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Multiple Genes Repress Motility in Uropathogenic *Escherichia coli* Constitutively Expressing Type 1 Fimbriae▽†

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Two surface organelles of uropathogenic *Escherichia coli* (UPEC), flagella and type 1 fimbriae, are critical for colonization of the urinary tract but mediate opposite actions. Flagella propel bacteria through urine and along mucus layers, while type 1 fimbriae allow bacteria to adhere to specific receptors present on uroepithelial cells. Constitutive expression of type 1 fimbriae leads to repression of motility and chemotaxis in UPEC strain CFT073, suggesting that UPEC may coordinately regulate motility and adherence. To identify genes involved in this regulation of motility by type 1 fimbriae, transposon mutagenesis was performed on a phase-locked type 1 fimbrial ON variant of strain CFT073 (CFT073 *fim* L-ON), followed by a screen for restoration of motility in soft agar. Functions of the genes identified included attachment, metabolism, transport, DNA mismatch repair, and transcriptional regulation, and a number of genes had hypothetical function. Isogenic deletion mutants of these genes were also constructed in CFT073 *fim* L-ON. Motility was partially restored in six of these mutants, including complementable mutations in four genes encoding known transcriptional regulators, *lrhA*, *lrp*, *slyA*, and *papX*; a mismatch repair gene, *mutS*; and one hypothetical gene, *ydiV*. Type 1 fimbrial expression in these mutants was unaltered, and the majority of these mutants expressed larger amounts of flagellin than the *fim* L-ON parental strain. Our results indicate that repression of motility in CFT073 *fim* L-ON is not solely due to the constitutive expression of type 1 fimbriae on the surfaces of the bacteria and that multiple genes may contribute to this repression.

Motility and adherence are two integral aspects of bacterial pathogenesis. Adherence, often mediated by fimbriae, permits bacteria to adhere to a cell surface and establish an infection, whereas flagellum-driven motility allows bacteria to move to more-advantageous sites for colonization. Both fimbriae and flagella have been shown to be important in the pathogenesis of uropathogenic *Escherichia coli* (UPEC). Type 1 fimbriae, expressed on the surfaces of *E. coli* and most of the *Enterobacteriaceae* (11), have been shown to be critical for colonization of the bladder by UPEC (27), and accordingly, genes encoding these fimbriae are highly expressed in vivo (53). While type 1 fimbriae allow UPEC to bind mannose-containing glycoprotein receptors, such as uroplakin (46, 61), and establish infection in the bladder, our laboratory (33) and another (51) have recently shown that flagella allow UPEC to ascend from the bladder to the kidneys. Furthermore, flagellum production contributes to the fitness of UPEC during murine urinary tract colonization (34, 60).

Flagella are complex organelles that are encoded by over 40 genes (reviewed in references 14 and 55). Genes for flagellum synthesis form a complex and tightly regulated transcriptional hierarchy consisting of three classes. The class I genes *flhDC* encode the master regulator $FlhD_2C_2$, required for transcription of the class II genes. Transcription of *flhDC* is modulated by a number of physiological and environmental signals, such as temperature (2), osmolarity (52), histone-like nucleoid-structuring protein (H-NS) (5), and autoregulation (31). Class II genes encode structural and assembly proteins needed for synthesis of the hook-basal body. In addition, two regulatory proteins, FliA (σ28) and FlgM (anti-σ28), are transcribed from class II promoters. FliA is the transcription factor required for expression from the class III promoters (41). These class III genes encode the flagellar filament proteins and other proteins needed for motility and the chemotaxis system.

Expression of type 1 fimbriae is phase variable and controlled by a promoter situated on an invertible element (IE) upstream of the type 1 fimbrial operon (*fimAICDFGH*) (1). The IE alternates between two orientations, phase ON and phase OFF. When the IE is positioned so that the promoter faces the *fim* operon, type 1 fimbriae are produced and the bacteria are considered phase ON. In the phase OFF conformation, the promoter faces away and transcription is blocked, resulting in no production of type 1 fimbriae. Switching of the IE between these two phases is mediated by the FimB and FimE recombinases (7, 30, 44) as well as the recently identified FimB- and FimE-like recombinases IpuA and IpbA (10). FimB promotes phase variation between both promoter orientations, while FimE promotes primarily ON-to-OFF switching of the IE (24, 30).

Reciprocal regulation is one mechanism by which bacteria reconcile the contradictory, yet necessary, actions of adherence and motility. It would not be beneficial for an organism tethered to a surface to suddenly attempt swimming or swarming. Also, high-level expression of fimbriae by a swimming organism could sabotage motility. Therefore, it is expected that a strongly fimbriated bacterium would not be highly motile and

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TABLE 1. Relevant bacterial strains and plasmids used in this study

Strain or plasmid	Description	Source or reference
E. coli		
CFT073	Wild-type pyelonephritis isolate (O6:K2:H1)	45
CFT073 *fim* L-ON	CFT073 *fim* IE phase locked ON; constitutive type 1 fimbrial expression	27
CFT073 *fim* L-ON Nal^r	Nal^r derivative of CFT073 *fim* L-ON	This study
CFT073 Δ*lrp*	CFT073 Δ*lrp*::*kan*; replacement of nucleotides 41 to 428 of *lrp* in CFT073 with the kanamycin resistance gene of pKD4	This study
CFT073 Δ*lrhA*	CFT073 Δ*lrhA*::*kan*; replacement of nucleotides 41 to 889 of *lrhA* in CFT073 with the kanamycin resistance gene of pKD4	This study
CFT073 Δ*mutS*	CFT073 Δ*mutS*::*kan*; replacement of nucleotides 99 to 2332 of *mutS* in CFT073 with the kanamycin resistance gene of pKD4	This study
CFT073 Δ*ydiV*	CFT073 Δ*ydiV*::*kan*; replacement of nucleotides 91 to 552 of *ydiV* in CFT073 with the kanamycin resistance gene of pKD4	This study
CFT073 Δ*slyA*	CFT073 Δ*slyA*::*kan*; replacement of nucleotides 46 to 345 of *slyA* in CFT073 with the kanamycin resistance gene of pKD4	This study
CFT073 Δ*papX*	CFT073 Δ*papX*::*kan*; replacement of nucleotides 1 to 550 of *papX* in CFT073 with the kanamycin resistance gene of pKD4	This study
CFT073 *fim* L-ON Δ*lrp*	CFT073 *fim* L-ON Δ*lrp*::*kan*; replacement of *lrp* (as in CFT073 Δ*lrp*) in CFT073 *fim* L-ON	This study
CFT073 *fim* L-ON Δ*lrhA*	CFT073 *fim* L-ON Δ*lrhA*::*kan*; replacement of *lrhA* (as in CFT073 Δ*lrhA*) in CFT073 *fim* L-ON	This study
CFT073 *fim* L-ON Δ*mutS*	CFT073 *fim* L-ON Δ*mutS*::*kan*; replacement of *mutS* (as in CFT073 Δ*mutS*) in CFT073 *fim* L-ON	This study
CFT073 *fim* L-ON Δ*ydiV*	CFT073 *fim* L-ON Δ*ydiV*::*kan*; replacement of *ydiV* (as in CFT073 Δ*ydiV*) in CFT073 *fim* L-ON	This study
CFT073 *fim* L-ON Δ*slyA*	CFT073 *fim* L-ON Δ*slyA*::*kan*; replacement of *slyA* (as in CFT073 Δ*slyA*) in CFT073 *fim* L-ON	This study
CFT073 *fim* L-ON Δ*papX*	CFT073 *fim* L-ON Δ*papX*::*kan*; replacement of *papX*, as in CFT073 Δ*papX*, in CFT073 *fim* L-ON	This study
Plasmids		
pUT/mini-Tn*5*Km2	Mini-Tn*5* Km2 transposon in pUT suicide vector (Kan^r Amp^r)	18
pKD4	Lambda red recombinase system template plasmid (Kan^r Amp^r)	17
pGEN-MCS	Derivative of pGEN222 with multiple cloning site replacing *gfpuv* (Amp^r)	33
pGEN-*lrp*	*lrp* (with native promoter) of CFT073 cloned into BamHI-SphI sites of pGEN-MCS (Amp^r)	This study
pGEN-*lrhA*	*lrhA* (with native promoter) of CFT073 cloned into BamHI-SphI sites of pGEN-MCS (Amp^r)	This study
pGEN-*mutS*	*mutS* (with native promoter) of CFT073 cloned into BamHI-SphI sites of pGEN-MCS (Amp^r)	This study
pGEN-*ydiV*	*ydiV* (with native promoter) of CFT073 cloned into BamHI-SphI sites of pGEN-MCS (Amp^r)	This study
pGEN-*slyA*	*slyA* (with native promoter) of CFT073 cloned into BamHI-SphI sites of pGEN-MCS (Amp^r)	This study
pLX3607	pQE60 cloning vector with *lacI*^{q} cloned into XhoI site (Amp^r)	37
pDRM001	*papX* of CFT073 cloned into NcoI–HindIII sites of pLX3607 (Amp^r)	37

