F11, F11Δ*hlyA*, or MG1655. Data were normalized to the mass of each mouse prior to gavage. ***, $p \leq 0.001$ with corrections when F11 is compared to either F11Δ*hlyA* or MG1655 by unpaired Student's *t* tests. In B-E, n=10 mice from two independent experiments.

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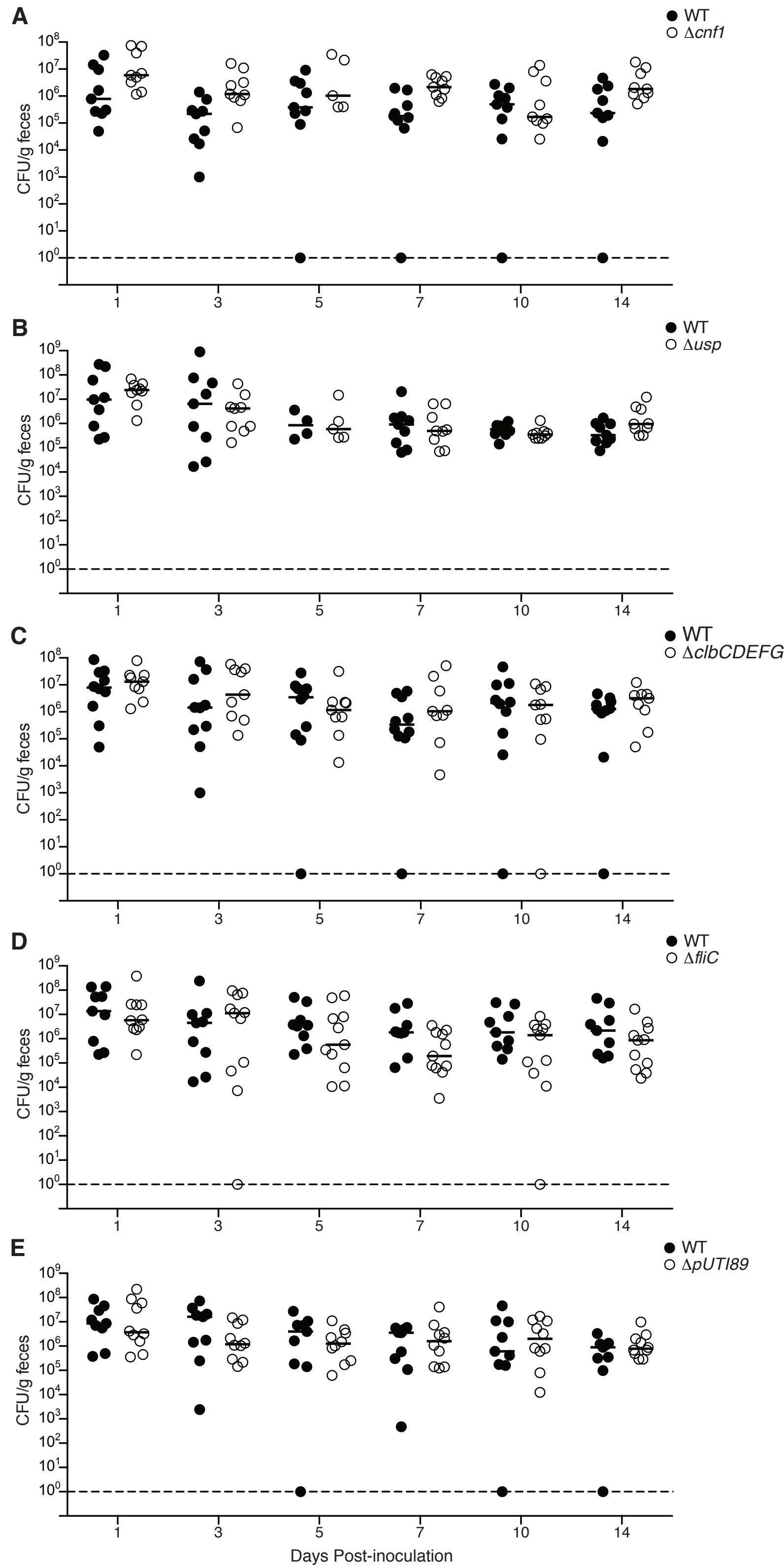


