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

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ABSTRACT
Extraintestinal pathogenic *Escherichia coli* (ExPEC) act as commensals within the mammalian gut, but can induce pathology upon dissemination to other host environments such as the urinary tract and bloodstream. ExPEC genomes are likely shaped by evolutionary forces encountered within the gut where the bacteria spend much of their time, provoking the question of how their extraintestinal virulence traits arose. The principle of coincidental evolution, in which a gene that evolved in one niche happens to be advantageous in another, has been used to argue that ExPEC virulence factors originated in response to selective pressures within the gut ecosystem. As a test of this hypothesis, the fitness of ExPEC mutants lacking canonical virulence factors was assessed within the intact murine gut in the absence of antibiotic treatment. We found that most of the tested factors—including CNF1, Usp, colibactin, flagella, and the plasmid pUTI89—were dispensable for gut colonization. Deletion of genes encoding the adhesin PapG or the toxin HlyA had transient effects, but did not interfere with longer-term persistence. In contrast, a mutant missing the type 1 pilus-associated adhesin FimH displayed somewhat reduced persistence within the gut. However, this phenotype varied dependent on the presence of specific competing strains and was partially attributable to aberrant flagellin expression in the absence of fimH. These data indicate that FimH and other key ExPEC-associated factors are not strictly required for gut colonization, suggesting that the development of extraintestinal virulence traits is not driven solely by selective pressures within the gut.
INTRODUCTION

The bacterium *Escherichia coli* was first described by Theodor Escherich in 1884, and has since become a critical model organism that has been used to understand the fundamentals of molecular biology (1). *E. coli* is able to live in a variety of locations, including the soil, water, and the human gut. Although it is a prominent member of the neonatal microbiota, it is quickly overshadowed by burgeoning anaerobic bacteria as oxygen becomes scarce within the gut following birth (2, 3). As the intestinal microbiota develops, *E. coli* and other facultative anaerobes preferentially colonize the mucus layer that lines the large intestine, where oxygen is most abundant (around 40 mm Hg) (1, 4). In contrast, most anaerobic bacteria occupy the lumen where oxygen is scarce (typically less than 1 mm Hg) (4). In adults, *E. coli* is present at about $10^7$ to $10^9$ Colony Forming Units (CFU)/g feces, a level that is 100-10,000-fold-lower than the resident anaerobes (5). Despite being a minor component of the microbiota, the estimated number of *E. coli* cells that are transmitted via fecal matter from each human being to the environment in a single day is staggering – about $10^{11}$ CFU (6).

Understanding the role of *E. coli* within the microbiota is complicated by the fact that *E. coli* is a very diverse species with a wide spectrum of phenotypes (1). Some *E. coli* strains live harmlessly in the gut, while others act as pathogens, causing diarrhea and hemorrhaging (7). A few have been linked with the development of Crohn’s Disease and colorectal cancer (8-10). One strain, Nissle 1917, acts as a probiotic that assuages inflammation in addition to inhibiting colonization by pathogens such as *Salmonella* (11, 12). A group of strains known as Extraintestinal Pathogenic *Escherichia coli* (ExPEC) generally act as commensals within the gut, but can disseminate to other host environments and subsequently cause disease (13). ExPEC include uropathogenic *E. coli* (UPEC), which cause the overwhelming majority of urinary tract
infections (UTIs) (14). These infections are especially prevalent among women, about half of
whom will have at least one UTI during their lifetime. ExPEC is also responsible for other, more
serious conditions, including sepsis and neonatal meningitis (13, 15).

The gut is thought to be the major ExPEC reservoir that seeds extraintestinal infections.
Evidence for this notion is that the same ExPEC strain can often be isolated from both the feces
and urine of individual patients suffering from UTIs (16-19). Indeed, ExPEC strains are
frequently difficult to clear from the gut with antibiotic treatments, even when the pathogens are
effectively eliminated from the urinary tract (20). Furthermore, E. coli strains belonging to
phylogenetic group B2, which includes many ExPEC isolates, are much more likely to be long-
term residents within the gut in comparison with other E. coli populations (21-23). The majority
of adults carry group B2 E. coli strains within the gut, irrespective of extraintestinal infections
(23). Cumulatively these observations suggest that ExPEC primarily inhabit the gut, with
sporadic departures to extraintestinal sites.

Given that ExPEC reside mostly within the gut, and that transmission of ExPEC among
individuals likely occurs chiefly through fecal-oral routes (5, 24-26), it is expected that ExPEC
genomes have been shaped in large part by the evolutionary pressures present within the gut.

How then, did extraintestinal virulence factors come into being? The hypothesis of coincidental
evolution has often been evoked to answer this question (27). In general terms, coincidental
evolution is when a factor evolves in one context, but happens to be useful in another context as
well (28). When the hypothesis of coincidental evolution is applied to the ExPEC life cycle, the
implication is that factors that promote virulence in extraintestinal niches evolved in the gut for a
function possibly unrelated to virulence.

Little concrete evidence has been put forth to support or contradict coincidental evolution
in the context of ExPEC infection, other than the fact that known extraintestinal virulence factors are often encoded by gut isolates (22, 27, 29). One prediction of this hypothesis is that extraintestinal virulence factors should play a role in gut colonization. To date, this possibility has only been addressed by one study in which an ExPEC mutant that lacks multiple pathogenicity-associated islands (PAIs) was found to be defective in its ability to persist within the murine intestinal tract (6). It is clear that more experimental work needs to be done to determine to what extent coincidental evolution applies to single virulence factors within ExPEC. To address this issue, several canonical virulence factors were individually deleted from a reference ExPEC isolate and the resulting mutants were tested for their ability to colonize and persist within the mouse gastrointestinal tract. Among eight virulence factors that were examined, only the type 1 pilus-associated mannose-binding adhesin FimH had a notable persistence defect within the gut. However, this defect was variable and partly contingent upon aberrant flagellin expression by the fimH mutant and the presence of specific competing strains. These findings are discussed in the context of both coincidental evolution and the development of anti-ExPEC therapeutics.