that a motile organism would not express large numbers of fimbriae. Accordingly, reciprocal regulation of motility and adherence has been observed in a number of pathogenic bacterial groups, including *Bordetella pertussis* (3), *Vibrio cholerae* (26), *Salmonella enterica* serovar Typhimurium (16), and *Proteus mirabilis* (37). In the uropathogens *P. mirabilis* and UPEC, proteins encoded within fimbrial operons have been shown to regulate motility. For example, overexpression of MrpJ, a protein encoded by the last gene of the MR/P fimbrial operon, inhibits both swimming and swarming motility in *P. mirabilis* (37). Also, PapX of UPEC, a homolog of MrpJ encoded by the *pheV*-associated but not the *pheU*-associated *pap* allele of CFT073, caused reduced motility when overexpressed in both *P. mirabilis* (37) and UPEC (A. Simms and H. L. T. Mobley, unpublished).

Recently, our laboratory (35) and another (10) have shown that constitutive production of type 1 fimbriae by a phase-locked ON variant of UPEC strain CFT073 (CFT073 *fim* L-ON) leads to a decrease in swimming motility in soft agar. Western blot analysis indicated that this repression of motility is due to a decrease in the level of flagella produced; also, real-time PCR studies demonstrated that constitutive expression of type 1 fimbriae results in decreased transcription of the major flagellin subunit gene, *fliC* (35). Deletion of the entire region of DNA carrying the structural and accessory genes of type 1 fimbriae partially restored motility in CFT073 *fim* L-ON (35), suggesting that repression of motility in this strain is not solely due to the constitutive expression of type 1 fimbriae on the surfaces of the bacteria. Here, we used transposon mutagenesis and isogenic deletion mutants of CFT073 *fim* L-ON to examine other possible mechanisms of regulation that repress motility when type 1 fimbriae are expressed.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *E. coli* CFT073 was initially isolated from the blood and urine of a patient with acute pyelonephritis (45). Additionally, the genome of *E. coli* CFT073 has been sequenced and annotated (59). All relevant *E. coli* strains and plasmids used in this study are listed in Table 1. *E. coli* was cultured on Luria-Bertani (LB) agar or in LB broth incubated at 37°C. Antibiotics were added as needed at the following concentrations: nalidixic acid (Nal), 50 μg/ml; ampicillin (Amp), 100 μg/ml; and kanamycin (Kan), 25 μg/ml. In vitro growth curves of *E. coli* in LB broth were generated in triplicate using a Microbiology Reader Bioscreen C (Oy Growth Curves AB Ltd.) in 0.2-ml volumes; optical density at 600 nm (OD_{600}) was recorded every 15 min.

Transposon mutagenesis of *E. coli* CFT073 *fim* L-ON and motility of the resultant Tn*5* mutants. Transposon mutagenesis was performed via a filter-mating conjugation protocol. Both donor [*E. coli* S17 λ *pir* (pUT/mini-Tn*5*Km2)] and recipient (*E. coli* CFT073 *fim* L-ON Nal^r) strains were cultured overnight in static LB broth cultures with appropriate antibiotics. No difference was observed in growth rate between the nalidixic acid-marked strain CFT073 *fim* L-ON Nal^r and CFT073 *fim* L-ON (data not shown). Cultures were gently mixed at a 1:4 donor/recipient ratio and placed onto one 0.2-mm-pore-size nitrocellulose filter on an LB agar plate. After 2 hours of incubation at 37°C, the filter was removed and bacteria on the filter were suspended by vigorous vortexing in a small amount of LB broth. This suspension was plated on LB agar containing nalidixic acid and

kanamycin to select for the recovery of kanamycin-resistant transposon mutants of CFT073 *fim* L-ON Nal^r.

To evaluate the motilities of kanamycin-resistant transposon mutants of CFT073 *fim* L-ON Nal^r, colonies from the initial mutagenesis were passaged to new LB agar plates containing nalidixic acid and kanamycin. Following overnight growth at 37°C, patched colonies were stabbed, using sterile toothpicks, into soft-agar plates (1% tryptone, 0.5% NaCl, 0.25% agar) without antibiotics. Care was taken to avoid touching the bottom of the plate to avoid possible spread by twitching motility. After 16 h of incubation at 30°C, the diameters of motility were measured for each strain. Motile strains were identified as those in which the diameter of motility was larger than that of the *E. coli* CFT073 *fim* L-ON parental control. Wild-type (WT) *E. coli* CFT073 and CFT073 *fim* L-ON were always included as controls, and three independent motility experiments for each motile transposon mutant were performed.