**Figure 2. Key ExPEC-associated factors are not required for gut colonization.** Mice were orally gavaged with ~$10^9$ CFU of WT F11 or isogenic mutant strains lacking (A) *cnf1*, (B) *usp*, (C) *clbCDEFG*, (D) *fliC*, or (E) plasmid p*UTI89*. At the indicated time points, feces were collected, homogenized, and plated on selective media to determine bacterial titers. Bars represent median values. n=9-11 mice from two independent experiments. No statistically significant differences between WT and mutant titers were discerned using Mann-Whitney U tests.

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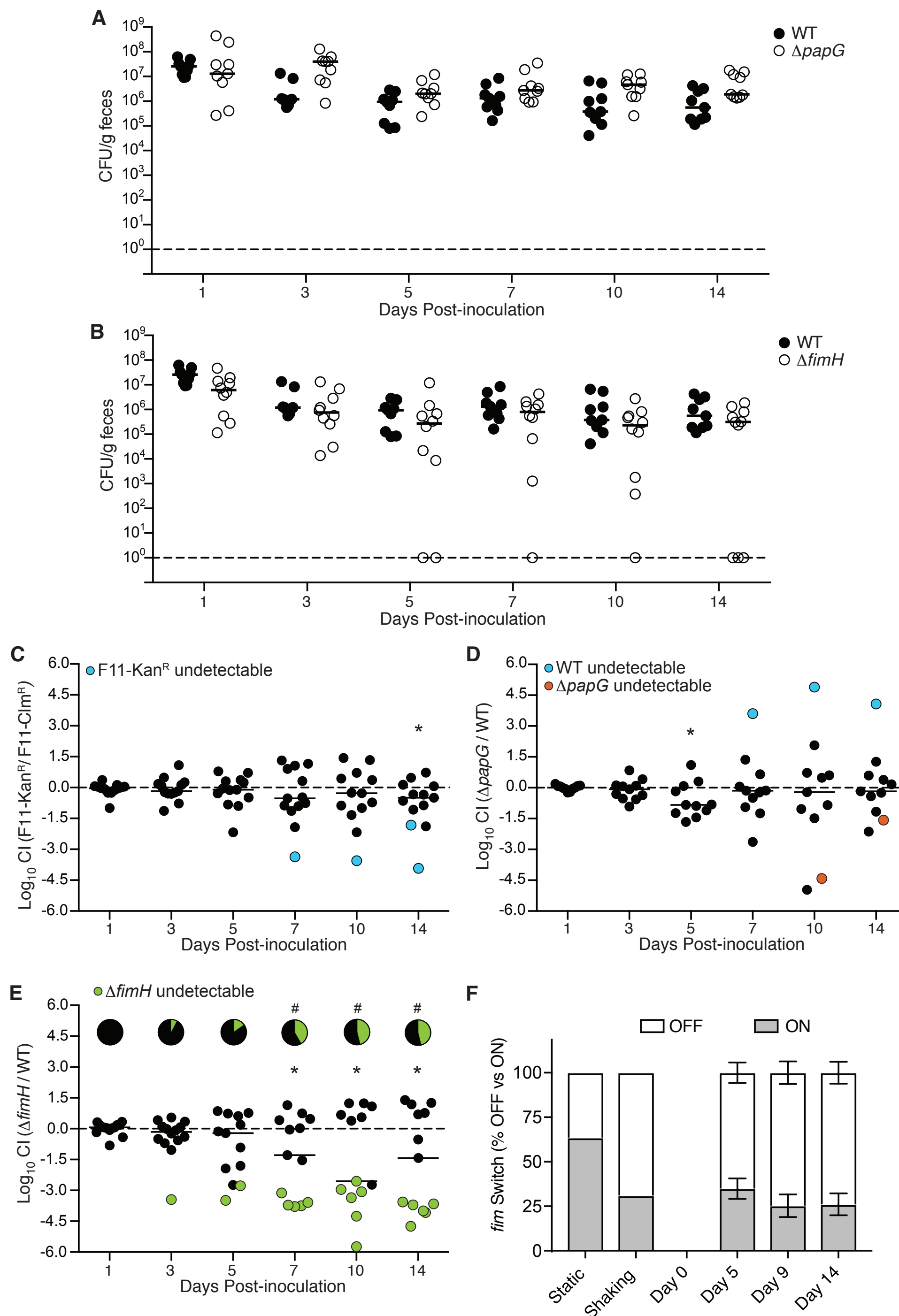