RESULTS

ExPEC stably colonizes the murine intestinal tract in the presence of the natural, intact microbiota. To examine ExPEC colonization of the intestinal tract, we employed a model in which adult specific pathogen free (SPF) Balb/c mice were intragastrically gavaged with ~10^9 CFUs of the reference cystitis isolate F11. At various time points post-gavage, feces were collected, and the numbers of viable ExPEC present were enumerated. F11 and other bacterial strains used in this study were engineered to express either chloramphenicol (Clm^R) or
kanamycin (Kan\textsuperscript{R}) resistance cassettes so that they could be easily identified by plating fecal homogenates on selective media. Following inoculation, the fecal titers of F11 remained fairly stable for up to 75 days, with median values ranging between $10^6$ and $10^7$ CFU/g feces after the first day (Fig. 1A). These data demonstrate that ExPEC can efficiently initiate and maintain colonization of the SPF mouse gut, in line with recent reports from our group and others (25, 30-32). Consistent with the observation that nonpathogenic \textit{E. coli} mostly reside within the large bowel (33), the cecum and colon carried the largest load of F11 at the 2-week time point, although considerable numbers of F11 were also present within the small intestine (Fig. 1B). Relatively few bacteria were recovered from the stomach, which is not thought to be a stable niche for \textit{E. coli}. Due to the coprophagic nature of mice, it is likely that the animals are ingesting fecal material containing shed F11 and that the low numbers of F11 recovered from the stomach simply represent bacteria that are in transit to the small intestine. The amounts of F11 shed in the feces did not allow for efficient intestinal colonization via mouse-to-mouse transmission through coprophagy (CW Russell, BA Fleming, and MA Mulvey, unpublished data). Importantly, in our assays, F11 fecal titers correlate well with the levels of F11 detected within the lower intestinal tract (Fig. 1C), indicating that the enumeration of fecal titers is a valid proxy for assessing gut colonization.

It is of note that the SPF mice utilized in these experiments were not treated with antibiotics and therefore each possesses an intact microbiota. This is in contrast to other commonly used mouse models in which mice are treated with streptomycin and/or other antibiotics in order to disrupt the intestinal microbiota and open up niches that can then be occupied by incoming microbes (33). Since antibiotic treatment was not required for consistent colonization of the gut by F11 (or by other ExPEC isolates (25, 30)), we wondered whether ExPEC is simply more adept at gut colonization than nonpathogenic \textit{E. coli} strains. As a test of
this idea, we first competed F11 head-to-head with MG1655, an often-used nonpathogenic E. coli K12 strain. Following oral gavage with equal numbers of F11 and MG1655, the K12 bacteria were cleared from all of the mice by day 3, while F11 stably persisted (Fig. 1D). When mice were gavaged with MG1655 alone, it was lost at a slower rate, but was still cleared from 80% of the mice by day 14 (Fig. 1E). This is in sharp contrast to the persistent phenotype observed with F11. Similar results were obtained in competitive assays using SPF C57Bl/6 mice (Fig. S1A).

To assess whether ExPEC colonization of the colon is marked by inflammation (colitis), the mice were weighed at several time points after colonization, as weight loss frequently accompanies colitis. Mice that were gavaged with F11 consistently experienced transient weight loss at the one-day time point, in comparison to mice that were inoculated with MG1655 (Fig. 1F). Since the ExPEC-associated pore forming toxin α-hemolysin (HlyA) was previously shown to induce inflammation within the gut in other mouse models (34-36), we hypothesized that HlyA contributes to the transient weight loss seen in F11-colonized animals. When mice were colonized with F11ΔhlyA, fecal titers of the mutant were notably lower relative to wild type (WT) F11 at 1 and 3 days post-inoculation (Fig. 1E, uncorrected $P = 0.015$ and 0.023, respectively). However, these differences were not significant when $P$ values were adjusted for multiple measurements. At later time points, the ΔhlyA mutant persisted at levels more like the WT strain, though median titers of mutant were always lower. These data, coupled with the observation that F11ΔhlyA does not elicit transient weight loss by the host (Fig. 1E), suggest that HlyA both enhances initial colonization of the gut by F11 and stimulates short-term inflammation that causes transient weight loss. By the 14-day time point, the colons of mice that were gavaged with WT F11, F11ΔhlyA, or MG1655 appear unperturbed, having normal crypt
architecture and no evidence of infiltrating immune cells, as assessed by histological analysis.

Altogether, these data indicate that F11 colonization induces transient inflammation of the intestinal tract with coordinate weight loss by the host. HlyA can facilitate these processes, but is not strictly required for the longer-term persistence of F11 within the gut.

Not all ExPEC-associated toxins promote ExPEC fitness within the gut. In light of the hypothesis of coincidental evolution and observations showing that HlyA can enhance initial colonization of the gut by F11 (Fig. 1E), we wished to examine possible roles for other canonical ExPEC-associated toxins as mediators of intestinal colonization. The first of these was cytotoxic necrotizing factor type 1 (CNF1), a secreted toxin that catalyzes the deamidation of a specific glutamine residue within Rho family GTPases (37). This causes constitutive activation of Rho GTPases, leading to aberrant host cytoskeletal rearrangements and multinucleation (38, 2, 39).

CNF1 has been linked with ExPEC strains in epidemiological studies (39), and can enhance host inflammatory responses and ExPEC virulence in mouse models of UTI (40) and prostatitis (41). However, the effects of CNF1 on ExPEC fitness within the host remain unclear, clouded somewhat by conflicting reports (40-42). To test if CNF1 plays a role in the gut, mice were gavaged with 10^9 CFU of either WT F11 or F11Δcnf1 and intestinal colonization levels were tracked by homogenizing and plating feces at various time points. Median bacterial titers for both the WT and mutant strains ranged between 10^5 and 10^6 CFU per gram of feces over the course of two weeks, with median levels of F11Δcnf1 being markedly higher at several time points (Fig. 2A). These results suggest that the absence of cnf1 can actually benefit the survival of F11 within the mouse gut.

Like HlyA and CNF1, Uropathogenic specific protein (Usp) is often encoded by ExPEC
isolates (43, 44). Usp is also associated with *E. coli* fecal isolates that are capable of long-term persistence within the human infant intestinal tract (45). Interestingly, Usp has genotoxic nuclease activity as well as homology to colicins, a group of toxins that can be used by bacteria to harm competing microbes (46-48). Given that interbacterial competition within the gut is commonplace, we tested whether Usp is important for ExPEC gut colonization. The F11Δusp mutant did not exhibit any notable defects within the gut, indicating that Usp is not required in this niche (Fig. 2B).

