

## Uropathogenic *Escherichia coli* Flagella Aid in Efficient Urinary Tract Colonization

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**In the murine model of urinary tract infections (UTI), cystitis by uropathogenic *Escherichia coli* (UPEC) occurs through an intimate relationship with the bladder superficial umbrella cell entailing cycles of adherence, invasion, intracellular bacterial community (IBC) formation, and dispersal (fluxing) from the intracellular environment. IBC dispersal is a key step that results in the spread of bacteria over the epithelial surface to initiate additional rounds of IBC formation. We investigated the role of flagella in mediating adherence and motility during UTI, hypothesizing that the dispersion of the IBC would be incomplete in the absence of motility, thus interrupting the IBC pathway and attenuating the infection. Using *gfp* reporter fusions, the expression of the flagellar class I *flhDC* and class III *fliC* genes was monitored to track key points of regulation throughout the pathogenic cascade. In vitro, growth under conditions promoting motility resulted in the robust expression of both fusions. In contrast, only the class I fusion produced significant expression throughout early stages of IBC development including the dispersion stage. Thus, unlike in vitro modeling of motility, the regulatory cascade appeared incomplete in vivo. Throughout IBC formation, nonmotile  $\Delta$ *fliC* mutants achieved the same number of IBCs as the wild-type (*wt*) strain, demonstrating that flagella are neither essential nor required for first- or second-generation IBC formation. However, in competition experiments between *wt* and  $\Delta$ *fliC* strains, the *wt* was shown to have a fitness advantage in persisting throughout the urinary tract for 2 weeks, demonstrating a subtle but measurable role for flagella in virulence.**

Urinary tract infections (UTI) rank third among the list of most common bacterial infections, with approximately 4.5 million UTI diagnosed annually (10, 55). Uropathogenic *Escherichia coli* (UPEC) is the predominant causative microorganism, accounting for 80% of all uncomplicated infections (10, 55), which are thought to occur in an ascending manner. Pathogens colonize the periurethral area, are introduced into the urinary tract, ascend the urethra into the bladder to cause cystitis, and ascend the ureters to reach the kidneys, producing pyelonephritis. The mechanism of ascension is currently unknown; however, motility may play a role, and flagella are reasonable candidates to participate in the ascending process.

Flagella may also have an important role in the complex life cycle of UPEC in the bladder. In a well-established UTI mouse model (34) in which bacteria are directly inoculated into the bladder, bypassing the urethra, UPEC invades superficial umbrella cells in a type 1 pilus-mediated process (27, 33, 36, 42). Due to the limitations of this model, any role that flagella may play in ascending from the perianal region to the periurethrum would not be observed. Invasion of *E. coli* into superficial umbrella cells activates a complex series of events leading to the formation of intracellular bacterial communities (IBCs), a protected niche with biofilm-like properties (1, 34). IBCs protect UPEC from innate host surveillance and defenses, antibiotic treatment, and clearance by micturition (34, 35). IBCs undergo a defined maturation and differentiation program cul-

minating in the dispersal of UPEC from infected host cells (fluxing), upon which the bacteria are able to initiate further rounds of binding, invasion, and IBC formation in naïve cells (21). Thus, IBC maturation is a process that is analogous to the growth of extracellular biofilms. Reentry of fluxed bacteria into the IBC developmental cascade is marked by slower kinetics. Ultimately, bacterial replication ceases, and a quiescent reservoir or persistent state is established in the bladder tissue that, in response to unknown signals, can reactivate and trigger a recurrent bacteruria (35).

Recently, the visual details of IBC dispersal were determined using high-resolution time lapse video microscopy (21). Green fluorescent protein (GFP)-producing UPEC bacteria were observed to detach and flux from mature IBCs at ~16 h postinfection and spread throughout the bladder, thus inferring a role for flagellum-mediated motility. Flagellum-mediated motility is conserved among UPEC strains, with 70% of clinical isolates obtained from women with acute, asymptomatic, or recurrent urinary tract infections demonstrating motility when grown in semisolid agar (K. Dodson, personal communication). Furthermore, flagella are known to play a role in *Proteus mirabilis* uropathogenesis (4, 41). Flagellar mutants of *P. mirabilis* are deficient in in vitro catheter-associated biofilm formation and also exhibit attenuated host urinary tract colonization (4, 19, 20). In addition to these roles, flagella function as adhesive and invasive organelles for some enteropathogenic *E. coli* (EPEC) strains (12) and *Salmonella* species, respectively (43, 51). With putative roles in adherence, ascension, biofilm formation, and dispersal, such as that of the IBCs, we hypothesized that flagella contribute to UPEC virulence during urinary tract infections.

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant genotype or features	Resistance(s)	Reference
<b>Strains</b>			
MC4100	<i>Escherichia coli</i> K-12		16
MG1655	<i>Escherichia coli</i> K-12		30
UTI89	<i>Escherichia coli</i> cystitis isolate		35
UTI89	UTI89 <i>att</i> <sub>HK022</sub> :: <i>COM-GFP</i>	Kan <sup>r</sup>	This work
UTI89/pVC	UTI89 <i>att</i> <sub>HK022</sub> :: <i>COM-GFP</i> /pBAD33	Kan <sup>r</sup> Cm <sup>r</sup>	This work
UTI89 $\Delta$ <i>fliC</i>	UTI89 $\Delta$ <i>fliC</i> $\Delta$ <i>kanR</i> <i>att</i> <sub>HK022</sub> :: <i>COM-GFP</i>	Kan <sup>r</sup>	This work
UTI89 $\Delta$ <i>fliC</i> /p <i>fliC</i>	UTI89 $\Delta$ <i>fliC</i> $\Delta$ <i>kanR</i> <i>att</i> <sub>HK022</sub> :: <i>COM-GFP</i> /pBAD33- <i>fliC</i>	Kan <sup>r</sup> Cm <sup>r</sup>	This work
UTI89 $\Delta$ <i>fliC</i> /pVC	UTI89 $\Delta$ <i>fliC</i> $\Delta$ <i>kanR</i> <i>att</i> <sub>hk</sub> :: <i>comGFP</i> /pBAD33	Kan <sup>r</sup> Cm <sup>r</sup>	This work
UTI89 promoterless GFP	UTI89 <i>att</i> <sub><math>\lambda</math></sub> :: <i>PSSH10-1</i>	Spec <sup>c</sup>	This work
UTI89 class I GFP	UTI89 <i>att</i> <sub><math>\lambda</math></sub> :: <i>P</i> <sub>flhDC</sub> -GFP	Spec <sup>c</sup>	This work
UTI89 class III GFP	UTI89 <i>att</i> <sub><math>\lambda</math></sub> :: <i>P</i> <sub>fliC</sub> - <i>FliC</i> <sub>1-15aa</sub> -GFP	Spec <sup>c</sup>	This work
<b>Plasmids</b>			
pCom-GFP	Source of <i>P</i> <sub>tac</sub> - <i>gfp</i>		6
pGREEN-TIR	Source of <i>gfp</i>		31
pAH69	HK022 integrase expression plasmid		15
pAH70	HK022 attachment integration plasmid		15
pAH70-COM-GFP	pAH70 with <i>P</i> <sub>tac</sub> - <i>gfp</i>		This work
pKD4	Antibiotic cassette template for red recombinase-mediated knockout		7
pKD46	Red recombinase expression plasmid		7
pCP20	<i>P</i> <sub>ara</sub> - <i>flp</i> ; <i>ara</i> -inducible Flp recombinase expression plasmid		7
pCD13SK	Lambda attachment integration vector		37
pPSSH10-1	pCD13SK with promoterless <i>gfp</i>		This work
pINT-TS	Lambda integrase expression		15
pVC	pBAD33; control vector	Cm <sup>r</sup>	13
p <i>fliC</i>	pBAD33 with <i>P</i> <sub>ara</sub> - <i>fliC</i> ; <i>Ara</i> -inducible <i>fliC</i> expression	Cm <sup>r</sup>	This work

In a series of experiments using a combination of reporter fusions and flagellar mutants, we show that, in contrast to in vitro expression, flagellar gene expression during the IBC pathway does not proceed to completion through the hierarchical regulatory steps, resulting in termination between class I and class III gene expression. Our experiments demonstrate that IBC formation, development, and dispersal do not require flagella. Although a prominent role for flagella is unclear in the most acute steps of cystitis, we show that flagella contribute to UPEC persistence in the bladder and kidney by use of a competition experiment between wild-type (*wt*) and  $\Delta$ *fliC* strains whereby the *wt* strain significantly outcompeted the flagellar mutant at 2 weeks postinfection. These data are consistent with studies by Lane et al., who have independently and concomitantly found increased fitness of another UPEC strain in comparison with an isogenic *fliC* mutant at 3 days postinfection (24). We conclude that while flagella do not play a central role in IBC development and dispersal, they enhance the fitness of UPEC throughout the urinary tract.