**Figure 3. The persistence of F11Δ*fimH*, but not F11Δ*papG*, within the gut is impaired in competitive assays.** Mice were gavaged with WT F11, (A) F11Δ*papG*, or (B) F11Δ*fimH* and fecal titers were determined at the indicated time points. In these non-competitive assays, no statistical significant differences were identified by Mann-Whitney U tests. For competitive assays, mice were gavaged with a 1:1 mixture of (C) F11-Kan$^R$ and F11-Clm$^R$, (D) WT F11 and F11Δ*papG*, (E) or WT F11 and F11Δ*fimH*. *, $p \leq 0.05$ by one sample *t* tests without correction for multiple comparisons. Pie charts in (E) indicate fraction of mice in which F11Δ*fimH* (green) was not detected. #, $p \leq 0.05$ by Fisher's exact tests without corrections. In A-E, n = 11-13 mice from two independent assays. (F) Graph shows fractions of the *fim* switch in the ON and OFF positions from fecal samples recovered from mice following oral inoculation with F11. Bars indicate mean values ± SEM; n=5-10 mice. Results from F11 grown in static or shaking LB broth are shown for comparison.

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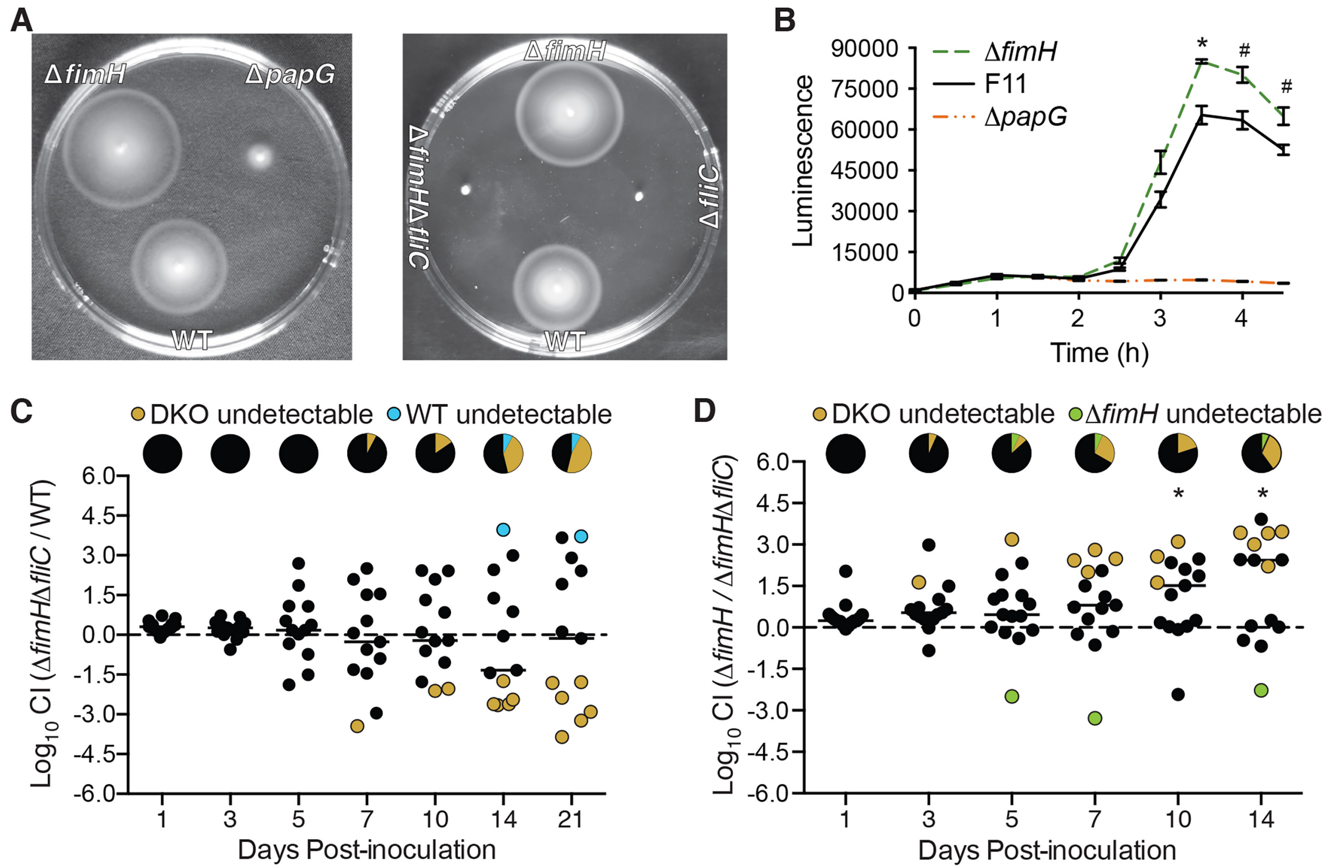