Another toxin that has been linked to ExPEC pathogenesis is colibactin, which is produced by a number of factors encoded by the polyketide synthase (*pks*) genomic island. The *pks* island is not typically carried by intestinal pathogenic *E. coli* strains, but is enriched in extraintestinal isolates relative to commensal fecal isolates (49). Colibactin induces DNA damage and cell cycle arrest in host cells (49, 50), and the presence of colibactin-producing bacteria has been linked with the development of colorectal cancer (51). In extraintestinal infections, colibactin-deficient bacterial strains are not as virulent as their WT counterparts. Mutation of the *pks* island reduces ExPEC translocation from the gut in neonatal rats (52) and reduces lymphopenia in septic mice (53). Whether the *pks* island also plays a role in bacterial fitness during gut colonization is not clear. Whereas one study observed decreased colonization of the small intestines of neonatal rats by colibactin mutants (52), another found no colonization differences between a *pks* mutant and WT bacteria (54). To test whether colibactin production is important in our adult mouse model of gut colonization, we orally inoculated mice with either WT F11 or F11ΔclbCDEFG, which lacks a key operon within the *pks* island (55). There was no difference in the colonization ability of the WT and mutant strains (Fig. 2C), suggesting that colibactin biosynthesis does not contribute to bacterial fitness in this model of gut colonization.
Flagella are not required for ExPEC gut colonization. Flagella are filamentous organelles comprised of polymers of the flagellin protein FliC (56). Though best known for their role in motility, flagella can also promote bacterial attachment and biofilm formation, and can potently stimulate host inflammatory responses (57, 58). Several studies have provided evidence that flagella enhance ExPEC colonization of the mouse urinary tract (59-62). In contrast, flagella are not critical for gut colonization by nonpathogenic *E. coli* in streptomycin-treated mice (63). To determine if flagella are required for gut colonization by ExPEC in the face of an intact microbiota, we inoculated adult Balb/c mice with WT F11 or an isogenic mutant lacking *fliC*. In these assays, no statistical differences between the WT strain and F11 Δ*fliC* were observed, though median titers for the mutant were lower at many of the time points assayed (Fig. 2D). These data suggest that deletion of *fliC* does not have a large influence on ExPEC fitness in the gut.

The pUTI89 plasmid is not important for gut colonization. Many ExPEC isolates, including F11, carry plasmids that are identical or closely related to the pUTI89 plasmid that was first identified in the cystitis isolate UTI89 (64, 65). This plasmid, which is roughly 114 kb in length, encodes conjugation machinery, numerous hypothetical genes, and several putative virulence factors such as the SenB enterotoxin and the *cjrABC* operon, which may encode for an iron uptake system (64). Loss of pUTI89 impairs the fitness and intracellular growth of UTI89 during the early stages of a UTI in adult mice (66). Likewise, deletion of a closely related plasmid from the neonatal meningitis *E. coli* isolate RS218 attenuates bacterial virulence during systemic infections in rat pups (65). In addition, a number of pUTI89-associated genes have been linked...
with ExPEC mucus and glucose metabolism in vitro (31). To determine if pUTI89 facilitates gut colonization by F11, an F11 derivative that was cured of the plasmid was tested in our mouse model. Though median titers of the cured strain were lower than WT F11 at early time points, no statistically significant differences between the two strains were discerned (Fig. 2E). These data suggest that pUTI89 is, for the most part, dispensable for ExPEC fitness within the gut.

Disruption of fimH reduces gut colonization fitness, whereas the lack of papG has no effect. ExPEC strains encode many types of hair-like adhesive organelles known as pili, or fimbriae. Two of the most often-studied ExPEC-associated adhesins are P and type 1 pili (T1P) (67). P pili terminate with the PapG adhesin, which binds host globoseries glycosphingolipids and can facilitate bacterial infection of the kidneys. T1P are capped by the mannose-binding adhesin FimH, which promotes biofilm formation as well as bacterial attachment to and invasion of bladder epithelial cells. In our non-competitive gut colonization assays in which the WT and mutant bacterial strains are kept separate, the deletion of either papG or fimH did not impair the ability of F11 to colonize the intestinal tract (Fig. 3A and B). Interestingly, like the Δcnf1 mutant, F11ΔpapG fared somewhat better than the WT strain at days 3, 10, and 14 post-inoculation. Notably, in these assays the ΔfimH mutant was cleared in 3 out of 10 mice – more than observed with any of the other tested mutants (Fig. 3B). These results prompted us to examine the fimH mutant further using competitive assays in which the WT and mutant strains are inoculated as a 1:1 mixture into mice via oral gavage. In these and other competitive assays, total ExPEC levels remain fairly steady over time at around 10^6 CFU/g feces, even when ratios of the individual competing ExPEC strains are variable.

As a control, we first competed Kan^R- and Clm^R-tagged F11 strains against one another
to assess if the resistance cassettes alone compromised bacterial fitness within the intestinal tract. In these control assays, F11-KanR exhibited a modest defect, amounting to about a 3-fold decrease relative to F11-ClmR on day 14, with a median competitive index (CI; see Methods) of 0.49 (Fig. 3C, uncorrected $P = 0.044$). Likewise, no striking differences between F11-ClmR and F11ΔpapG (KanR) were observed in competitive assays, with the exception of a transient 6.8-fold decrease (median CI of -0.83) in prevalence of the papG mutant on day 5 (Fig. 3D, uncorrected $P = 0.038$). In contrast, when F11ΔfimH (KanR) was competed against F11-ClmR, the ΔfimH mutant became progressively worse than the control strain starting at day 7 post-inoculation (Fig. 3E). By day 10, there was about a 360-fold reduction in the relative levels of F11ΔfimH recovered in the feces, reflecting a median CI of -2.55 (uncorrected $P = 0.039$). In addition, the ΔfimH mutant began to be cleared as early as day 3, and was not detected in the feces of nearly half of the mice by day 10 (green, Fig. 3E). In comparison, F11ΔpapG dropped below levels of detection in only one mouse during the 14-d time course of these competitive assays (red, Fig. 3D). The phenotype observed with the ΔfimH mutant suggests that disruption of this gene can markedly impair ExPEC persistence within the gut in competitive assays.