Identification of the location of the Tn*5* insertion in motile transposon mutants of CFT073 *fim* L-ON. Sites of transposon insertion were amplified by arbitrary primed PCR, as described by Burall et al. (12). Arbitrary primed PCR was performed in two sequential amplification steps, with primers complementary to mini-Tn*5* ends reading outward and arbitrarily designed primers reading inward from the unknown sequence. For arbitrary PCR 1, primer Arb1 paired with primer 1955 was used. Primer sequences are listed in Table S1 in the supplemental material. The template for these PCRs consisted of a single-colony suspension of mutant *E. coli* in 25 μl of double-distilled water (ddH_2O) boiled for 10 min. PCR amplifications were completed in a DNA Engine PTC-200 thermal cycler (MJ Research). After the initial denaturation for 5 min at 95°C, amplification was completed by cycling 29 times at 95°C for 30 seconds, followed by variable annealing at 53° to 60°C for 30 seconds and 72°C for 90 seconds. The reaction was completed with a final elongation step at 72°C for 5 min. For arbitrary PCR 2, the reagents and conditions were the same, except that the template was a 2-μl sample from arbitrary PCR 1 and different primer sets were used. For arbitrary PCR 2, primers complementary to sequences nested outside the PCR 1 primers, primer 1954 paired with Arb2 (the constant region of primer Arb1), were used (12). Arbitrary PCR 2 products were analyzed by electrophoresis on a 1% (wt/vol) agarose gel, and PCR products were directly cloned into pCR2.1-TOPO (Invitrogen) per the manufacturer's protocols. These reaction products were then transformed into electrocompetent *E. coli* Top10 (Invitrogen). Plasmid DNA was extracted using a QIAprep Spin mini prep kit (Qiagen), and nucleotide sequencing using the M13 universal forward and reverse primers was performed by the University of Michigan DNA Sequencing Core on purified plasmid DNA.

Construction and complementation of isogenic mutants of CFT073 and CFT073 *fim* L-ON. Deletion mutants of genes identified in the transposon screen or other genes of interest were generated in CFT073 or CFT073 *fim* L-ON, using the lambda red recombinase system designed by Datsenko and Wanner (17). Primers containing sequences homologous to the 5′ and 3′ regions of the targeted genes were designed (H1P1 and H2P2) and used to amplify the kanamycin resistance gene from the template plasmid pKD4 (see Table S1 in the supplemental material). Lambda red-mediated recombination then replaced the specific gene(s) with this resulting PCR product. Kanamycin was used for selection of all mutants. Primers that flank the target gene sequence were designed (see Table S1 in the supplemental material) and used to confirm by PCR whether the kanamycin resistance cassette recombined within the target gene site as described previously (34).

To complement selected mutants of CFT073 and CFT073 *fim* L-ON, the *lrp*, *lrhA*, *mutS*, *ydiV*, and *slyA* genes, with associated promoters, were amplified using primers listed in Table S1 in the supplemental material. Each PCR fragment was digested with BamHI and SphI, followed by ligation into BamHI-SphI-cut pGEN-MCS. pGEN-MCS is a derivative of plasmid pGEN222 (22) in which the *gfpuv* gene is replaced with a multiple cloning site (33). The resultant plasmid constructs were used to transform their respective mutants, with selection for ampicillin resistance. The constructs were also confirmed by isolation of plasmid DNA, followed by both digestion with the above-mentioned restriction enzymes and PCR using primers outside the BamHI and SphI sites of pGEN-MCS (see Table S1 in the supplemental material). Both *papX* mutants of CFT073 and CFT073 *fim* L-ON were complemented by transformation with the plasmid pDRM001 (37). This plasmid contains *papX* under the control of an IPTG (isopropyl-β-D-thiogalactopyranoside)-inducible promoter. However, no IPTG was used in our assays presented here, as we have determined that the background expression of *papX* from this promoter is sufficient for repression of motility in CFT073 (A. Simms and H. L. T. Mobley, unpublished). Complementation was assessed by restoration of repression of motility in each of the mutants, using the phenotypic assays described below.

Motility of isogenic mutants of CFT073 and CFT073 *fim* L-ON. Motility was evaluated using soft-agar plates as described previously (34). Briefly, a sample of overnight culture of each mutant was used to inoculate 5 ml of sterile LB broth, followed by incubation at 37°C with aeration (200 rpm) to an OD_{600} of 1.0 to 1.2. Cultures were standardized to an OD_{600} of 1.0 and stabbed into the middles of soft-agar plates by using a sterile inoculating needle. Care was taken to avoid touching the bottom of the plate to avoid possible spreading from twitching motility. The plates were incubated for 16 h at 30°C, after which the diameter of motility was measured. Our laboratory has previously shown that CFT073 *fim* L-ON is heavily fimbriated and abundantly expresses FimH at 30°C throughout the motility assays (35), as seen under regular growth conditions at 37°C. WT *E. coli* CFT073 and CFT073 *fim* L-ON were always included as controls. The motilities of the complemented mutants of CFT073 and CFT073 *fim* L-ON were determined as described above except that the cultures and soft-agar plates contained ampicillin for maintenance of the complementation plasmid. Results of the motility agar assay were confirmed for all mutants by phase contrast microscopy. Wet mounts of bacterial cultures grown to an OD_{600} of 0.3 to 0.4, corresponding to optimal WT motility (35), were viewed at ×400 magnification by using a Zeiss Axioplan microscope. In this assay, CFT073 *fim* L-ON exhibits reduced motility compared to the WT strain (35).

Detection of flagellum production. Bacteria were cultured for optimal WT motility as mentioned previously (35), followed by standardization to an OD_{600} of 0.35. Aliquots of these standardized suspensions (1.5 ml) were centrifuged at 3,500 rpm for 10 min at room temperature to pellet the bacteria. Whole-cell lysates were prepared for electrophoresis by gentle resuspension of the bacterial pellets in 100 μl of ddH_2O and 20 μl of 6× sodium dodecyl sulfate (SDS) sample buffer, followed by boiling for 10 min. Samples were electrophoresed under denaturing conditions on a 10% SDS-polyacrylamide gel and transferred to a polyvinylidene difluoride membrane (Immobilon-P; Millipore Corp.). The blot was incubated with a 1:40,000 dilution of rabbit polyclonal antiserum to H1 flagella (Statens Serum Institute; Copenhagen, Denmark), followed by a 1:25,000 dilution of peroxidase-conjugated goat anti-rabbit immunoglobulin G (Sigma). The blot was developed using chemiluminescence according to the manufacturer's instructions (Amersham ECL Plus; GE Healthcare Life Sciences).

Detection of type 1 fimbrial expression. Type 1 fimbrial expression was first examined in all mutants of CFT073 *fim* L-ON, using a mannose-sensitive hemagglutination assay as described previously (54). Briefly, approximately 1×10^9 CFU of bacteria, cultured statically overnight in LB broth, were resuspended in phosphate-buffered saline (PBS) and serially diluted twofold in round-bottom 96-well microtiter plates. An equal amount of a 3% (vol/vol) solution of guinea pig erythrocytes (Rockland Immunochemicals) with or without 25 μg α-methyl mannoside was mixed with the bacterial suspension. A diffuse mat of cells across the bottom of the well indicated positive hemagglutination.