**Figure 4. Flagellin expression impacts the efficacy of gut colonization by F11Δ*fimH*.** (A) WT F11 and the indicated mutant derivatives were inoculated into motility agar to assess swimming. Images of swim plates were taken at 8-10 h post-inoculation and are representative of results from three independent assays. (B) Plot shows results from *fliC* expression reporter assays with WT F11, F11Δ*fimH*, and F11Δ*papG* carrying p*fliC-lux*. Lines indicate mean luminescence values ± SEM from three independent assays performed in triplicate. $p \leq 0.05$ by multiple *t* tests with (*) or without (#) corrections for multiple comparisons. (C-D) Graphs show results from competitive assays in which mice were orally gavaged with a 1:1 mixture of (C) WT F11 and F11Δ*fimH*Δ*fliC* (DKO) bacteria or (D) F11Δ*fimH* and F11Δ*fimH*Δ*fliC* (DKO). Fecal titers of each strain were determined at the indicated time points by plating on selective media. *, $p \leq 0.05$ by one sample *t* tests with corrections for multiple comparisons. Pie charts in (C-D) denote the fraction of mice in which the DKO mutant (yellow), WT F11 (blue), or F11Δ*fimH* (green) were not detected. No significant differences were discerned by Fisher's exact tests. n=13-15 mice from at least two independent assays.