**T1P expression by F11 is modest following excretion from the gut.** T1P are phase variable, being turned ON or OFF through recombinase-mediated flipping of an invertible promoter element within the fim gene cluster (67). The orientation of this fim switch can be monitored and quantified by PCR as a means to assess levels of T1P expression (68, 69). Within the feces of mice that are colonized by F11, we found that the fim switch is in the ON position in about 25 to 35% of the excreted bacteria recovered on days 5, 9, and 14 post-inoculation (Fig. 3F). This is on par with results from shaking broth cultures, in which the fim switch is skewed towards the OFF
These data indicate that T1P expression by feces-associated ExPEC is limited, which may enable the pathogen to better disseminate either within the intestinal tract or after being discharged from the host.

**Increased flagellin expression partially explains the colonization defect observed with F11ΔfimH.** T1P, and FimH in particular, may enhance ExPEC persistence within the gut via multiple mechanisms, such as aiding the formation of protective biofilm-like communities or facilitating bacterial attachment to intestinal tissues (58, 70, 71). However, it is conceivable that disruption of fimH could also reduce ExPEC fitness within the gut via effects on other bacterial or host processes. Specifically, previous work showed that deletion of the entire fim operon in the ExPEC isolate UTI89 causes the upregulation of FliC with a coordinate increase in swimming motility (72). This could be problematic for fim mutants since it is known that aberrant overexpression of FliC can impair ExPEC colonization of the gut (6), possibly as a consequence of FliC-mediated stimulation of host inflammatory responses (57). Furthermore, within the intestinal tracts of germ-free or streptomycin-treated mice, mutations that reduce bacterial motility and FliC expression are selected and can promote the persistence of the K12 strain MG1655 (73-75). Together, these observations led us to question if defects in the ability of F11ΔfimH to survive within the gut in competitive assays are associated with altered FliC expression.

In assessing this possibility, we first noted that deletion of fimH does increase the motility of F11 in swim agar plates (Fig. 4A). Complementation of the ΔfimH mutant with a plasmid that expresses FimH in trans restored motility to WT levels (Fig. S2). The increased motility of F11ΔfimH correlates with augmented FliC expression (Fig. 4B), as measured by use of the low-
copy reporter construct pfliC-lux in which the luxCDABE gene cluster encoding bacterial luciferase is transcriptionally fused with the conserved fliC promoter (62). These results mirror those reported for a UTI89 mutant lacking the entire fim operon (72). Interestingly, in our assays the deletion of papG in F11 had nearly the opposite effect of the fimH deletion, greatly reducing the motility of F11 and ablating FliC expression (Fig. 4A-B).

To test if FliC contributes to the defects in gut colonization observed with F11ΔfimH, we generated a double knockout (DKO) mutant lacking both fimH and fliC. This mutant (F11ΔfimHΔfliC) is non-motile in swim plates, similar to the single ΔfliC mutant strain (Fig. 4A, right panel). In competitive gut colonization assays with WT F11, the ΔfimHΔfliC mutant exhibited less pronounced deficiencies than F11ΔfimH (Fig. 4C; compare with Fig. 3E). The greatest defect was observed at day 14 post-inoculation, at which point F11ΔfimHΔfliC was not detected in the feces of 5 of the 13 mice. Relative to the WT strain, F11ΔfimHΔfliC titers were reduced by about 21-fold on day 14, corresponding with a median CI of -1.33 (uncorrected P = 0.711). This defect was substantially smaller than the maximal ~360-fold reduction seen with the single ΔfimH mutant in competition with WT F11 on day 10 post-inoculation (see Fig. 3E, uncorrected P = 0.046). In addition, clearance of F11ΔfimHΔfliC was not observed until day 7 post-inoculation (Fig. 4C), whereas loss of F11ΔfimH was evident starting at day 3 (Fig. 3E). In light of the delayed defects seen with F11ΔfimHΔfliC, we extended the assays to day 21. At this point, the median CI value returned close to 0, though F11ΔfimHΔfliC was still undetectable in feces from nearly half of the mice while the WT strain was present in all but one sample (Fig. 4C). Differences between F11ΔfimHΔfliC and the WT strain at later time points were not statistically significant. The less conspicuous defects seen with F11ΔfimHΔfliC in these assays suggest that aberrant FliC expression does contribute to the compromised persistence of
F11ΔfimH within the gut.

F11ΔfimH outcompetes F11ΔfimHΔfliC within the gut. To better understand the effects that the loss of fimH and fliC have on bacterial fitness during gut colonization, the ΔfimHΔfliC and ΔfimH mutants were directly competed. We hypothesized that the ΔfimH strain, with heightened FliC expression, would be outcompeted by the ΔfimHΔfliC DKO mutant. However, F11ΔfimHΔfliC exhibited clear and statistically significant defects in comparison with F11ΔfimH (Fig 4D). Fecal titers of the DKO mutant were below levels of detection in one out of 15 mice on day 3 post-inoculation, and by day 14 the DKO mutant was undetectable in the feces of a third of the animals. At this point, F11ΔfimH was about 270-fold more abundant than the DKO mutant, corresponding with a median CI of 2.43 (corrected P = 0.029). These data, as well results from non-competitive assays (see Fig. 3B), indicate that the importance of FimH to ExPEC survival within the intestinal tract is dependent upon the nature of the competing microbes that are present.

WT F11 and F11ΔfimH can outcompete one another in reciprocal serial colonization assays. To further evaluate FimH requirements within the intestinal tract, we carried out serial colonization assays in which Balb/c mice were first orally gavaged with WT F11 before the introduction of F11ΔfimH 7 d later. Though fecal titers of the ΔfimH mutant were initially high and on par with the WT strain, levels of the mutant dropped precipitously and were below the limits of detection in most mice by day 3 post-inoculation (Fig. 5A). In reciprocal experiments, in which F11ΔfimH was allowed to colonize the mice prior to instillation of the WT strain, F11ΔfimH persisted while WT F11 was cleared from most of the mice by day 10 (Fig. 5B).
These data show that FimH is not strictly required for ExPEC to prevent colonization of the gut by a new competing strain, though the adhesin does seem to aid this process. In addition, these results indicate that WT F11 and F11ΔfimH likely vie for the same intestinal niches, with the first strain established having the upper hand irrespective of FimH expression.

**DISCUSSION**

The concept of coincidental evolution, coupled with phylogenetic analyses, suggests that the extraintestinal success of ExPEC strains is a by-product of their ability to colonize the gut (27). A corollary of this hypothesis is that extraintestinal virulence and fitness factors promote ExPEC colonization of the gut. In support of this possibility, researchers previously showed that deletion of the seven major pathogenicity islands (PAIs) of the ExPEC isolate 536 not only reduced the virulence of this pathogen in a murine sepsis model, but also attenuated pathogen persistence within the gut (6). Here, we set out to determine if the principle of coincidental evolution could be applied to individual virulence and fitness determinants encoded by the reference ExPEC strain F11.