For Western blotting, bacteria were cultured for optimal WT motility as described above, followed by standardization to an OD_{600} of 0.35. Aliquots of these standardized suspensions (1.5 ml) were centrifuged at 10,000 rpm for 10 min at room temperature to pellet the bacteria. Whole-cell lysates were prepared for electrophoresis by gentle resuspension of the bacterial pellets in 100 μl of acidified ddH_2O (pH 1.8), followed by boiling for 10 min. After boiling, 20 μl of 6× SDS sample buffer was added to each fimbrial sample, followed by neutralization with 1 N NaOH. Samples were electrophoresed under denaturing conditions on a 10% SDS-polyacrylamide gel and transferred to a polyvinylidene difluoride membrane (Immobilon-P; Millipore Corp.). The blot was incubated with a 1:5,000 dilution of mouse polyclonal antiserum against FimC and FimH (courtesy of S. Langermann), followed by a 1:100,000 dilution of peroxidase-conjugated goat anti-mouse immunoglobulin G (Sigma). The blot was developed using chemiluminescence according to the manufacturer's instructions (Amersham ECL Plus; GE Healthcare Life Sciences).

Indirect fluorescent antibody staining. Detection of H1 flagellin on the surfaces of CFT073, CFT073 *fim* L-ON, and the six isogenic deletion mutants of CFT073 *fim* L-ON was performed by a sequential staining procedure. Bacteria, cultured to optimal motility in LB (35), were spotted onto Teflon-printed indirect fluorescent-antibody slides (Electron Microscopy Sciences), dried, and fixed in PBS with 4% formaldehyde at 4°C. Fixative was washed off the slides with PBS. The slides were incubated with a polyclonal rabbit serum raised against purified FliC protein of CFT073 (1:1,000). Goat anti-rabbit IgG (Alexa 488 conjugate; Molecular Probes) (1:5,000) was used as the secondary antibody. All antisera were diluted in PBS containing 4% fetal bovine serum, and all incubations were done for 1 h at room temperature. The slides were rinsed three times with PBS after each incubation. Propidium iodide was used to stain bacterial nucleic acids by diluting a 1.0-mg/ml solution (Sigma) 1:10,000 in the first PBS wash after incubation with the secondary antibody. The slides were mounted with ProLong Gold Antifade mounting medium (Invitrogen). The slides were examined

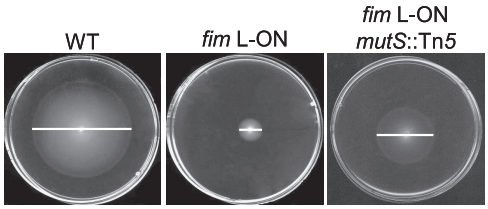

FIG. 1. Screen for restoration of swimming motility in transposon mutants of *E. coli* CFT073 constitutively expressing type 1 fimbriae (*fim* L-ON). Motilities of the WT, *fim* L-ON, and a representative motile transposon mutant of *fim* L-ON (*fim* L-ON *mutS*::Tn*5*) in soft agar are shown. White bars indicate the diameter of motility of each strain (WT, 44 mm; *fim* L-ON, 10 mm; *fim* L-ON *mutS*::Tn*5*, 25 mm).

at ×1,000 magnification with an Olympus BX60 system microscope equipped for fluorescence with filters for fluorescein isothiocyanate and Texas Red detection. All images were obtained and analyzed with an Olympus DP70 color digital video camera and DP Controller/Manager software.

Statistical analysis. For motility assays, a paired Student *t* test was used to determine significant differences in motility between strains (InStat; GraphPad Software).

RESULTS

Screening for genes involved in repression of motility in CFT073 constitutively expressing type 1 fimbriae (*fim* L-ON). Our laboratory (35) and another (10) have previously shown that constitutive expression of type 1 fimbriae leads to a repression of motility and chemotaxis in UPEC strain CFT073. This regulation appears to occur at the transcriptional level; it was demonstrated by real-time PCR studies that constitutive expression of type 1 fimbriae results in decreased transcription of the major flagellin subunit gene, *fliC*, leading to a subsequent decrease in the level of flagella produced (35). Therefore, we hypothesized that UPEC possesses defined regulatory pathways by which it controls the transition from adherent to motile states and vice versa. To test this hypothesis, transposon mutagenesis was performed on a phase-locked type 1 fimbrial ON variant of UPEC strain CFT073 (CFT073 *fim* L-ON), followed by a screen for restoration of swimming motility in soft agar (Fig. 1). CFT073 *fim* L-ON contains a mutation in the left inverted repeat of the type 1 fimbrial IE that blocks DNA inversion by FimB, FimE, or other recombinases, thus locking the type 1 fimbrial promoter in an ON conformation (27). CFT073 *fim* L-ON constitutively expresses type 1 fimbriae on its surface.

The genome of UPEC CFT073 has been fully sequenced and annotated and contains 5,379 open reading frames (59). Consequently, to achieve ≥90% genome saturation as calculated using the formula of Zilsel et al. (62), the swimming motilities of 12,000 Kan^r Tn*5* mutants of CFT073 *fim* L-ON Nal^r in 0.25% tryptone agar were examined. There was no detectable difference in growth or motility between CFT073 *fim* L-ON Nal^r and CFT073 *fim* L-ON. Forty-eight of 12,000 Kan^r Tn*5* mutants of CFT073 *fim* L-ON Nal^r exhibited reproducible motility phenotypes greater than that of the CFT073 *fim* L-ON parent strain. Interestingly, however, all of these mutants were less motile than the CFT073 WT strain (data not shown). The locations of the Tn*5* insertion in all 48 of these mutants were identified (Table 2). In 22 of the 48 mutants, the transposon inserted itself into the type 1 fimbrial operon, specifically within *fimA*, *fimI*, *fimC*, and *fimD* (see below). Other classes of genes disrupted in the remaining 26 mutants included those involved with metabolism, DNA mismatch repair, transcriptional regulation, and transport (Table 2). A large number of mutants contained the transposon in hypothetical genes. Also, multiple mutants in which the transposon had inserted itself in the same gene or region of the chromosome were isolated (Table 2) (*mutS*, hypothetical gene c1234, and the intergenic region between hypothetical genes c1204 and c1205).