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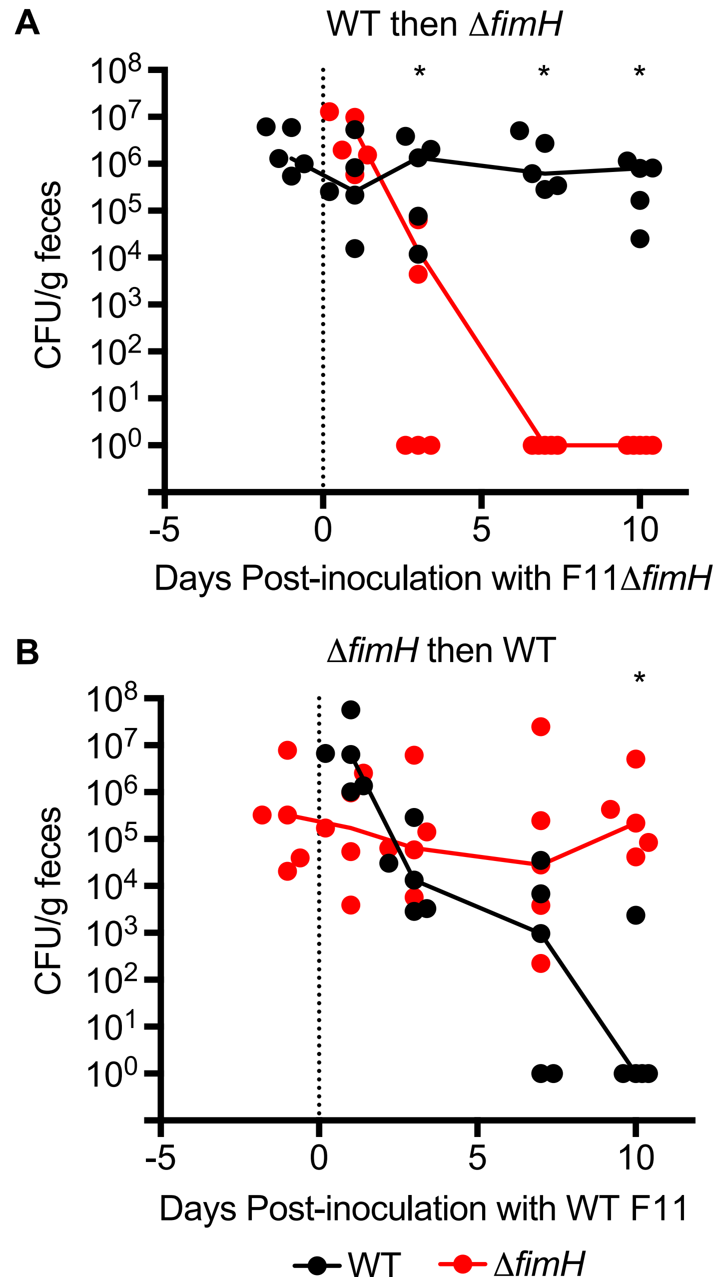


**Figure 5. Pre-colonization of mice with F11Δ*fimH* effectively limits colonization by the WT strain, and vice versa.** Balb/c mice were inoculated via oral gavage with (A) WT F11 (specifically F11-$Clm^R$) and then with F11Δ*fimH* ($Kan^R$) 7 d later. (B) Alternatively, mice were inoculated with F11Δ*fimH* followed 7 d later by the WT strain. Solid lines connect median fecal titers of each strain over time. The zero time point (dotted line) indicates when the second strain (WT F11 or F11Δ*fimH*) was introduced. *, $p<0.05$ by Mann-Whitney U tests with corrections for multiple measurements; n=5 mice.