In non-competitive assays using adult SPF mice, we observed no major defects in intestinal colonization or the persistence of ExPEC mutants lacking Usp, colibactin, flagellin, or the plasmid pUTI89 (see Fig. 2). F11ΔhlyA exhibited early colonization defects, but these did not have a statistically significant effect on longer-term survival of the ExPEC strain within the gut (Fig. 1E). In contrast, F11Δcnf1 tended to colonize the gut at somewhat higher levels than WT F11 (Fig. 2A). These results indicate that cnf1 may negatively impact ExPEC survival in some situations, perhaps by eliciting localized inflammation within the intestinal tract. Like F11Δcnf1, a mutant lacking the adhesin papG also colonized the murine intestinal tract at moderately higher
levels than the WT strain (Fig. 3A), though in competitive assays F11ΔpapG had a transient colonization defect (Fig. 3D). In total, these results indicate that at least some ExPEC-linked genes can influence bacterial fitness within the gut, though the effects may be modest.

Discerning more unequivocal phenotypes for individual ExPEC-associated loci within the gut can be complicated and context-dependent, as exemplified by the analysis of ΔfimH mutants. In non-competitive assays, we observed no significant differences between WT F11 and F11ΔfimH, although the mutant was cleared from a few mice over the course of the 14-d experiments (Fig. 3B). This prompted us to test F11ΔfimH further using competitive assays with the WT strain. In these assays, differences between the ΔfimH mutant and WT strains were more distinct, but not significantly so if the data are corrected for multiple comparisons (Fig. 3E). Still, the fact that F11ΔfimH titers within the feces from nearly half the mice fell below detectable levels by day 10 post-inoculation suggests that FimH can contribute to the persistence of ExPEC within the gut. These findings are in line with recently published data showing that intestinal persistence of the ExPEC isolate UTI89 within streptomycin-treated mice is enhanced by FimH expression (76). This study, as well as new results with the ExPEC isolate CP9 and other work with enteric *E. coli* pathogens (71, 77-79), indicate that FimH can promote bacterial interactions with intestinal epithelial cells. In our mouse models, we observed that F11 is localized primarily within the lumen of the colon, though substantial numbers of the pathogen are also associated with the colonic tissue (see Fig. S1B). In addition to facilitating ExPEC interactions with the intestinal epithelium (76, 79), the FimH adhesin may also influence pathogen persistence within the gut via effects on other processes, including biofilm development and the modulation of innate host defenses (58, 70, 80, 81).

Defining the contributions of FimH to ExPEC fitness within the gut is further
complicated by the observation that the deletion of fimH enhances FliC expression by F11 and increases motility (Fig. 4). Analysis of a fliC and fimH DKO mutant in competition with WT F11 indicated that aberrant FliC expression is at least partially responsible for the colonization defects observed with the ΔfimH mutant (Figs. 3E and 4C). This situation mirrors results reported for the ExPEC strain 536, in which reduced intestinal persistence of the mutant lacking seven PAIs was attributed to FliC overexpression (see above, (6)). The expression of flagella is generally thought to have mostly detrimental effects on E. coli fitness within the gut, possibly due to an increased burden on bacterial metabolism and the ability of FliC to stimulate host inflammatory pathways (57, 73-75). However, in competitive assays F11ΔfimH outperformed F11ΔfimHΔfliC, even though the single mutant is hypermotile (Fig. 4D). One potential explanation for this finding is that flagella expression might at times be an advantage for E. coli within one or more intestinal niches, as previously suggested (75). This possibility is difficult to reconcile with the observation that F11ΔfliC has no substantial defects in our noncompetitive assays (Fig. 2D). It is plausible that crosstalk among bacterial regulators of motility, T1P expression, and other adhesins also contribute to the phenotypes observed in our assays with the fimH mutants and other F11 derivatives (82-86), but this will require additional studies to tease apart.

The ability of distinct types of bacteria to utilize different spatial and nutritional niches within the gut allows for the coexistence of the diverse organisms that comprise the intestinal microbiota, and helps provide a barrier against colonization by newly arriving microbes (87). This latter effect, known as colonization resistance, is one reason that it is generally necessary to treat mice with an antibiotic like streptomycin to open up niches for incoming bacteria that are delivered into the gastrointestinal tract by oral gavage (33). A striking feature of our
experimental system is that F11 is able to colonize and persist indefinitely within the intestinal
tract of conventional SPF mice without the need to first administer antibiotics (Fig. 1A). Our
group and others have recently reported similar results with distinct ExPEC isolates in different
mouse strain backgrounds (25, 30, 32, 88). ExPEC may be able to effectively colonize our
untreated mice because they have very low numbers of endogenous E. coli and other
Enterobacteriaceae based on 16S rRNA gene sequencing and selective plating assays (CW
Russell and MA Mulvey, unpublished data). Nevertheless, these animals are still resistant to
colonization by the K12 strain MG1655, indicating that MG1655 lacks one or more genes that
ExPEC employs to persist within the gut. When in competition with F11, MG1655 is cleared
from the gut at a notably faster rate than in noncompetitive assays (see Fig. 1), suggesting that
these two strains compete for the same intestinal niches.

A better understanding of the survival mechanisms used by ExPEC within the intestinal
tract may aid the development of more efficacious probiotics, while also elucidating new
therapeutic strategies to combat ExPEC before it is able to diseminate and cause disease at sites
beyond the intestinal tract. The effectiveness of such approaches may be dependent on multiple
variables, including timing, the makeup of the microbiota, and the presence of specific
competing strains that can alter ExPEC requirements for individual fitness determinants. For
example, in contrast to the situation in competitive assays, F11ΔfimH has no trouble colonizing
the gut in noncompetitive experiments and, once established, this mutant can even persist when
challenged with the WT strain (see Figs. 3B and 5B). Thus, while FimH can facilitate ExPEC
persistence within the gut in some settings, it is not always an absolute requirement. This
conclusion may help reconcile results from older studies in which the expression of T1P was
found to be unnecessary for E. coli persistence within the intestinal tracts of rodents and human
infants (89-91).