#### MATERIALS AND METHODS

**Bacterial strains and culture conditions.** The strains used in this study are shown in Table 1. UTI89, a cystitis-derived isolate of serotype O18:K1:H7, has been described previously (35). Bacterial strains were grown using standard techniques. For all in vitro and in vivo studies, overnight (typically 16-h), aerated cultures were diluted 1:250 into fresh Luria-Bertani (LB) broth and grown statically at 37°C for 20 to 24 h to induce type 1 piliation. Antibiotics and supplements were used at the following concentrations where appropriate (for K-12 and UTI89 strains, respectively): spectinomycin, 37.5 and 50  $\mu$ g/ml; kanamycin, 25 and 50  $\mu$ g/ml; chloramphenicol, 20  $\mu$ g/ml; and 5 mM L-(+)-arabinose.

**Construction of UTI89 *att*<sub>HK022</sub>::*COM-GFP*.** A GFP-marked isogenic derivative of UTI89 was constructed as follows. Plasmid pCOM-GFP (6) was digested with MluI and SphI to obtain a fragment of approximately 1.1 kb containing *gfp* under the control of the *tac* promoter; this fragment was ligated into compatibly

cut pAH70 (15) to create pAH70-COM-GFP (Table 1). This vector was subsequently electroporated into MG1655/pAH69 (15), expressing the HK022 phage integrase. Chromosomal integrants were selected on LB-kanamycin (25  $\mu$ g/ml) plates. Strains were screened via PCR for single-copy integration using combinations of primers P1 to P4 (Table 2). A single-copy integrant was chosen for P1 lysis and transduction of the HK locus into UTI89, creating strain UTI89 *att*<sub>HK022</sub>::*COM-GFP*.

**Construction of a nonmotile UPEC mutant.** UTI89  $\Delta$ *fliC* was constructed according to the method of Datsenko and Wanner (7) using primers FLiCKO1 and FLiCKO2 (Table 2) and the template pKD4 (7) to first delete the *fliC* gene in MC4100 (16). Gene deletion was confirmed with the *fliC*-flanking primers FliC 1 and FliC 2 (Table 2). UTI89 was transduced with a P1 lysate derived from MC4100  $\Delta$ *fliC*, followed by excision of the kanamycin cassette by introduction of the Flp recombinase-expressing vector pCP20 (7). To facilitate microscopy, the  $\Delta$ *fliC* derivative was marked by GFP expression using transduction with a P1 lysate strain prepared from MG1655 *att*<sub>HK022</sub>::*COM-GFP*. The final strain is referred to as UTI89  $\Delta$ *fliC*.

**Construction of *gfp* reporter strains.** For construction of the *gfp* expression vector pPSSH10-1 (Table 1), the ~750-bp SmaI fragment from pGREEN-TIR (31) containing *gfp* was ligated into SpeI-cut, filled-in, and calf intestinal phosphatase-treated pCD13SK (37) to produce a *pir*-dependent vector carrying the  $\lambda$  *att* site with versatile multicloning sites upstream of *gfp*.

pPSSH10-1 and its derivatives were integrated into MG1655 (30) at the  $\lambda$  *att* site using integrase (Int) expressed from vector pINT-TS (15) and selected on LB-spectinomycin (37.5  $\mu$ g/ml) plates. Clones were tested by PCR for single-copy integration at the lambda phage attachment site using primer pairs AB, AC, BD, and CD (Table 2) (37). UTI89 was transduced with a P1 phage lysate of MG1655 *att* <sub>$\lambda$</sub> ::*PSSH10-1* and selected on LB-spectinomycin (50 or 100  $\mu$ g/ml) plates to create UTI89 *att* <sub>$\lambda$</sub> ::*PSSH10-1*. An additional PCR was performed as described above for confirmation of the new locus posttransduction.

**p*fliC* construction.** p*fliC*, an L-(+)-arabinose-inducible flagellin expression vector, was constructed as follows. *fliC* was amplified from UTI89 genomic DNA by PCR (Platinum *Pfx*; Invitrogen) with primers FliC 3 and FliC 4, restriction endonuclease digested with NcoI and BglII, ligated into compatibly digested pBAD33 (13), and confirmed by restriction endonuclease digestion and DNA sequencing. p*fliC* (Table 1) and pBAD33 were individually transformed into UTI89  $\Delta$ *fliC* by electroporation to create the complemented (UTI89  $\Delta$ *fliC*/p*fliC*) and vector control (UTI89  $\Delta$ *fliC*/pBAD33) strains, respectively.

**Hemagglutination assay for type 1 piliation.** Strains were grown statically at 37°C for 24 h as described above to induce production of type 1 pili. Hemagglutination assays with guinea pig erythrocytes (Colorado Veterinary Products) were performed according to published protocols (18). Results are representative of three independent experiments.

**Western blotting.** For immunoblot analysis of bacterial strains, gels were prepared as follows. Equivalent numbers of cells (~10<sup>8</sup>) from 24-h static cultures were suspended in 1× Laemmli buffer (23), acidified with 1 M hydrochloric acid, boiled for 3 to 5 min, neutralized, and resolved by 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Proteins were transferred to nitrocellulose membranes by semidry electroblotting (Bio-Rad), blocked, probed with either an anti-FimCH (type 1 pilus marker; a kind gift from H. Mobley) or an anti-H7 (flagellar marker; Difco) antiserum followed by alkaline phosphate-conjugated anti-mouse or anti-rabbit whole-molecule immunoglobulin G, and incubated with CDP-Star (Tropix, Bedford, MA). Finally, specific proteins were visualized by exposure to photographic film.

**Motility assay.** Strains were stabbed into 0.25% LB agar supplemented with 2,3,5-triphenyltetrazolium chloride to better visualize motility, grown at 37°C for 20 h, and qualitatively assessed for motility. When necessary, LB agar was supplemented with antibiotics alone or with both antibiotics and L-(+)-arabinose to maintain plasmids or induce expression, respectively. To quantify motility, radii of motility circles were measured for all strains at 8 h postinoculation; then areas of swimming were calculated by the formula  $A = \pi r^2$ , normalized to the area of motility of UTI89, and expressed as a percentage of the motility of UTI89.

**EM and negative staining.** Strains were cultured as described under “Bacterial strains and culture conditions” above and were prepared for electron microscopy (EM) as follows. Bacteria were allowed to be absorbed onto Formvar-carbon-coated copper grids for 1 min. Grids were washed in distilled H<sub>2</sub>O and stained with 1% aqueous uranyl acetate (Ted Pella Inc., Redding, CA) for 1 min. Excess liquid was gently wicked off, and grids were allowed to air dry. Samples were viewed on a JEOL 1200EX transmission electron microscope (JEOL USA, Peabody, MA) at an accelerating voltage of 80 kV.

**Construction of chromosomal promoter-GFP fusion strains.** To permit visualization of the *flhDC* (class I flagellar genes) and *fliC* (class III flagellar gene) promoters in vivo, transcriptional *gfp* fusions were constructed. The promoter regions were identified based on published data (45, 47) and amplified from UTI89 genomic DNA using primer pairs FlhDC1-FlhDC2 and FliC5-FliC6. Amplicons were restriction endonuclease digested and cloned into the ClaI/BamHI or HindIII/BamHI site of pPSSH10-1 (for class I and class III, respectively) using standard cloning techniques. The amplified products were sequenced for confirmation. Integrations of each fusion into MG1655 and P1 transduction into UTI89 were performed as described above. For construction of a class III translational fusion, a two-step PCR was used to fuse the *fliC* promoter and the first 45 nucleotides of *fliC* coding sequence to the entire coding sequence of *gfp* using primers FliC7 to FliC10. The class III translational fusion UTI89 strain was prepared by integration and P1 transduction as above.