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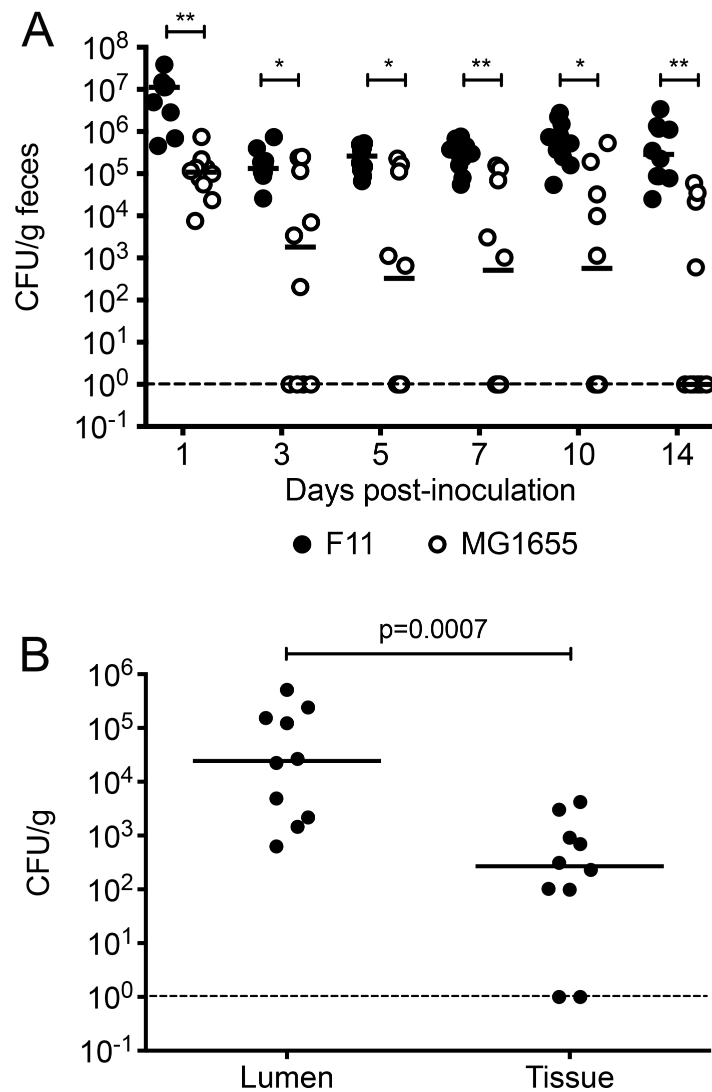


**Supplemental Figure S1. F11, but not MG1655, effectively colonizes the intestinal tract of SPF C57Bl/6 mice.** Adult female SPF C57Bl/6 mice were inoculated via oral gavage with ~$10^9$ CFU of either F11 or MG1655. (A) Graph shows titers of F11 and MG1655 recovered from the feces at various time points post-gavage. n=10 mice from two independent non-competitive assays. **, $p<0.01$ by Wilcoxon signed-rank tests, with corrections for multiple comparisons. (B) F11 titers recovered in association with the colonic tissue or within the lumen of the colon at 14 d post-gavage. *P* value determined by Mann-Whitney test; n=10 mice.

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Supplemental Table S1. Bacterial strains used in this study