Disruption of type 1 fimbrial genes partially restored motility in CFT073 *fim* L-ON Nal^r. Our laboratory showed previously that deletion of the type 1 fimbrial operon in CFT073 *fim* L-ON partially restored motility and flagellum expression (35). In this earlier study, the major structural and assembly genes of type 1 fimbriae were deleted to create a *fim*-null mutant of CFT073 *fim* L-ON, CFT073 Δ*fimAICDFGH*. The motility of CFT073 *fim* L-ON Δ*fimAICDFGH* was significantly higher than that of its CFT073 *fim* L-ON parent but not of the same level as that of WT CFT073 (35). In the study presented here, 22 of 48 motile Tn*5* mutants of CFT073 *fim* L-ON Nal^r analyzed contained the transposon insertion in the type 1 fimbrial operon (Table 2). In all of these mutants, the disruption occurred downstream of the IE containing the type 1 fimbrial promoter. A PCR-based IE assay described previously (39) was used to determine that the type 1 fimbrial promoter was unaffected and remained in the locked ON orientation in all of the type 1 fimbrial Tn*5* mutants (data not shown). Also, these mutants were shown, via mannose-sensitive hemagglutination and Western blots, to express no detectable type 1 fimbriae (data not shown). The increased motilities of these Tn*5* fimbrial mutants of CFT073 *fim* L-ON compared to that of the parent strain, as well as our previous observation that deletion of the type 1 fimbrial operon partially restores motility to CFT073 *fim* L-ON (35), indicated that the production of type 1 fimbriae in CFT073 *fim* L-ON partially inhibits motility. This partial inhibition of motility, along with the isolation of motile Tn*5* mutants with insertions in nonfimbrial genes, suggested that factors other than the production of type 1 fimbriae also contribute to the repression of motility in CFT073 *fim* L-ON.

Six nonfimbrial genes are involved in repression of motility in CFT073 *fim* L-ON. As presented above, this 90% saturation transposon screen identified 21 nonfimbrial loci that contributed to the regulation of motility and type 1 fimbrial expression. The level of flagellin protein produced by these mutants

TABLE 2. Location of Tn*5* insertions in motile CFT073 *fim* L-ON Nal^r transposon mutants

Function	Gene(s) or locations[a]
Attachment	*fimA* (2), *fimI* (4), *fimC* (5), *fimD* (11)
Metabolism	*tyrB*, *nrdF*, c3405
DNA mismatch repair	*mutS* (2)
Transcriptional regulation	*lrp*, *lrhA*, *slyA*, *ybdO*, *yjjQ*
Transport	*yrbE*
Hypothetical	*yjdA*, *yjdM*, *ydiV*, *ykgI*, c0322, c1234 (3), c4376, c2400, c2295, region upstream of c1204 and c1205 (3), region upstream of *yhbT* and *yhbU*

[a] The numbers in parenthesis represent the numbers of motile transposon mutants of CFT073 *fim* L-ON Nal^r in which the Tn*5* cassette had inserted itself in that particular gene or location.

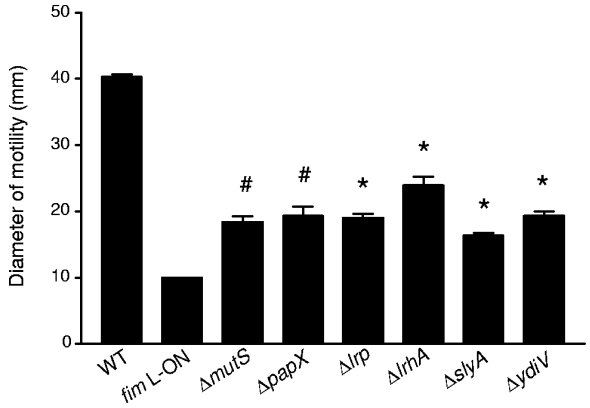

FIG. 2. Diameters of swimming motility of motile deletion mutants of CFT073 *fim* L-ON. Data represent the averages of three separate experiments. Error bars represent the standard errors of the means (SEM). Significant differences in motility from the *fim* L-ON parent were determined using a paired Student *t* test. #, $P < 0.05$; *, $P < 0.01$.

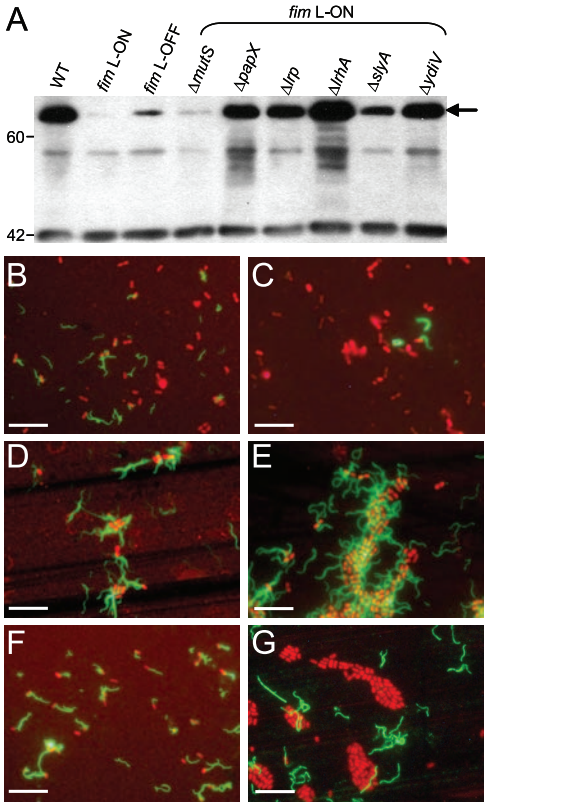

FIG. 3. Flagellum production in motile deletion mutants of CFT073 *fim* L-ON. (A) Western blot analysis of flagellin production. Whole-cell lysates from standardized mid-log-phase cultures were prepared, electrophoresed onto SDS-polyacrylamide gel electrophoresis gels, and subjected to Western blot analysis with antiserum specific to H1 flagella. The WT and the phase-locked type 1 fimbrial mutants CFT073 *fim* L-ON and *fim* L-OFF are included as controls. The arrow indicates the 60.9-kDa FliC protein. Molecular mass markers are indicated on the left in kDa. (B-G) Indirect immunofluorescence analysis of flagellum expression. CFT073 (B), CFT073 *fim* L-ON (C), CFT073 *fim* L-ON Δ*lrhA* (D), CFT073 *fim* L-ON Δ*slyA* (E), CFT073 *fim* L-ON Δ*ydiV* (F), and CFT073 *fim* L-ON Δ*mutS* (G) were spotted onto microscope slides and stained with antibody specific to H1 flagellin of CFT073 (green). Bacterial nucleic acids were visualized by staining with propidium iodide (red). Scale bars indicate 10 μm. The flagellar phenotypes of the *papX* and *lrp* mutants of CFT073 *fim* L-ON as determined by this analysis were similar to that of CFT073 *fim* L-ON *ydiV* (F).

was different in each strain, in direct correlation with the motility results (data not shown). Also, in all mutants, the IE containing the type 1 fimbrial promoter remained in the ON position. The majority of these Tn*5* mutants were shown to express type 1 fimbriae by mannose-sensitive hemagglutination and Western blotting (data not shown). Two mutants that were shown not to produce any detectable type 1 fimbriae contained the transposon in either c2295 or the region in between c1204 and c1205. However, the other two mutants isolated in which the transposon had also inserted itself into the region in between c1204 and c1205 were shown to produce type 1 fimbriae (data not shown).