Context-dependent variability in the phenotypic effects of fitness determinants like FimH may complicate treatment approaches, as well as our ability to discern how life within the gut affects the evolution of ExPEC virulence traits. Data presented here indicate that the principle of coincidental evolution does not apply to all ExPEC-associated genes individually, though there are caveats to this conclusion. Our analysis of F11ΔfimH and F11ΔhlyA shows that the phenotypic effects of some genes within the gut can be modest and variable, dependent on the experimental system that is used and the time points that are assayed. In future work, varying other experimental parameters (such as inoculum dose, the genetic backgrounds of the host and ExPEC strain, and competition with other types of bacteria) could reveal additional bacterial genes and gene sets that can promote ExPEC fitness within the gut. In the present study, the lack of easily discernable phenotypes for multiple ExPEC-associated virulence genes within the gut suggests that the evolution of these loci is driven in large part by selective forces encountered outside of the intestinal tract. For example, extraintestinal virulence has been correlated with the ability of ExPEC strains to resist killing by amoebae (92), while T1P expression has been linked with the transmission of ExPEC between individuals by promoting transient colonization of the oropharynx (89). In total, the results presented here show that piecing together the evolutionary history of ExPEC virulence and fitness traits is a complicated task. However, continuing efforts to resolve this problem will provide a more detailed picture of ExPEC ecology and may help identify niche-specific fitness determinants that could be attractive targets for therapeutic intervention.
MATERIALS AND METHODS

Bacterial strains. The cystitis isolate F11 and the K-12 strain MG1655 were genetically modified using lambda Red recombination that was facilitated by the pKM208 plasmid (93). Most of the constructs used for recombination were created by PCR using either pKD4 or pKD3 as a template to amplify a kanamycin or chloramphenicol resistance cassette, respectively, flanked by ~40 base pairs of DNA with homology to the target insertion site. In some cases, longer homology regions were required, and three-part PCR was performed. This was done by PCR amplification of an antibiotic resistance cassette and regions that are upstream and downstream of the target gene. Primers used were designed to contain sections of homology to allow the three PCR products to be stitched together in a single ligation reaction.

The pCP20 plasmid was used to remove the resistance cassette as necessary (94). To cure F11 of the pUTI89 plasmid, the ccdAB toxin-antitoxin system was replaced with a tetA-sacB construct and spontaneous loss of the plasmid was selected for on LB agar plates containing fusaric acid and sucrose, as explained previously (95). The strains used in this study are listed in Table 1 along with the primers used to create them. Prior to lambda Red recombination, bacteria were grown shaking in LB broth at 37°C. All growth in petri dishes was done using LB agar supplemented with chloramphenicol (20 µg/ml), kanamycin (50 µg/ml), or ampicillin (50 µg/ml), as appropriate.

Mouse gut colonization. Mice were handled in accordance with protocols approved by the Institutional Animal Care and Use Committee at the University of Utah (Protocol number 15-12015), following US federal guidelines indicated by the Office of Laboratory Animal Welfare (OLAW) and described in the Guide for the Care and Use of Laboratory Animals, 8th Edition. Prior to inoculation into mice, bacteria were grown statically from frozen stocks for 24 h.
at 37°C in 250 ml flasks containing 20 ml of modified M9 media [MgSO₄•7H₂O (1 mM), CaCl₂•2H₂O (0.1 mM), D-(-)-glucose (0.1%), nicotinic acid (0.00125%), thiamine HCl (0.00165%), casamino acids (0.2%), Na₂HPO₄ (6g/L), KH₂PO₄ (3 g/L), NH₄Cl (1 g/L), and NaCl (0.5 g/L) in water]. A total of 12 ml of culture (6 ml of each culture for competitive experiments) was centrifuged at 8,000 X g for 10 min. Bacterial pellets were then washed once and resuspended in 0.5 ml of PBS. To inoculate the mouse gastrointestinal tract, 7-8 week old female, specific pathogen free (SPF) Balb/c or C57Bl/6 mice (The Jackson Laboratory) were orally gavaged with 50 µl PBS containing ~10⁹ CFU of bacteria. At various time points post-inoculation, individual mice were briefly (3 to 15 min) placed into unused takeout boxes for weighing and feces collection. Freshly deposited feces were collected from the boxes and immediately added to 1 ml of 0.7% NaCl, weighed, and set on ice. The samples were homogenized shortly thereafter to break up the fecal pellets, and then briefly centrifuged to pellet any insoluble debris. Supernatants were serially diluted and spread onto LB agar plates containing either chloramphenicol (20 µg/ml) or kanamycin (50 µg/ml) to select for growth of the relevant bacterial strains. Prior to gavage, fecal samples were analyzed to ensure that there were no endogenous bacteria present that were resistant to chloramphenicol or kanamycin. Mice were housed 3-5 per cage, and were allowed to eat (irradiated Teklad Global Soy Protein-Free Extruded chow) and drink antibiotic-free water ad libitum. Competitive indices (CIs) were calculated as the ratio of mutant over WT bacteria recovered in the feces divided by the ratio of mutant over WT bacteria in the inoculum, as noted previously (31).

To determine F11 (specifically F11-Clm⁵) titers within the various regions of the gastrointestinal tract at 14 d post-inoculation, mice were anaesthetized via isofluorane, euthanized by cervical dislocation, and the cecum, colon, small intestine, and stomach were
removed. The small intestine was divided into thirds, with the portion closest to the stomach labeled “proximal” and the portion closest to the cecum labeled “distal”. A part of each organ was weighed and placed into a Safe-Lock tube (Eppendorf) with three 3.2 mm stainless steel beads, and homogenized using the Bullet Blender (Next Advance). The homogenates were then serially diluted and plated onto LB agar plates containing chloramphenicol (20 µg/mL) to quantify the levels of F11-clmR present.

For histology, colon tissues were fixed in 10% neutral buffered formalin and submitted to the University of Utah Research Histology core for processing and staining with hematoxylin and eosin (H&E). Uninfected intestinal tissues and tissues recovered from mice at 14 d post-inoculation with either MG1655 or F11ΔhlyA were used for comparison. Random colon tissue sections from 5 or more mice in each experimental group were analyzed in a blinded fashion by a trained pathologist (M.P. Bronner).

**Motility Assays.** To test the swimming ability of particular strains, motility agar plates were made by pouring 25 mL of LB soft agar (0.1% agar) into a petri dish. Bacteria (1.5 µl from an overnight culture) were dispensed just below the surface of the plate, which was then incubated at 37°C for 5-10 h prior to imaging with a Stratagene Eagle Eye II System.