| Strain | Description | Primers* used to create strain, or reference |
|---|---|---|
| MG1655::*clm* | MG1655 derivative in which a chloramphenicol resistance cassette has been inserted at the *attTn7* site | F: AGGATGTTTGATTAAAAACATAACAGGAAGAAAAATGCTGTGTAGGCTGGAGCTGCTTCG<br>R: ATCGGTTACGGTTGAGTAATAAATGGATGCCCTGCGTAAGCATATGAATATCCTCCTTAG |
| F11::*clm* (F11-Clm$^R$) | F11 with a chromosomal insertion of a chloramphenicol resistance cassette at the intergenic region between genes *EcF11_2526* and *xseA* | 1 |
| F11::*kan* (F11-Kan$^R$) | F11 with a chromosomal insertion of a kanamycin resistance cassette at the intergenic region between genes *EcF11_2526* and *xseA* | F: TCTGGCGTAGCCTGGGAGTTATTGCCGGATGCGATGCTGGTGTGTAGGCTGGAGCTGCTTCG<br>R: TCACGTAAAAAAACGTCTAATCCGTAGACCGGATAAGAGGCATATGAATATCCTCCTTAG |
| F11Δ*clbCDEFG*::*clm* | F11 derivative in which the *clbCDEFG* operon has been replaced with a chloramphenicol resistance cassette | F1: TCGGGCGATCGATAGATTAG<br>R1: CGAAGCAGCTCCAGCCTACACAGCTTGCGTATTCCATAAACTTC<br>F2: TGTGTAGGCTGGAGCTGCTTCG<br>R2: CATATGAATATCCTCCTTAG<br>F3: CTAAGGAGGATATTCATATGCCCGTCACGCCATTTTACGT<br>R3: TAATATACGCCAGTTGCCGC |
| F11Δ*cnf1*::*kan* | F11 derivative in which the *cnf1* gene has been replaced with a kanamycin resistance cassette | F: GATAAGGTGTAGTAAAATATTAATCTTCACAGAGGAGTGTGTAGGCTGGAGCTGCTTCG<br>R: GGAGTAACTATAACAATGGCCAATAAATAATTTCCCGAACATATGAATATCCTCCTTAG |
| F11Δ*fimH*::*kan* | F11 derivative in which the *fimH* gene has been replaced with a kanamycin resistance cassette | F: TTATTGATAAACAAAAGTCACGCCAATAATCGATTGCATGTGTAGGCTGGAGCTGCTTCG<br>R: ATGAAACGAGTTATTACCCTGTTTGCTGTACTGCTGATGGCATATGAATATCCTCCTTAG |
| F11Δ*fimH*Δ*fliC*::*clm* | F11 derivative in which the *fimH* gene has been deleted, and the *fliC* gene has been replaced with a chloramphenicol resistance cassette | See *fimH* and *fliC* single knockout primers |
| F11Δ*fliC*::*kan* | F11 derivative in which the *fliC* gene was replaced with a kanamycin resistance cassette | F: ATGGCACAAGTCATTAATACCAACAGCCTCTCGCTGATCTGTGTAGGCTGGAGCTGCTTCG<br>R: TTAACCCTGCAGCAGAGACAGAACCTGCTGCGGTACCTGGCATATGAATATCCTCCTTAG |
| F11Δ*hlyA*::*kan* | F11 derivative in which the *hlyA* gene has been replaced with a kanamycin resistance cassette | 2 |
| F11Δ*papG*::*kan* | F11 derivative in which the *papG* gene has been replaced with a kanamycin resistance cassette | F: ATGTTTTACTCGTTTAATGATAACATTTATCGTCCTCATGTGTAGGCTGGAGCTGCTTCG<br>R: TTATGGCAATATCATGAGCAGCGTTGCTGAACCAGATAGTCATATGAATATCCTCCTTAG |
| F11Δ*usp*::*kan* | F11 derivative in which the *usp* gene has been replaced with a kanamycin resistance cassette | F: GTGGGCGATATTGTTTACCTGAGAATAATCGGTGAGAATGTGTAGGCTGGAGCTGCTTCG<br>R: TTATCTCCTGTAGTGAATCTCATCGTGTAGTCTGGGGGTACATATGAATATCCTCCTTAG |
| F11::*kan* Δ*pUTI89* | A derivative of F11::*kan* which was cured of the pUTI89 plasmid by replacement of *ccdAB* with *tetA-sacB* followed by counterselection | F1: CTGTTCGTTTATTACGCCG<br>R1: GATAGAGTGTCAACAAAAATTAGGAATGTCAGGCTCCGTTATACAC<br>F2: TCCTAATTTTTGTTGACACTCTATC<br>R2: TTAATCAAAGGGAAAACTGTCCATATGC<br>F3: GCATATGGACAGTTTTCCCTTTGATTAAAGCACACCTCTTTTTGACATACT<br>R3: GTTGCTATTTCTGGCTTAGTCAG |

References:


(1) Russell CW, Mulvey MA. 2015. The extraintestinal pathogenic *Escherichia coli* factor RqlI constrains the genotoxic effects of the RecQ-like helicase RqlH. *PLoS Pathog.* 11(12):e1005317.

(2) Wiles TJ, Bower JM, Redd MJ, Mulvey MA. 2009. Use of zebrafish to probe the divergent virulence potentials and toxin requirements of extraintestinal pathogenic *Escherichia coli*. *PLoS Pathog.* 5(12):e1000697.


* In some cases longer homology arms were created by three-part PCR. In these instances, three primer sets are listed.