**Mouse infections.** Seven- to eight-week-old wild-type C3H/HeN female mice were obtained from Harlan Sprague-Dawley. Twenty-four-hour static cultures of the UTI89, UTI89  $\Delta$ *fliC*, UTI89 class I GFP, or UTI89 class III GFP strain were pelleted by centrifugation at ~3,000 × g for 15 min and resuspended in sterile phosphate-buffered saline (PBS) to a concentration of ~2 × 10<sup>8</sup> CFU/ml. For competition experiments, UTI89 and UTI89  $\Delta$ *fliC* at ~2 × 10<sup>8</sup> CFU/ml were mixed at a ratio of 1:1. Mice were anesthetized by inhalation of isoflurane and infected via transurethral catheterization with 50  $\mu$ l of the bacterial suspension (~1 × 10<sup>7</sup> to 2 × 10<sup>7</sup> CFU). At the indicated times postinfection, mice were sacrificed by cervical dislocation under anesthesia, and the bladders were immediately harvested and processed either for microscopy or for bacterial titer determinations as described in the next two sections.

**Confocal microscopy.** For in vivo transcriptional profiling of class I and class III promoter-*gfp* fusion strains, infected bladders were harvested at the indicated times, bisected, splayed by pinning under sterile PBS, gently washed with PBS, fixed with 3% paraformaldehyde (PFA; EM grade; EMS)-PBS for 45 to 60 min at room temperature protected from light, washed with PBS, and stained with TO-PRO-3 iodide. For in vivo IBC imaging, bladders infected with UTI89 or UTI89  $\Delta$ *fliC* were bisected, splayed by pinning under sterile PBS, gently washed with sterile PBS, stained with Alexa Fluor 633-conjugated wheat germ agglutinin (WGA) (Molecular Probes), washed with PBS, and fixed with 3% PFA (EM grade; EMS)-PBS as described above. Prepared bladders were mounted in a large drop of antifade (Prolong; Molecular Probes) and a coverslip placed on top. Microscopy was performed on a Zeiss (Thornwood, NY) LSM 510 Meta Laser Scanning inverted confocal microscope using a 63× oil immersion objective. One hundred percent of the bladder surface was imaged for each animal,

Primer name

Sequence (5'→3')

P1.....	GGAATCAATGCTGAGTG
P2.....	ACTTAACGGCTGACATGG
P3.....	ACGAGTATCGAGATGGCA
P4.....	GGCATCAACAGCACATTC
FliCKO1.....	AAACCCAAATACGTAATCAACGACCTTGCAATATAGGATAACGAATCGTGTAGGCTGGAGCTGCTTC
FliCKO2.....	GCAATTTGGCGTTGGCCGTCAGTCTCAGTTAATCAGGTTAACAGGACATATGAAATATCTCCTTAG
FliC1.....	GTTAGCTGCCACCCCTCATGCTG
FliC2.....	CAGCATGAAGGGGTGGCAGCTAAC
A.....	CGCCCTGAAATCGTGTATG
B.....	CACTCTCTGGCAAGGCG-CTC
C.....	TCAAATGCTTGAGGCCAGTTTGCTCAGGCT
D.....	GCTCTC-CGGCTTCTCGCTC
FliC3.....	AATGTACCATGGGGTGTGTTTACGACAGACGATTAACAGG
FliC4.....	TTAAGTAGATCTGTTGGCCGTCAGTCTCAGTTAATCAG
FliDC1.....	CGAACTATCGATCGCAATCCCACTCAGTTAAACAGG
FliDC2.....	CCCATAGGATCCGCTTCCGGCAGTTCTTTATAGAG
FliC5.....	ACCCTGGAAGCTGTACTGTGCTGTGGCGATC
FliC6.....	TCGTTGGATCCCCAACCTGAAATTTTCATCGAACCGTTC
FliC7.....	ACCCTGGAAGCTGTACTGTGCTGTGGCGATC
FliC8.....	ATGGCACAAGTCATTAATACCAACAGCCTCTCGCTGATCACTCAATGATGAAGGAGAAGAACTTTTCACTGAGTTGCCAATTCTT
FliC9.....	AAGAAATGGGACCACTCCAGTGAAMAAGTCTCTCTTACTCAATTTGAGTATGATCAGAGAGGCTGTTGGTAAATGAGACTTGTGCCAT
FliC10.....	CCACTATCTAGATTATTTGTATAGTTTCATCCATGCAATGTTAATCCAGAGCTGT

TABLE 2. Primers used in this study

and images were acquired using accompanying Zeiss software. GFP was imaged at 488-nm excitation and 505- to 530-nm emission. TO-PRO-3 iodide (Molecular Probes) was imaged at 633-nm excitation with emission filters collecting between the wavelengths of 650 and 690 nm. Alexa Fluor 633-conjugated WGA was imaged at 633 nm excitation with emission filters collecting between 650 and 800 nm.

**Tissue bacterial titer determinations.** To enumerate the bacteria present, bladders and kidneys were aseptically harvested 2 weeks postinfection, homogenized in PBS containing 0.025% Triton X-100, serially diluted, and plated onto LB agar plates. CFU was enumerated after 24 h of growth at 37°C. For competition experiments, the relative bacterial loads for wild-type and mutant bacteria were determined by replica plating onto LB and LB-kanamycin (50 µg/ml) plates. Total CFU was determined from LB plates, and the number of kanamycin-resistant (nonmotile UTI89  $\Delta fliC$ ) CFU was determined for bacteria that grew on LB-kanamycin (100 µg/ml) plates. Kanamycin-sensitive CFU (wild-type UTI89) represents bacteria that do not grow on LB-kanamycin (100 µg/ml) plates. Competition indices (CI) were calculated similarly to Freter et al. (11) by using UTI89 as the reference strain, as follows:  $CI = (CFU_{UTI89}/CFU_{UTI89 \Delta fliC} \text{ recovered from mice}) / (CFU_{UTI89}/CFU_{UTI89 \Delta fliC} \text{ present in the initial inoculum})$ .

**Statistical analysis.** CFU/bladder or CFU/pair of kidneys was calculated as described in "Tissue bacterial titer determinations" above and analyzed for significance using the nonparametric Mann-Whitney U test (InStat; GraphPad Software) to compare bladder and kidney colonization levels between *wt* UTI89 and UTI89  $\Delta fliC$ .

## RESULTS

**Temporal and spatial expression of class I and class III flagellar genes in vitro and in vivo.** Flagella are produced from more than 40 genes which are organized into hierarchal classes defined by sequence of activation (5, 48). Class I genes, *flhDC*, encode the master regulator of flagellar biosynthesis and are activated first in response to various environmental cues. Class II and class III genes are activated subsequently. To determine if flagella are expressed during IBC formation, strains carrying single-copy chromosomal *gfp* reporter fusions to class I and class III flagellar genes were monitored during acute cystitis. Expression of *flhDC* and *fliC*, class I and class III flagellar genes, respectively, was chosen because *flhDC* has been shown in vitro to be the checkpoint for activation of and commitment to flagellar biosynthesis (48), and *fliC* encodes a highly abundant class III gene product and thus was expected to be detectable in vivo. The reporter fusions were integrated at the  $\lambda$  *att* site for stability, single copy number, and avoidance of the native gene loci where insertions may perturb expression. Prior studies (unpublished data) show that integration at this site does not adversely affect UTI pathogenesis.