**Luciferase assays.** The pflC-lux construct was kindly provided by the Mobley lab (62). Overnight shaking cultures of F11, F11ΔpapG, and F11ΔfimH carrying pflC-lux were diluted 1:100 into 1 ml of fresh tryptone broth (Fisher Scientific) containing ampicillin (50 µg/ml). Three 100-ul-aliquots of each culture were then transferred to a white 96-well polystyrene plate (Dynex Technologies) and grown statically at 37°C. Luminescent emission spectra were collected every 30 minutes for 4.5 hours using Gen5 Software with a BioTek Synergy H1 plate reader. The instrument was set to integrate readings over 10 seconds using a gain of 135 and...
height of 1 mm. Before each reading, the plates were shaken for 1 second. Corresponding growth
curves, which were obtained by taking OD600 measurements of cultures grown statically in clear
96-well polystyrene plates (CytoOne), showed that the strains used in these assays grew
similarly.

**Analysis of the fim switch.** Quantification of the fim switch in the ON and OFF positions
was DNA was carried out essentially as previously described (68, 69). DNA was purified from
feces using the ZR Fecal DNA MiniPrep kit, whereas DNA was purified from broth cultures
using the Promega Wizard Genomic DNA Purification kit. To skew the fim switch towards the
ON position, F11 was grown statically at 37°C in 20 mL LB broth in 250 mL Erlenmeyer flasks
for 24 h, sub-cultured 1:1000 into fresh LB broth, and then incubated for another 24 h. To drive
the fim switch towards the OFF position, bacteria were grown shaking in LB broth to exponential
phase. The primers F11_fimS_F (TACCGCCAGTAATGCTGCTC) and
F11_fimS_R (GTCCCACCATTACCGTCGT) were used to amplify the fim switch region by
PCR using the following thermocycler conditions: 95°C for 5 minutes, 10 cycles (95°C for 30
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,
72°C for 40 sec), and ending with a 5 min incubation at 72°C. The reaction products were
column purified, digested with HinfI for 1 h at 37°C, resolved in 1% agarose gels, and imaged.
Bands representing the fim switch in the ON and OFF positions were quantified using ImageJ.

**Statistical Analysis.** All statistical tests were carried out using GraphPad Prism or
Stata/IC-14 software, and corrections for multiple comparisons were made using the Hochberg
procedure (96). P values ≤ 0.05 were considered significant.
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**Table 1. Bacterial strains and plasmids used in this study**

| Strain      | Description                                                                 | Primers* used to create strain or source reference                                                                 |
|-------------|-----------------------------------------------------------------------------|-------------------------------------------------------------------------------------------------------------------|
| MG1655::clm | MG1655 with a chloramphenicol resistance cassette inserted at the attTn7 site | AGGATGTTTGTATTAAAACATAACAGGAAGA AAAATGCTGTGTAGGCTGGAGCTGCTTCG ATCGGTTACGGTTGAGTAATAAATGGATGCC TGCAGTAAGCATATGAAATATCCTCCTTAG |
| F11         | *E. coli* cystitis isolate (O6:K2:H31)                                       | (97)                                                                                                             |
| F11::clm    | F11 with a chloramphenicol resistance cassette inserted at the attTn7 site  | (32)                                                                                                             |
| F11::kan    | F11 with a kanamycin resistance cassette inserted at the attTn7 site         | TCTGGCGTAGCCTGGAGTTATTGGCCGATGC GATGCTGGTGTAGGCTGGAGCTGCTTCG TCACGTAAMAAAAACGTCTAATCCGTAGACGG ATAAAGAGCATATGAATATCCTCCTTAG |
| F11 ∆clbCDE FG::clm | F11 with the *clbCDEFG* operon replaced with chloramphenicol resistance | TCGGGCGATCGATAGATAGTTGGGCTGGAGTTATTGGCCGATGC GATGCTGGTGTAGGCTGGAGCTGCTTCG TCACGTAAMAAAAACGTCTAATCCGTAGACGG ATAAAGAGCATATGAATATCCTCCTTAG |
| F11 ∆cnf1::kan | F11 with the *cnf1* operon replaced with kanamycin resistance               | GAAAGGCTTGAATATTATTAATGTCGATGCTTCG GAAGGCTTGAATATTATTAATGTCGATGCTTCG GAAGGCTTGAATATTATTAATGTCGATGCTTCG |
| F11 ∆fimH::kan | F11 with the *fimH* operon replaced with kanamycin resistance               | TTATTGATAAAACAAAAGTCACGCIAATAATCG AGGATGTTTGTAGGCTGGAGCTGCTTCG AGGATGTTTGTAGGCTGGAGCTGCTTCG |
| F11 ΔfimH ΔfliC::clm | F11 derivative in which the *fimH* gene has been deleted, and the *fliC* gene has been replaced with a chloramphenicol resistance | See *fimH* and *fliC* single knockout primers                                                                   |
F11 \(\Delta fliC::\text{kan}\) F11 derivative in which the \(fliC\) gene was replaced with a kanamycin resistance cassette

\[
\begin{align*}
\text{ATGGCACAAGTCATTAATACCAACAGCCTCTCGCT} \\
\text{GATCTGTGGCTGGAGCTGCTCTCG} \\
\text{TTAACCTGCAGCAGACAGAACACCTGCTGCGGT} \\
\text{ACCTGGCATATGAAATATCTCCTCCTTAG}
\end{align*}
\]

F11 \(\Delta hlyA::\text{kan}\) F11 derivative in which the \(hlyA\) gene has been replaced with a kanamycin resistance cassette

\[
\begin{align*}
\text{ATGTTTTACTCGTTTAATGATAACATTTATCGTCCT} \\
\text{CATGTGTAGGCTGGAGCTGCTTCG} \\
\text{TTATGGCAATATCATGAGCAGCGTTGCTGAACCAG} \\
\text{ATAGTCATATGAAATATCTCCTCCTTAG}
\end{align*}
\]

F11 \(\Delta papG::\text{kan}\) F11 derivative in which the \(papG\) gene has been replaced with a kanamycin resistance cassette

\[
\begin{align*}
\text{ATGTTTTACTCGTTTAATGATAACATTTATCGTCCT} \\
\text{CATGTGTAGGCTGGAGCTGCTTCG} \\
\text{TTATGGCAATATCATGAGCAGCGTTGCTGAACCAG} \\
\text{ATAGTCATATGAAATATCTCCTCCTTAG}
\end{align*}
\]