This observation, as well as the known polarity of mini-Tn*5* transposon insertions (18), led us to construct isogenic in-frame deletion mutants of CFT073 *fim* L-ON in genes identified in the transposon screen as well as in other genes of interest known to affect flagellum expression. In all mutants constructed, the IE containing the type 1 fimbrial promoter remained in the ON position and these mutants were shown to express type 1 fimbriae by mannose-sensitive hemagglutination and Western blotting (data not shown). Examination of the motilities of these 34 isogenic deletion mutants of CFT073 *fim* L-ON revealed six nonfimbrial genes important for repression of motility during constitutive expression of type 1 fimbriae: *mutS*, *papX*, *lrp*, *lrhA*, *slyA*, and *ydiV*. As seen with the type 1 fimbrial mutant (35), single-gene-deletion mutants of these six candidate genes in CFT073 *fim* L-ON exhibited increased motility compared to the *fim* L-ON parent strain (Fig. 2). However, motility was not fully restored to WT levels (Fig. 2). Also, when the strains were cultured in broth to optimal motility (35), the amounts of flagellin protein produced in the *papX*, *lrp*, *lrhA*, *slyA*, and *ydiV* mutants were greater than that in the *fim* L-ON parent (Fig. 3A). As expected based on the motility results, these mutants expressed greater numbers of flagella on their surfaces than the *fim* L-ON strain (Fig. 3C to F). Interestingly, the Western blot analysis indicated that the level of flagellin made by CFT073 *fim* L-ON Δ*mutS* was comparable to that seen in the *fim* L-ON parental strain (Fig. 3A) and, accordingly, this mutant appeared to express less surface flagella than the other mutants of CFT073 *fim* L-ON (Fig. 3G). These results indicate that the increase in motility seen in this mutant may be due to factors other than a simple increase in the production of flagella.

Complementation of mutants. The MutS protein recognizes and binds to mismatched DNA sequences and is critical for DNA mismatch repair in prokaryotes (reviewed in reference 28). Therefore, it is possible that the deletion of *mutS* in CFT073 *fim* L-ON Δ*mutS* led to an increase in random mutation events during DNA replication and cell division. To address this possibility, as well as to determine whether the mutations made in the other mutants were nonpolar and disrupted only the genes of interest, *mutS*, *papX*, *lrp*, *lrhA*, *slyA*, and *ydiV*, with their respective promoters, were cloned into the low-copy-number plasmid pGEN-MCS and transformed into

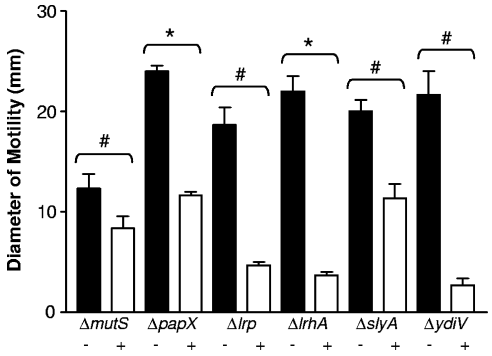

FIG. 4. Diameters of swimming motility of complemented mutants of CFT073 *fim* L-ON as well as mutants carrying the vector plasmid only. The + and − signs indicate mutants transformed with the complementation plasmid and the vector plasmid, respectively. Data represent the averages of three separate experiments. Error bars represent the SEM. Significant differences in motility between complemented mutants and mutants carrying the vector plasmid were determined using a paired Student *t* test. #, $P < 0.05$; *, $P < 0.01$.

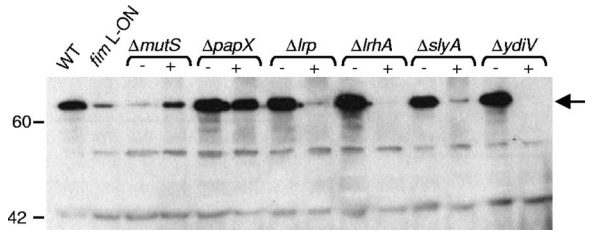

FIG. 5. Western blot analysis of flagellum production in complemented motile mutants of CFT073 *fim* L-ON. Whole-cell lysates from standardized mid-log-phase cultures were prepared, electrophoresed onto SDS-polyacrylamide gel electrophoresis gels, and subjected to Western blot analysis with antiserum specific to H1 flagella. The + and − signs indicate mutants transformed with the complementation plasmid and the vector plasmid, respectively. WT CFT073 and the parental phase-locked type 1 fimbrial mutant *fim* L-ON are included as controls. The arrow indicates the 60.9-kDa FliC protein. Molecular mass markers are indicated on the left in kDa.

the respective mutant strains. Motility, flagellar, and type 1 fimbrial phenotypes were determined for each complemented mutant strain and compared to those from each mutant strain transformed with the vector alone. In each mutant examined, complementation with the plasmid-expressed gene led to a significant decrease in motility in soft agar compared to that of the vector-only controls (Fig. 4). In the *papX*, *lrp*, *lrhA*, *slyA*, and *ydiV* mutants, this decrease in motility after complementation coincided with a reduction in flagellin protein (Fig. 5), indicating that products of these genes directly affected production of flagella in UPEC. However, in the *mutS* mutant of CFT073 *fim* L-ON, complementation with WT *mutS* led to an increase in the amount of flagellin protein produced (Fig. 5).

Deletion of identified genes increases motility in WT CFT073. We identified the contribution of six genes to motility in a variant of UPEC strain CFT073 constitutively expressing type 1 fimbriae (CFT073 *fim* L-ON). The remaining question involved examining the effect on the motilities of these genes in the WT strain, in which type 1 fimbrial genes can phase vary without restriction. Therefore, single-isogenic-deletion mutants of *mutS*, *papX*, *lrp*, *lrhA*, *slyA*, and *ydiV* were made in CFT073. The motility of each of these mutants was increased compared to that of CFT073 (Fig. 6A), indicating that the products of these genes repress motility in CFT073. However, in contrast to the results seen with the majority of the *fim* L-ON mutants, the levels of flagellin protein produced in these mutants of CFT073 were not largely increased compared to those in the WT parent (Fig. 6B). Only slight increases in flagellin protein are seen in the *slyA* and *ydiV* mutants, while the motilities were increased almost twofold. Also, the motility of CFT073 Δ*papX* was only slightly increased compared to that of WT CFT073, but the level of flagellin protein appears to be much higher in the mutant strain. Complementation of the mutants of CFT073 further supported the observation that these genes repress motility. As expected from the results with the *fim* L-ON strains, complementation with the plasmid-expressed gene led to a significant decrease in motility in soft agar compared to those of the vector-only controls (Fig. 6C). Also, as seen with the mutants of CFT073 *fim* L-ON, in the *papX*, *lrp*, *lrhA*, *slyA*, and *ydiV* mutants, the decrease in motility after complementation coincided with a reduction in flagellin protein (Fig. 6D), while in the *mutS* mutant of CFT073, complementation with WT *mutS* led to an increase in the amount of flagellin protein produced (Fig. 6D).