The *gfp* reporter fusion strains were initially analyzed in vitro to ensure that *gfp* expression correctly correlated with the motile, flagellum-positive phenotype and that the strains responded appropriately to environmental signals known to influence motility, including glucose and temperature (25). Motility and flagellar gene expression were demonstrated to be repressed as a function of increasing temperature and elevated glucose concentrations. Each fusion-bearing strain exhibited wild-type motility in LB or minimal medium soft agar and, like the *wt* unmodified strain, repressed motility in minimal medium in the presence of 1.5% glucose (data not shown). Fluorometry measurements of the class I promoter fusion strain relative to the promoterless reporter strain yielded a 5.5-fold reduction in GFP fluorescence for growth at 37°C versus 25°C on LB agar plates. However, under all of the conditions assayed, the fluorescence of the class III reporter strain was

never significantly greater than that of the promoterless *gfp* control strain (data not shown). We hypothesized that UTI89 may produce low levels of *fliC* transcript coupled with efficient translation. Thus, a *fliC::gfp* translational reporter strain was constructed by fusing the promoter, ribosome binding site, and codons 1 to 15 of *fliC* to codon 2 of *gfp*. GFP production was strongly evident in this strain during growth favoring motility (0.25% motility agar) but not under repressive conditions (shaken growth, LB) (data not shown). Epifluorescent and light microscopy further confirmed that *fliC::gfp* expression correlated with motility. These in vitro data validated and demonstrated the accuracy and robustness of the UTI89 *flhDC* transcriptional and *fliC* translational *gfp* reporter strains for in vivo studies.

The bladders of mice infected transurethrally with the flagellar gene-specific and promoterless *gfp* fusions were examined at time points through the course of acute infection representing different stages of primary and secondary IBC development: 6 h for early-stage, 16 h for mid- to late-stage, and 30 h for second-generation IBCs. At 6 h postinfection, *flhDC* expression was heterogeneous among different IBCs, ranging from entire IBCs lacking expression (Fig. 1A) to IBCs containing small GFP-positive populations adjacent to those with abundant expression involving most of the bacteria (Fig. 1B). Of early-stage IBCs containing the *flhDC-gfp* strain, roughly 50% were highly active. Three-dimensional reconstruction of Z stack images facilitated the complete examination of each IBC for reporter expression. At 16 h postinfection, *flhDC-gfp* expression within all IBCs observed was uniformly active and robust (Fig. 1D and E). At both 6 (Fig. 2A to C) and 16 (Fig. 2D to F) hours, *fliC-gfp* expression was not observed within IBCs, although the surface bacteria and filaments (fluxed organisms) (Fig. 2D) exhibited background fluorescence. At 30 h postinfection, 76% of IBCs observed (75/99) were completely inactive (Fig. 2G), 20% (20/99) contained a minority of GFP-positive bacteria (Fig. 2H), and 4% (4/99) had a mixed population of randomly distributed GFP-positive bacteria throughout the IBC (Fig. 2I). At both 6 and 16 h, the promoterless reporter showed no appreciable emission (Fig. 1C and F). The lack of overlapping expression profiles by comparison of the class I and class III fusions at these time points implied that the flagellar regulatory cascade is incomplete without a commitment to the expression of the structural components required for functional flagella. As a consequence, these data suggest that flagella may not perform a critical role in these early steps of acute cystitis.

**In vitro characterization of UTI89  $\Delta fliC$ .** If the conclusions from the prior experiments were valid, namely, that flagella are not widely expressed during IBC development, dispersal, or luminal colonization, then nonmotile strains should yield similar burdens of bacteria and numbers of IBCs during the same time frame. We subsequently produced a flagellum-null isogenic derivative of UTI89 to test this hypothesis. The flagellum is a proteinaceous organelle powered by an ATP-dependent motor that propels the bacterium forward during the swim phase of swim-tumble cycles of flagellum-mediated motility (17). The flagellar filament is composed of the major structural subunit, flagellin (or FliC), and its deletion renders the bacterium nonmotile (25). We sought to engineer a completely immotile strain and validate its phenotype in vitro; thus, *fliC*

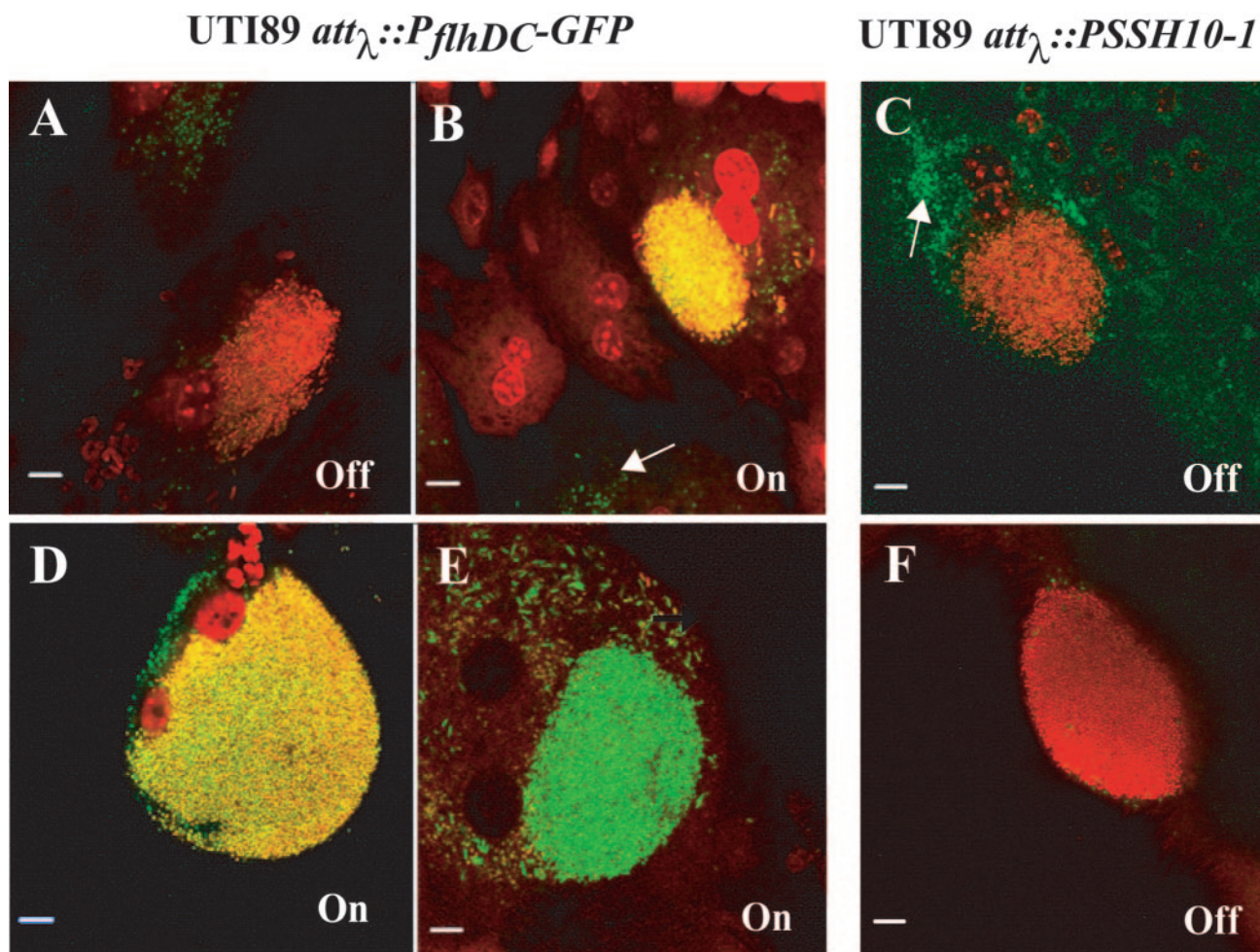


FIG. 1. In vivo *fliC* (class I flagellar gene) transcriptional activity. The bladders of mice infected with  $10^7$  bacteria of either the UTI89 class I GFP strain (UTI89 *attλ::P<sub>fliC</sub>*) (A, B, D, and E) or the UTI89 promoterless GFP reporter strain (UTI89 *attλ::PSSH10-1*) (C and F) were collected 6 (A to C) or 16 (D to F) hours postinfection, splayed by pinning, fixed with PFA, stained with TO-PRO-3 iodide (nuclear stain; red), and imaged by confocal microscopy at  $\times 63$  magnification. Yellow/green staining indicates active transcription. Red staining indicates no transcription. Bar, 10  $\mu$ m. White arrows indicate autofluorescent vacuoles normally present in superficial facet cells. The UTI89 class I GFP reporter strain resulted in both transcriptionally active and inactive IBCs at 6 h and only active IBCs at 16 h, whereas the control, UTI89 promoterless GFP strain exhibited background GFP levels at both time points.

was targeted for complete in-frame deletion to determine the role of flagellum-mediated motility during UPEC pathogenesis.