F11 \(\Delta usp::\text{kan}\) F11 derivative in which the \(usp\) gene has been replaced with a kanamycin resistance cassette

\[
\begin{align*}
\text{ATGTTTTACTCGTTTAATGATAACATTTATCGTCCT} \\
\text{CATGTGTAGGCTGGAGCTGCTTCG} \\
\text{TTATGGCAATATCATGAGCAGCGTTGCTGAACCAG} \\
\text{ATAGTCATATGAAATATCTCCTCCTTAG}
\end{align*}
\]

F11::\text{kan} \(\Delta p\text{UTI89}\) A derivative of F11::\text{kan} which was cured of the pUTI89 plasmid by replacement of \(ccdAB\) with \(tetA\)-\(sacB\) followed by counterselection

\[
\begin{align*}
\text{CTGTTCGTTTATTACGCG} \\
\text{GATAGAGTGTCAACAAAAATTAGGAATGTCAGGC} \\
\text{TCCGTTATACAC} \\
\text{TCCTAATTTTTTGACACTCTATC} \\
\text{TTAATCAAGGGAAACTGTCATATGC} \\
\text{GCATATGGACAGTTTTTCCCTTGGATTAAAGCA} \\
\text{CACCTCTTTTTGACATACT} \\
\text{GTTGCTATTTTTGGCTTATGTCAG}
\end{align*}
\]

| Plasmid       | Description                                                                 | Source reference |
|---------------|-----------------------------------------------------------------------------|----------------|
| pHJ20         | Encodes \(fimH\) under control of the \(tac\) promoter                     | (99)           |
| pHJ19         | Same as pHJ20, except that \(fimH\) is positioned in the opposite orientation | (100)          |
| p\(fliC\)-lux | Carries the lux\text{CDABE} gene cluster (encoding bacterial luciferase) transcriptionally fused with a conserved \(fliC\) promoter | (62)           |
| pKM208        | Encodes IPTG-inducible lambda Red recombinase                               | (93)           |
| Plasmid | Description | Reference |
|---------|-------------|-----------|
| pKD3    | Template plasmid used to amplify the Clm<sup>R</sup> cassette | (94) |
| pKD4    | Template plasmid used to amplify the Kan<sup>R</sup> cassette | (94) |
| pCP20   | Flippase expression construct | (94) |

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

**Figure 1.** ExPEC colonizes and persists within the gut of SPF mice without causing serious long-term 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 tissues from the intestinal tract at 14 d post-gavage. (C) F11 titers within the feces relative to titers within the large intestines (colon with cecum and associated fecal matter), determined 24 h post-gavage (n=13). (D) 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 < 0.05 by Wilcoxon signed-rank tests with corrections for multiple comparisons. (E) Fecal titers from non-competitive assays in which mice were orally inoculated with F11, F11ΔhlyA, or MG1655. ****, P < 0.0001 by Mann-Whitney U Tests with corrections for multiple comparisons. *, P < 0.05 by Mann-Whitney U Tests, only without corrections. In A-D, bars indicate median values. (F) Relative weights (mean values ± SD) of mice following oral inoculation with F11, F11ΔhlyA, or MG1655. Data were normalized to the mass of each mouse prior to gavage. ***, P ≤ 0.005 with corrections when F11 is compared to either F11ΔhlyA or MG1655 by multiple t tests. In B-F, n ≥ 10 mice from at least two independent experiments.

**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 pUTI89. 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. *, P < 0.05 by
Mann-Whitney U Tests. In (A), the $P$ value at the 14-d time point is not significant when adjusted for multiple comparisons.

Figure 3. The persistence of F11ΔfimH, but not F11ΔpapG, within the gut is impaired in competitive assays. Mice were gavaged with WT F11, (A) F11ΔpapG, or (B) F11ΔfimH and fecal titers were determined at the indicated time points. *, $P < 0.05$, by Mann-Whitney U tests in these non-competitive assays. For competitive assays, mice were gavaged with a 1:1 mixture of (C) F11-KanR and F11-CmR, (D) WT F11 and F11ΔpapG, (E) or WT F11 and F11ΔfimH. *, $P < 0.05$ by one sample $t$ tests. Pie charts in (E) indicate fraction of mice in which F11ΔfimH (green) was not detected. *, $P < 0.05$ by Fisher’s exact tests without corrections. In A-E, $n = 11-13$ mice from two independent assays. For all data in A-E, only the $P$ value for the 3-d time point in (A) is significant when corrections are made for multiple comparisons. (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.

Figure 4. Flagellin expression impacts the efficacy of gut colonization by F11ΔfimH. (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, F11ΔfimH, and F11ΔpapG carrying pFC-lux. Lines indicate mean luminescence values ± SEM from three independent assays performed in triplicate. $P < 0.05$ by multiple $t$ tests. (C-D) Graphs show results from competitive assays in which mice were orally gavaged...
with a 1:1 mixture of (C) WT F11 and F11ΔfimHΔfliC (DKO) bacteria or (D) F11ΔfimH and
F11ΔfimHΔfliC (DKO). Fecal titers of each strain were determined at the indicated time points
by plating on selective media. *, P < 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 F11ΔfimH (green) were not detected. No significant differences were
discerned by Fisher’s exact tests. n = 13-15 mice from at least two independent assays. The P-
values for data at the 3-h time point in (B), the 1-d time point in (C), and days 1, 3, 10, and 14 in
(D) are significant when adjusted for multiple comparisons.

**Figure 5. Pre-colonization of mice with F11ΔfimH effectively limits colonization by the WT
strain, and vice versa.** Balb/c mice were inoculated via oral gavage with (A) WT F11
(specifically F11-ClnR) and then with F11ΔfimH (KanR) 14 d later. (B) Alternatively, mice were
inoculated with F11ΔfimH followed 14 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 F11ΔfimH) was introduced. At the end points, P = 0.0256 (*) or P = 0.0003 (**), as
determined by Mann-Whitney U tests. n = 10 mice from two independent assays.
A) WT before F11ΔfimH

B) F11ΔfimH before WT

Days Post-inoculation with F11ΔfimH

Days Post-inoculation with WT F11

- CFU/g Feces

- WT

- F11ΔfimH