DISCUSSION

The abilities to adhere to host epithelia and to move via flagella are important yet opposite factors in the pathogenesis of UPEC. Previously, it was determined that constitutive expression of type 1 fimbriae leads to a repression of motility and chemotaxis in UPEC strain CFT073, suggesting that UPEC may coordinately regulate motility and adherence (10, 35). Furthermore, our laboratory showed that deletion of the entire region of DNA carrying the structural and accessory genes of type 1 fimbriae can only partially restore motility in this phase-locked *fim* ON mutant, CFT073 *fim* L-ON (35), suggesting that repression of motility in this strain is not solely due to the constitutive expression of these fimbriae on the surfaces of the bacteria. Here, we present studies that attempt to identify other genes involved in this regulation of motility by type 1 fimbriae. We initially identified 48 transposon mutants of CFT073 *fim* L-ON, corresponding to 27 genes, for which motility in soft agar was partially restored. Non-fimbria-encoding genes identified were knocked out in single-deletion mutants of CFT073 *fim* L-ON, and six genes that restored motility to CFT073 *fim* L-ON in our soft-agar screen were identified. These genes, all complementable, included the mismatch repair gene *mutS*; four known transcriptional regulators, *lrhA*, *lrp*, *slyA*, and *papX*; and one gene of hypothetical function, *ydiV*. BLASTx analysis revealed 95% amino acid identity between YdiV and a putative diguanylate phosphodiesterase (PDE) of *E. coli* strain B. This is interesting in that PDEs degrade the second messenger, cyclic di-GMP (c-di-GMP), which is known to regulate the transition between motility and biofilm formation (reviewed in reference 29). YdiV contains the prototypical EAL domain necessary for this hydrolysis of c-di-GMP. In many bacterial species, c-di-GMP inversely regulates adherence and motility in that low concentrations of c-di-GMP are associated with motile bacteria, whereas high

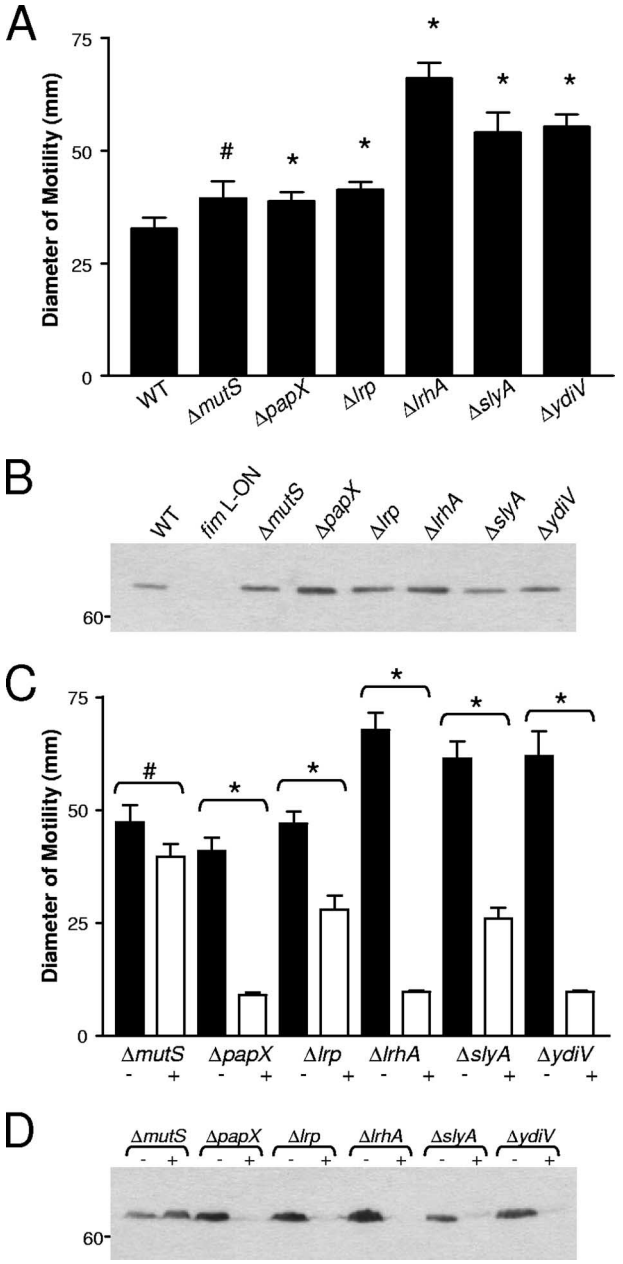

FIG. 6. Analysis of motility and flagellin production in mutants of WT CFT073. (A) Diameters of swimming motility of motile deletion mutants of CFT073. Data represent the averages of three separate experiments. Error bars represent the SEM. Significant differences in motility from the WT parent were determined using a paired Student *t* test. #, $P < 0.05$; ∗, $P < 0.01$. (B) Western blot analysis of flagellum production in motile deletion mutants of CFT073. The WT and the phase-locked type 1 fimbrial mutant CFT073 *fim* L-ON are included as controls. Molecular mass markers are indicated on the left in kDa. (C) Diameters of swimming motility of complemented mutants of CFT073 as well as mutants carrying the vector plasmid only. The + and − signs indicate mutants transformed with the complementation plasmid and the vector plasmid, respectively. Data represent the averages of three separate experiments. Error bars represent the SEM. Significant differences in motility between complemented mutants and mutants carrying the vector plasmid were determined using a paired Student *t* test. #, $P < 0.05$; ∗, $P < 0.01$. (D) Western blot analysis of flagellum production in complemented motile mutants of CFT073. Molecular mass markers are indicated on the left in kDa.

concentrations of this second messenger promote adherence and biofilm formation. However, our results with the *ydiV* mutant of CFT073 are not consistent with these observations. In our study, we saw the opposite in that deletion of *ydiV* in both WT CFT073 and CFT073 *fim* L-ON increased motility. Reasons for this discrepancy between our study and the previous literature could be that the EAL domain of *ydiV* is nonfunctional or that YdiV may have other, as-yet-unidentified interactions with other protein systems. Further studies are needed to determine whether *ydiV* acts as a functional PDE in CFT073 and what role it plays in regulation of motility of UPEC.

LrhA (*Lys*R *h*omolog *A*) has been shown to repress type 1 fimbrial expression and biofilm synthesis. LrhA acts upon the expression of type 1 fimbriae by binding the promoters of the recombinase genes *fimE* and *fimB* as well as the actual promoter of the type 1 fimbrial operon (*fimAICDFGH*) itself (8). FimB and FimE drive the inversion of the IE containing the promoter for the type 1 fimbrial operon, thus determining whether type 1 fimbriae are expressed by the bacterium. FimB can switch the IE in both directions, while FimE predominantly switches the IE from ON to OFF (24, 30). LrhA exhibits higher affinity for the *fimE* gene than *fimB* and therefore, by activating transcription of the *fimE* gene, represses synthesis of type 1 fimbriae (8). In our study, expression of type 1 fimbriae was locked ON, due to mutations generated in the IE, rendering it unsusceptible to inversion by the *fim* recombinases. However, it is not surprising that we isolated a mutant of this gene, given that LrhA has also been shown previously to repress motility in UPEC. LrhA represses expression of the global regulator $FlhD_2C_2$ by binding directly to the *flhDC* promoter (36). Similar to our results generated with an *lrhA* mutant constructed in the *fim* L-ON background of UPEC CFT073, an *lrhA* mutant of the laboratory *E. coli* strain K-12 exhibited greater swimming and swarming motility than the WT parent strain.