Immunodetection of flagella in strains grown under motility-inducing conditions using H7 anti-flagellin sera showed that the  $\Delta$ *fliC* strain did not produce flagellin while the *wt* and complemented strains did (Fig. 3C). While the growth of the different strains was unaffected in both single and mixed (1:1) growth curves (data not shown),  $\Delta$ *fliC* was nonmotile compared to the highly motile *wt* parent in 0.25% LB agar (Fig. 3A). Complementation of  $\Delta$ *fliC* with *fliC* expressed in *trans*, but not with the control vector, restored motility to  $\sim 60\%$  of UTI89 levels, thus confirming that loss of motility was due to the absence of *fliC* and not to a polar effect on downstream gene expression (Fig. 3B). Flagella and pili have been suggested to be reciprocally regulated (26). Since type 1 pili are required for adherence and invasion in cystitis, we ensured that the deletion of *fliC* would not adversely affect type

1 production. Anti-FimCH antisera revealed in an immunoblot that adhesive pili were expressed in the  $\Delta$ *fliC* strain. In addition, the cultures were hemagglutination positive (and mannose inhibitable), and EM analysis with negative staining on 24-h static LB broth cultures revealed pili on both *wt* and  $\Delta$ *fliC* strains (Fig. 3D to G). In addition, pili and flagella were expressed simultaneously on individual bacteria. These studies argued strongly that type 1 pilus expression was not affected by the flagellar mutation. These data, like those of the reporter strain experiments, emphasize the marked regulatory differences between K-12 laboratory strains and UPEC isolates.

**UTI89  $\Delta$ *fliC* and IBC maturation during acute cystitis.** To determine whether flagella play a role in the IBC developmental cascade, the ability of UTI89  $\Delta$ *fliC* to form IBCs was analyzed relative to that of UTI89 at 6 (early IBC), 16 (mid- to late IBC), and 30 (predominantly second-generation IBC) hours during the acute phase of murine cystitis. Confocal imaging of the infected, splayed bladders showed that UTI89  $\Delta$ *fliC*

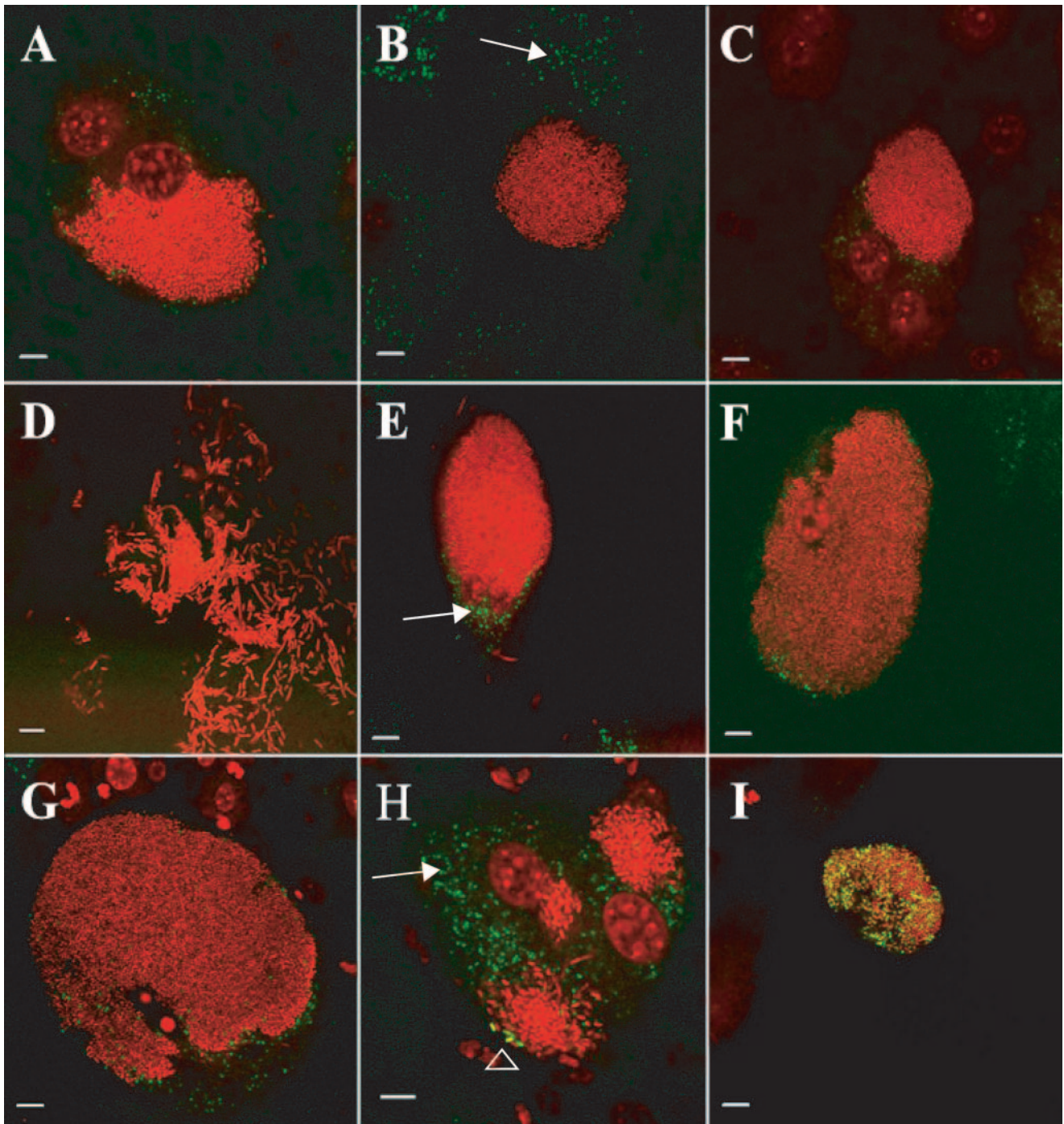


FIG. 2. In vivo *fliC* (class III flagellar gene) transcriptional activity. The bladders of mice infected with  $10^7$  UTI89 class III GFP bacteria (UTI89 *attλ::P<sub>fliC</sub>-FliC<sub>1-15aa</sub>-GFP*; translational fusion) were collected 6 (A to C), 16 (D to F), or 30 (G to I) hours postinfection, splayed by pinning, fixed with PFA, stained with TO-PRO-3 iodide (nuclear stain; red), and imaged by confocal microscopy at  $\times 63$  magnification. Yellow/green staining indicates active transcription. Red staining indicates no transcription. Bar, 10  $\mu$ m. White arrow indicates autofluorescent vacuoles normally present in superficial facet cells. Open arrowhead indicates GFP-positive bacteria. The UTI89 class III GFP reporter strain resulted primarily in transcriptionally inactive IBCs; however, transcriptionally active bacteria were detected in IBCs from bladders harvested 30 h postinfection.

formed IBCs at each time point (Fig. 4D to F) grossly indistinguishable in both morphology and number from the *wt* IBCs (Fig. 4A to C). These data suggest that the flagellar filament does not have an essential role in primary or secondary IBC formation and maturation. These data also demonstrate that

motility is not essential for host colonization during acute infection in this murine model.

**Flagella and persistent cystitis.** Although the flagellum was not required for the acute steps of cystitis, including IBC formation, we hypothesized that it may provide an advantage at

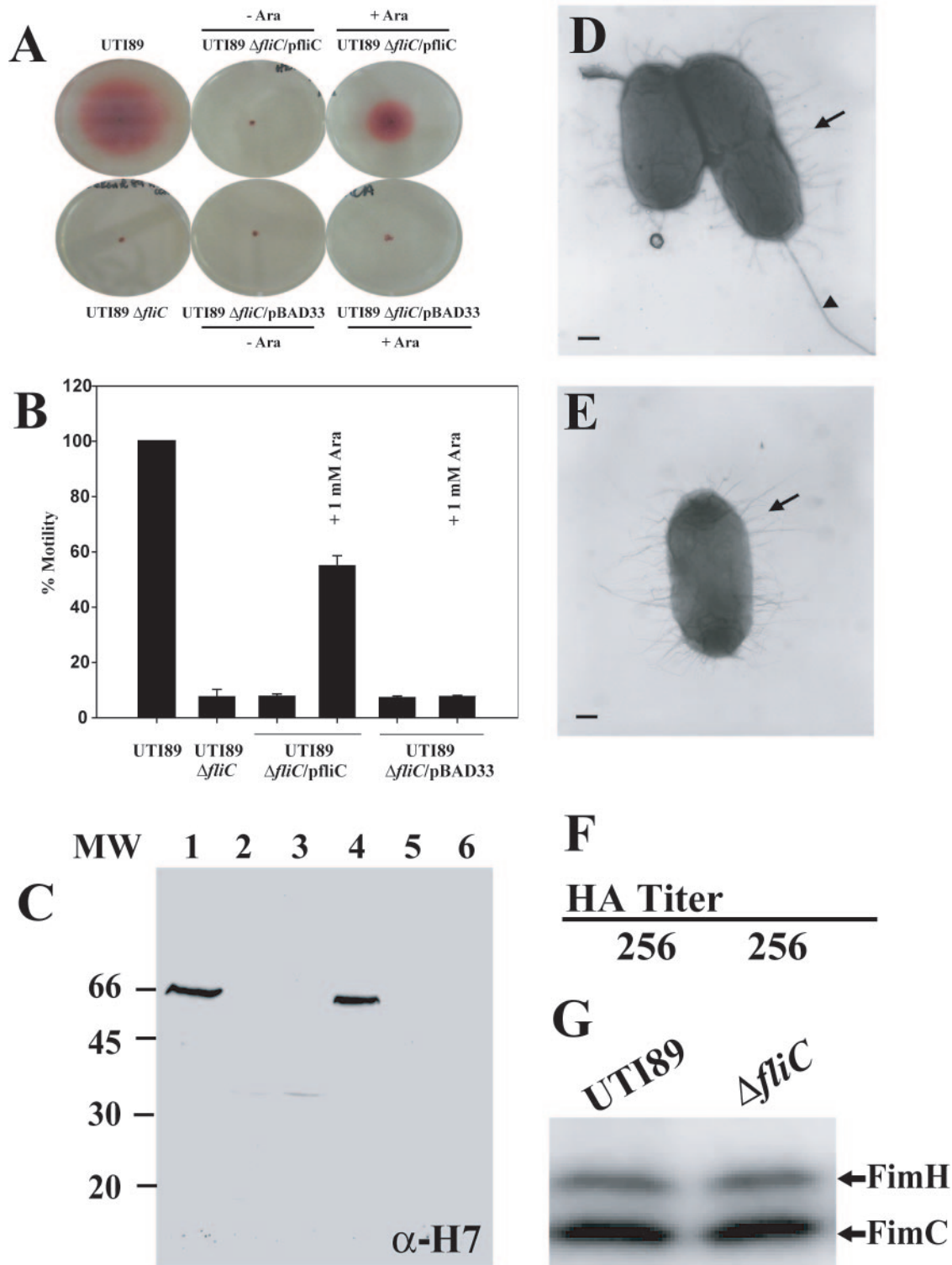


FIG. 3. In vitro characterization of the nonmotile UPEC strain UTI89  $\Delta$ fliC. (A) Motility analysis of UTI89  $\Delta$ fliC, UTI89  $\Delta$ fliC/pfliC, and UTI89  $\Delta$ fliC/pVC. Strains were grown statically in LB broth and analyzed relative to the similarly grown wild-type UPEC strain UTI89 and UTI89/pVC for motility in 0.25% LB agar alone or supplemented with chloramphenicol (pfliC- and pVC-containing strains). Wells on the right contain 5 mM L-(+)-arabinose, in contrast to the center wells, in which strains are grown without arabinose. (B) Quantified motility. Motility was measured as described in Materials and Methods. (C) Flagellin (H7) immunoblot analysis. Lane 1, UTI89; lane 2, UTI89  $\Delta$ fliC; lane 3, UTI89  $\Delta$ fliC/pfliC without arabinose; lane 4, UTI89  $\Delta$ fliC/pfliC with arabinose; lane 5, UTI89  $\Delta$ fliC/pVC without arabinose; lane 6, UTI89  $\Delta$ fliC/pVC with arabinose. MW, molecular weight (in thousands). (D and E) Negatively stained electron micrographs of wild-type UTI89 (D) and UTI89  $\Delta$ fliC (E). Arrows indicate pili, and the arrowhead indicates the flagellum. (F) Guinea pig erythrocyte hemagglutination (HA). (G) Representative FimCH immunoblot ( $n = 6$ ). Left lane, UTI89; right lane, UTI89  $\Delta$ fliC.