The transcriptional regulator SlyA was initially discovered in the chromosome of *Salmonella enterica* serovar Typhimurium and was presumed to encode a hemolytic and cytolytic protein (38); however, it was later shown to be a regulator of a cryptic hemolysin (HlyE/ClyA/SheA) in *E. coli* (42). In addition, SlyA has been shown to regulate the expression of a number of genes required for acid and heat resistance and also for survival of bacteria within phagocytic cells (56). The relationship between SlyA and motility in *S. enterica* and enteropathogenic *E. coli* has been examined. In *S. enterica*, production of flagellin was decreased in a *slyA* mutant, indicating a positive regulation of motility by SlyA in this organism (56). Deletion of *hosA*, a homolog of SlyA, in enteropathogenic *E. coli* also leads to a reduction in flagellin production (21). However, these results are opposite of what we observed in UPEC strain CFT073, where deletion of *slyA* led to an increase in flagellin production. As discussed above, SlyA regulates the expression of the pore-forming toxin HlyE (ClyA/SheA) (49). Expression of *hlyE* is activated by SlyA and repressed by H-NS. SlyA and H-NS both bind the promoter of *hlyE* at overlapping sites (40). This competition for binding to the promoter leads to the differential regulation. Interestingly, H-NS is a positive regulator of flagellum expression in *E. coli*. Deletion of *hns* confers a nonmotile phenotype in *E. coli* that can be partially complemented

by the introduction of plasmid-borne *hns* (5). Taken together with our results, these data suggest that SlyA may compete with H-NS for binding sites present on the *flhDC* promoter in CFT073. Also, H-NS represses phase variation of type 1 fimbriae in that mutations in *hns* increase the expression of the *fimB*, *fimE*, and *fimA* genes (20, 48). Phase variation of type 1 fimbrial expression is sensitive to temperature. Switching from phase ON to phase OFF, mediated by *fimE*, declines as the temperature increases, while switching from phase OFF to phase ON (*fimB*) increases to its optimal level at 37°C (23). Deletion of *hns* abrogated this temperature sensitivity of both *fimE* and *fimB* expression (48). It remains to be seen if SlyA itself regulates the expression of type 1 fimbriae in UPEC or whether this regulation is mediated through antagonism of the function of H-NS.

The leucine-responsive regulatory protein (Lrp) has previously been implicated in phase variation of type 1 fimbriae of *E. coli*. Lrp is a global regulator that influences the transcription of over 40 genes, commonly known as the leucine-Lrp regulon (reviewed in references 13 and 47). Lrp is involved in the regulation of a number of fimbrial operons in *E. coli*, including S fimbriae (*sfa*) (58), P fimbriae (*pap*) (9), and type 1 fimbriae (*fim*) (6). In regulation of type 1 fimbriae, Lrp binds directly to the IE containing the *fimA* promoter, affecting both *fimE*- and *fimB*-mediated switching (25). The amino acids leucine, isoleucine, valine, and alanine also contribute to this *lrp*-stimulated inversion (23). However, in our study, the promoter of *fimA* was locked in the ON confirmation, negating any effect Lrp should have had on its transcription. As mentioned above, *lrp* is involved in the regulation of P fimbriae of UPEC. Lrp is required for transcription of the *pap* operon in that the *pap* operon is locked OFF in an *lrp*-null mutant of UPEC (57). Therefore, it is interesting to speculate that *lrp* may repress expression of motility through regulation of the *pap* operon. Contributing to this idea is our observation that a *papX* mutant of CFT073 *fim* L-ON is more motile than its parental strain. PapX is a protein encoded within the *pheV*-associated *pap* operon of CFT073. However, *papX* is not required for P fimbrial biogenesis (43). Also, it has been previously shown that overexpression of *papX* in *P. mirabilis* leads to a repression of motility (37). Furthermore, a *papX* mutant of UPEC CFT073 exhibits a slight but significant increase in motility and flagellin expression compared to the WT strain. We are currently investigating the role of PapX in the transcription of type 1 fimbriae and regulation of motility in UPEC.

With the exception of *mutS*, the levels of flagellin produced in the other five mutants were increased significantly compared to that in the *fim* L-ON parent strain, indicating that the regulation imparted by the products of these genes involved production of the flagellum. However, we do not know at what step in the flagellar gene cascade this regulation occurred in each mutant and if this regulation occurred during transcription or translation. In the *mutS* mutant, the level of flagellin protein was not changed compared to that of the WT and, interestingly, complementation with *mutS* on a low-copy-number plasmid led to an actual increase in the amount of flagella produced. These results suggest that the increase in motility seen in the *mutS* mutant is not due to direct actions of *mutS* on flagellum synthesis and that *mutS* may act further downstream in the flagellar cascade, perhaps by altering the flagellar motor or chemotaxis systems. MutS is a key part of the DNA mismatch repair system (reviewed in reference 28). It has been previously shown in the laboratory strain *E. coli* K-12 that deletion of *mutS* alone does not confer many differences in gene expression in this strain (50). However, the transcript levels of both *fliC* and the gene carrying the flagellar hook subunit *flgE* were increased in the *mutS* mutant compared to those in the WT strain (50). In our study, we did not see an increase in FliC protein, but did see an increase in motility in our *fim* L-ON *mutS* mutant. It is possible that a transient increase in the expression of *fliC* occurred in our *mutS* mutant, which we would not have identified by the methods used. There is a high frequency of strains possessing a defective DNA mismatch repair system among UPEC isolates (19). These mutator strains have an advantage in vivo in late infection of the murine urinary tract and in in vitro urine culture (32). If the increase in motility were due to random mutations created by loss of the mismatch repair system in our mutant, we would have expected the repression of the motility phenotype to not be restored by complementation with *mutS*. However, this was not the case, indicating that in some way, MutS itself is involved in regulation of motility in our constitutively expressing type 1 fimbrial strain.

In summary, our data strongly suggest that UPEC possesses several methods for coordinately regulating the expression of type 1 fimbriae and flagella. We have identified six genes that appear to regulate motility in UPEC when type 1 fimbriae are constitutively expressed. Deletion of these genes also increases motility in the WT strain, where phase variation of type 1 fimbriae can occur. It remains to be determined whether these genes regulate expression of type 1 fimbriae in UPEC. Our laboratory is currently examining this possibility as well as examining the mechanism(s) these genes employ to regulate motility in UPEC.

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