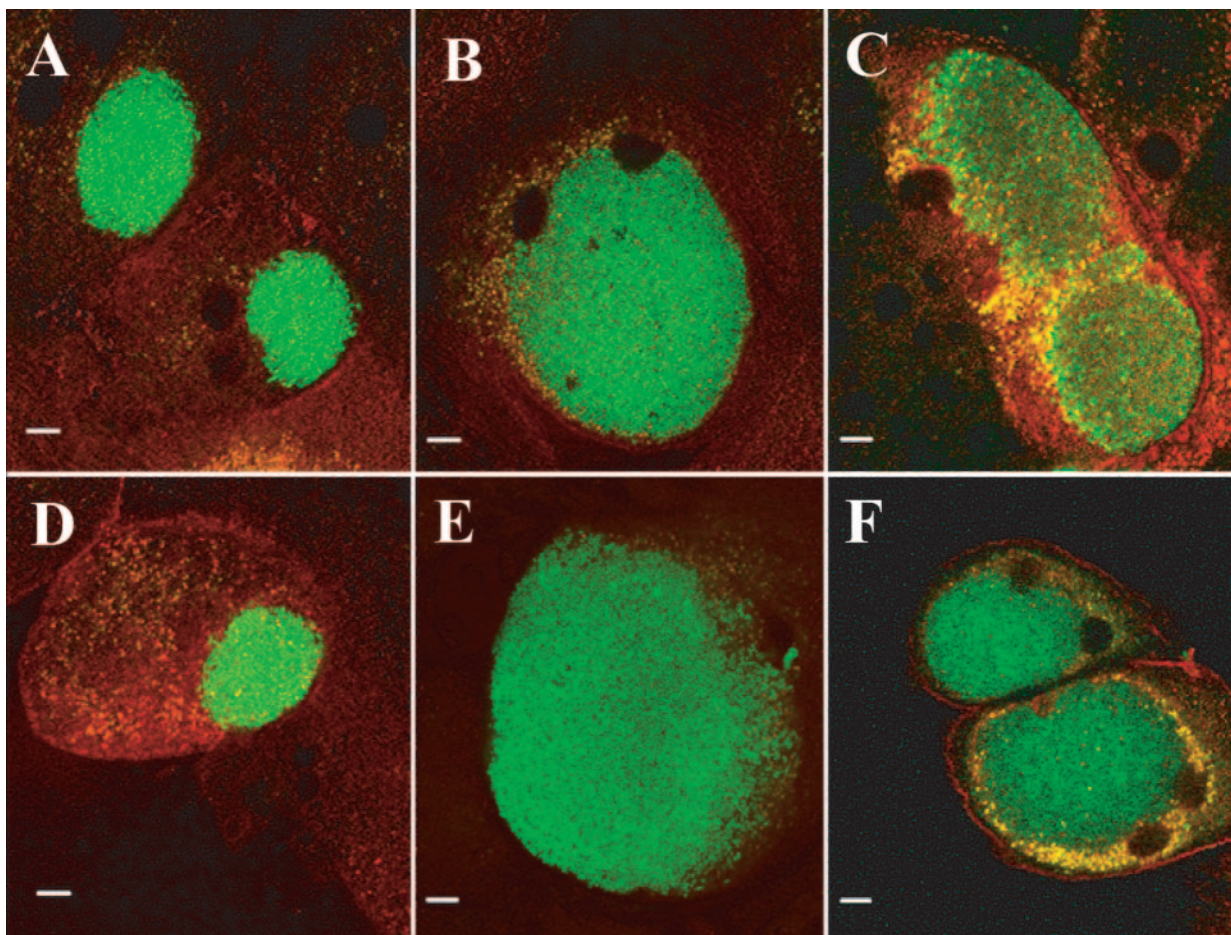


FIG. 4. IBC formation by the wild-type UPEC strain UTI89 and the nonmotile UPEC strain UTI89  $\Delta fliC$  as a function of time. The bladders of mice infected with either wild-type UTI89 (A to C) or the nonmotile UPEC strain (D to F), UTI89  $\Delta fliC$ , were collected 6 (A and D), 16 (B and E), and 30 (C and F) hours postinfection, splayed by pinning, stained with Alexa Fluor 633-conjugated WGA, fixed with PFA, and imaged by confocal microscopy at  $\times 63$  magnification. Bar, 10  $\mu\text{m}$ . Green staining indicates bacteria. Yellow staining indicates autofluorescent vacuoles. Red staining indicates WGA localization. IBCs arising from the nonmotile UPEC strain UTI89  $\Delta fliC$  were indistinguishable from those arising from the wild-type strain UTI89 at all acute cystitis time points examined.

later time points in the pathogenic cascade, namely, reservoir formation or spread to the kidney. Single infections with UTI89 or UTI89  $\Delta fliC$  produced equivalent bladder ( $P = 0.5476$ ) and kidney ( $P = 0.3095$ ) colonization at 2 weeks postinfection (Fig. 5A). However, competition experiments at 2 weeks postinfection demonstrated a competitive advantage of the wild-type bacteria over nonmotile  $\Delta fliC$  mutant UPEC in both bladder and kidney colonization. A 20-fold-lower bladder titer was seen for the nonmotile  $\Delta fliC$  mutant than for a competing wild-type strain. This defect was even more pronounced in the kidney, yielding a kidney titer for UTI89  $\Delta fliC$  300-fold-lower than that for UTI89. Using competition tissue titers, competition indices were calculated (see Materials and Methods) for each sample and “averaged” by determining the geometric mean for each group. By definition, a competition index of  $>1$  indicates outcompetition of the mutant strain (UTI89  $\Delta fliC$ ) by the wild-type reference strain (UTI89). A competition index equal to 1 indicates no difference in colonization, and a competition index of  $<1$  indicates outcompetition of the wild-type reference strain (UTI89) by the mutant

(UTI89  $\Delta fliC$ ). Geometric mean CI were calculated to be 19 for bladders and 140 for kidneys (Fig. 5B), indicating that the *wt* strain outcompetes the isogenic nonmotile  $\Delta fliC$  derivative. These data show that flagella and motility confer a fitness advantage in persistence throughout the urinary tract.

## DISCUSSION

Urinary tract infections occur as a continuum of steps by ascension of UPEC from the perineum through the urethra to the bladder, passing through the ureters to the kidneys. Clinically, the symptoms of cystitis, dysuria, and frequency often precede those of upper tract disease such as flank pain and chills. The bladder is a key intermediate or end organ of disease where UPEC can adhere via type 1 pili and amass (27, 33, 36, 42). UPEC bacteria, however, do not reside exclusively in the lumen of the bladder but instead rapidly gain access to the epithelium, where they proliferate in an intracellular sanctuary, protected from the massive inflammation associated with cystitis (34, 35). Their intracellular proliferation results in com-



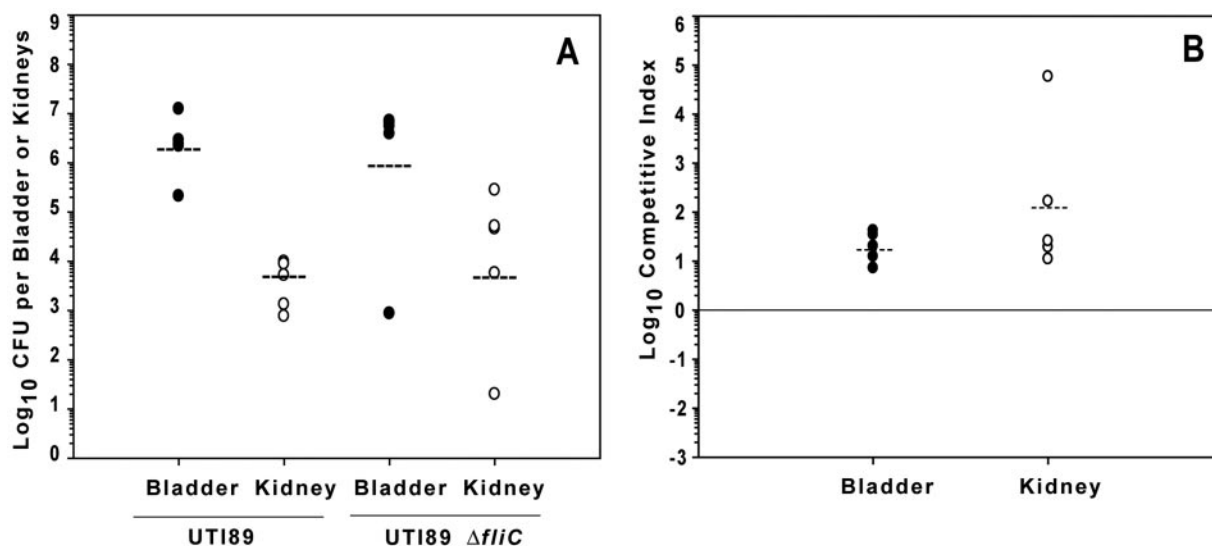


FIG. 5. Competitive infection of a prototypic cystitis strain, UTI89, and a nonmotile UPEC strain, UTI89  $\Delta$ *fliC*, in the bladders and kidneys of mice. (A) Mice were transurethurally inoculated with either UTI89 or UTI89  $\Delta$ *fliC*, and CFU in the bladders (●) and kidneys (○) was enumerated 2 weeks postinfection. (B) Mice were inoculated with a mixed inoculum (1:1 ratio) of both UTI89 and UTI89  $\Delta$ *fliC*, and CFU were enumerated in bladders and kidneys by replica plating onto LB or LB-kanamycin (50  $\mu$ g/ml) plates 2 weeks postinfection. The CI of bacteria recovered from the organs were calculated as described in Materials and Methods. Horizontal bars indicate the geometric mean titer or CI of the sample group indicated ( $n = 5$  mice per strain). During the competitive infection, recovery of UTI89  $\Delta$ *fliC* was significantly lower in the bladder ( $P = 0.008$  by the Mann-Whitney U test), indicating a competitive disadvantage during persistent cystitis, while a trend toward significance in the kidney was seen ( $P = 0.1$  by the Mann-Whitney U test).

munal formations with biofilm-like properties—the IBCs. The bacteria thrive tightly enmeshed in the protective matrix of the IBC within the host epithelium. Ultimately, they need to be released and dispersed in order to exit the infected cell and find new naïve cells for residence. The dispersion and the exiting of the host cell are, therefore, central steps in the UPEC life cycle in the bladder (termed the IBC pathway).

Flagella play a key role in the living dynamic of biofilms, having a strong influence over the ultrastructure and spread of biofilms (22, 38, 50). In *Pseudomonas aeruginosa* biofilms, nonmotile strains produce greater local biomasses but less surface coverage (22). Biofilm formation on living tissues may be even more dependent on motility, since the adherent surface may be dynamic, including the death or loss of the host tissue engaged in the interaction. The luminal surface of an infected bladder shows bacteria distributed widely but not equally over the epithelium (34, 35). While some bacteria reside singly, most are in small formations of clusters akin to early biofilm formation. The complex molecular cross talk at the host-pathogen interface likely influences the biofilm pathway, igniting genetic programs that differ from biofilm formation on an innate surface. Assimilating our knowledge of luminal colonization and the IBC pathway, we reasoned that flagella may play a role in multiple steps of UTI pathogenesis by assuming a central role in virulence during cystitis. We approached the problem by first evaluating if flagellar gene expression was active during cystitis, anticipating that this expression may correlate with a role in pathogenesis, and then examining the impact of the loss of motility on the course of cystitis.

Using promoter-*gfp* fusions to the class I-regulated *flhDC* operon and the class III-regulated *fliC* gene, we found that the fusions were highly active under in vitro conditions that pro-

mote motility. We found, however, that in contrast to findings of in vitro studies involving laboratory *E. coli* strains, *fliC* transcription in our prototypic UPEC strain, UTI89, is minimal even under optimal induction of motility. UTI89 seems to have modified its regulation, relying on efficient translation or increased stability of *fliC* transcripts to ensure the production of the abundant flagellin required for the assembly of flagella. In vivo, the class I genes were robustly and homogeneously transcribed within IBCs by 16 h postinfection; however, the expression of *fliC* could be detected within only 26% of the IBCs observed, arguing that the commitment to make flagella, as evidenced by the robust expression of class I genes, is interrupted or at least delayed in vivo. Class III genes may in fact be expressed in bacteria within IBCs; however, the expression may be transient or may be produced at time points not examined in this study, either of which possibilities would account for the infrequent detection of class III genes.

The regulation of flagellar expression is arguably the most complex network of genes and environmental inputs mapped in prokaryotes. The production of flagella is the culmination of environmental cues, such as pH, osmolarity, temperature, and cyclic AMP, intersecting with the hierarchical stages of expression. Thus, the differential expression of different gene classes may provide insights into the physiology of individual bacteria and the IBC as a whole. In addition to highlighting the physiological status of the bacteria and IBC, these studies are novel and represent the first in vivo flagellar class I and class III gene profiling. Our observed activation of the class I *flhDC* promoter may reflect declining nutrient bioavailability or altered physiological environments. For instance, the rapid production of the large IBC biomass probably does not result in a significant production of acid resulting in a lower pH, since *flhDC*

expression is reported to be repressed by low pHs (49). However, the comprehensive signals required for expression of the later gene products do not seem to be present *in vivo*, such that flagellar regulon activation is delayed or stops early in the hierarchy.

Both CsrA and DnaK have been shown to play roles in regulating flagella (9, 28, 44). CsrA plays a central role as a carbon storage regulator but has multiple effects on the regulation of biofilm-associated proteins such as flagella (9, 28). It has been shown that mutations in *dnaK* result in decreased expression of *flhDC*, causing a nonmotile phenotype (44). Late IBC formation is temporally associated with neutrophil infiltration and epithelial cell death, both contributing to potential oxidative stress in the bacteria. Reactive oxygen species have been shown to reversibly decrease levels of DnaK while up-regulating Hsp33 (52). Thus, it is possible that under the stress from host factors, DnaK becomes titrated, resulting in dysregulation of flagellar expression. Similarly, changes in carbon availability *in vivo* but not *in vitro* may affect the CsrA pathway and account for the results observed.

The incomplete regulatory circuit from class I to class III activation may reflect the conservation of resources, since production and powering of this organelle may divert important assets from otherwise essential activities. Alternatively, only the hook and basal body may be expressed as part of a virulence program. Since flagella are thought to be evolutionary relatives of type III secretion systems, the hook and basal body could produce a structure analogous to a secretion pore through which bacterial effectors interact with the host cell to promote IBC development and maintenance. Further studies will elucidate whether the hook and basal body are produced and functional during IBC formation.

The flagella of UPEC (12) and *Salmonella* spp. (43, 51) function not only in motility but also as adhesive and invasive organelles, respectively, to adhere to epithelial cells and promote biofilm formation (39, 40). However, the nonmotile UTI89  $\Delta$ *fliC* produced IBCs similar in number and morphology to those formed by UTI89 at all time points examined, suggesting that the flagellar filament does not function as an adhesive organelle to aggregate bacteria within the IBC. Furthermore, the filament was not required for invasion, as evidenced by the fact that bacteria were observed intracellularly. These results are consistent with the minimal expression or lack of expression of *fliC* in IBCs. The 30-h UTI89  $\Delta$ *fliC* IBCs observed may be first-generation IBCs halted from progressing, or UPEC may disperse from an IBC by an alternative mechanism. Without flagellum-mediated dispersal and fluxing, nonmotile bacteria could be passively released by cell death and deterioration of eukaryotic cellular membranes during exfoliation in response to infection. This mechanism is supported by the lack of *gfp* expression or minimal *gfp* expression from the class III-*gfp* reporter in early, mid-, and late IBCs in contrast to its robust activity under *in vitro* conditions favorable to motility. However, when mice were infected simultaneously with wild-type UTI89 and UTI89  $\Delta$ *fliC* at a ratio of 1:1, 20-fold and 300-fold-lower titers in the bladder and kidney, respectively, were seen for the nonmotile *fliC*-negative mutant relative to the motile parent strain. Thus, a *fliC* deletion mutant had a competitive disadvantage in both the bladder and the kidneys 2 weeks postinfection, suggesting a role for flagella

in the pathogenic cascade downstream of IBC formation. Similar results were obtained using UPEC CFT073 in the CBA mouse model of UTI (14); whereas the CFT073 *fliC* mutant was able to colonize the bladder to levels not significantly different from those of the *wt* in single infections, wild-type CFT073 outcompeted a *fliC* mutant in cochallenge experiments (24).

In contrast to the known UPEC virulence factor, type 1 pili, which are up-regulated to promote virulence, we hypothesize flagella to be a UPEC virulence factor which is primarily down-regulated in the urinary tract environment. The concept of negatively regulating a virulence factor as a function of environment is a known mechanism in *Haemophilus influenzae*, which down-regulates HMW1 for immune system evasion in the inner ear (8). Flagellin from many bacterial species, including *E. coli*, elicits a strong proinflammatory activity by epithelial cells, which in turn promotes bacterial clearance by innate host defenses (3, 29, 32, 53, 54). Down-regulation of flagella at appropriate times may provide UPEC an additional defense mechanism that contributes to persistence within the urinary tract. Induction of flagellar expression in response to specific environmental cues may provide motile bacteria with subtle advantages.

**Concluding remarks.** Flagella have been established as an important virulence factor in numerous bacterial pathogenesis models, but their requirement and specific role in UTI have not been elucidated previously, particularly in light of the recent description of IBCs and their dispersal. The paradigm of UTI pathogenesis, an ascending infection against the counter-current of urinary flow, suggests an overt and even essential role for flagella in producing disease. However, the studies described in this report suggest that the role is subtle. In support of a subtle role for flagella in UPEC pathogenesis are the recent reports by Bahrani-Mougeot et al. and Snyder et al. (2, 46). Signature-tagged mutagenesis screens in an ascending UTI mouse model and transcriptome microarray gene profiling of bacteria recovered from the urine of mice with active UTI failed to identify flagella as a key virulence factor. In fact, transcriptome profiling demonstrated flagella and chemotaxis genes to be down-regulated *in vivo*, further supporting the results presented in this study. While nonmotile UPEC can unmistakably advance through the stages of UTI in both the bladder and the kidney, the studies reported here clearly show that bacteria with the full capacity to produce flagella are at a competitive advantage in both the bladder and the kidney, although the mechanism by which this advantage is conferred is still unclear. Notably, our study of flagellar expression during the course of IBC development has revealed the first *in vivo* example of the checkpoints present in flagellar production, previously detailed through *in vitro* genetic studies. The expression of selective gene classes *in vivo* may provide important leads regarding the physiological states of the IBC and the host epithelial cell.

Studies of the roles of flagella in UPEC UTI pathogenesis may need to be revisited if true models of ascension are to be developed. Since most UPEC strains are motile, flagellar function appears to be evolutionarily conserved. Perhaps the role of flagella is in maintenance in the enteric or genitourinary tracts, allowing emergence into the periurethral region and ascent into the urethra for the initiation of UTI. Flagella may

also have a role in dissemination from the urinary tract in urosepsis or in the ascension from the bladder to the kidneys. Only true models of UPEC recirculation between extraurinary sites of colonization and the urinary tract will serve to answer these questions.

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