

PapX, a P Fimbrial Operon-Encoded Inhibitor of Motility in Uropathogenic *Escherichia coli*[∇]

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Received 22 May 2008/Returned for modification 2 July 2008/Accepted 6 August 2008

Motility and adherence are two integral aspects of bacterial pathogenesis. Adherence, often mediated by fimbriae, permits bacteria to attach to host cells and establish infection, whereas flagellum-driven motility allows bacteria to disseminate to sites more advantageous for colonization. Both fimbriae and flagella have been proven important for virulence of uropathogenic *Escherichia coli* (UPEC). Reciprocal regulation is one mechanism by which bacteria may reconcile the contradictory actions of adherence and motility. PapX, a P fimbrial gene product of UPEC strain CFT073, is a functional homolog of MrpJ of *Proteus mirabilis*; ectopic expression of *papX* in *P. mirabilis* reduces motility. To define the connection between P fimbria expression and motility in UPEC, the role of *papX* in the regulation of motility of strain CFT073 was examined. Overexpression of *papX* decreased motility of CFT073, which correlated with both a significant reduction in flagellin protein synthesized and flagella assembled on the cell surface. Conversely, an increase in motility and flagellin production was seen in an isogenic *papX* deletion mutant of CFT073. Microarray and quantitative reverse transcription-PCR analysis indicated that repression of motility of CFT073 by PapX appears to occur at the transcriptional level; expression of many motility-associated genes, including *flhDC*, the master regulator of motility, is decreased when *papX* is overexpressed. Transcription of motility genes is increased in the *papX* mutant compared to wild type. Electrophoretic mobility gel shift analysis revealed that PapX binds to the *flhD* promoter. We conclude that synthesis of P fimbriae regulates flagellum synthesis to repress motility via PapX.

Uropathogenic *Escherichia coli* (UPEC) is the most common cause of uncomplicated urinary tract infection (UTI) in humans (16). It is estimated that 40% of all women will experience at least one UTI during their lifetime, leading to an estimated 8 million physician visits annually in the United States. Also, one in four women has a chance of experiencing a recurrent UTI within 6 months of the primary infection (7). In general, UTIs develop after periurethral colonization, after which bacteria ascend the urethra to the bladder, causing cystitis. If left untreated, bacteria can then ascend the ureters into the kidneys, causing pyelonephritis.

The abilities of UPEC to swim using flagella and to adhere by fimbriae are important in the establishment and maintenance of UTI. Flagella propel bacteria through urine and mucus layers. Fimbriae anchor bacteria to carbohydrate or proteinaceous receptors expressed on the surface of uroepithelial cells, thus immobilizing the organism. P fimbriae, encoded by the *pap* (named for pyelonephritis-associated pili) genes (reviewed in reference 19), allow UPEC to adhere to Gal α (1-4) β Gal moieties present in the P blood antigen glycosphingolipids present on the surface of kidney epithelial cells (26, 30). Epidemiological studies have shown a link between P-fimbria-producing UPEC and pyelonephritis (20); however, the role of P fimbriae in the pathogenesis of UPEC is not as clear. Human volunteer studies have implicated P fimbriae as important in the establishment of UTI (49), but animal model data are

inconclusive, and complementation has not been performed. The role of flagella in the pathogenesis of UPEC is more clear. It has recently been shown in ascending models of UTI that flagella allow UPEC to ascend from the bladder to the kidneys (23, 41). Furthermore, flagellum production contributes to the fitness of UPEC during murine urinary tract colonization (24, 48).

Reciprocal regulation of fimbriae and flagella expression allows the bacterium to maintain a balance between these two contradictory, yet necessary processes. For example, it would not be advantageous for an organism tethered to a surface to suddenly attempt swimming or swarming. Also, high expression of fimbriae by a swimming organism could sabotage motility. Therefore, it is logical that a highly fimbriated bacterium would not be highly motile and that a motile organism would not express large numbers of fimbriae. As expected, reciprocal regulation of motility and fimbria-mediated adherence has been observed in a number of pathogenic bacteria. In *Bordetella* species, for example, the two-component regulatory system BvgAS has been shown to induce the expression of adherence factors needed for colonization, while repressing the synthesis of flagella (1). Also, in a different form of regulation, mutations altering motility in *V. cholerae* directly feed back to the ToxR regulatory system, altering the production of the toxin-coregulated pili (8). Interestingly, proteins encoded within fimbrial operons have also been shown to repress motility. Increased expression of FimZ, encoded by the type 1 fimbrial operon in *Salmonella enterica* serovar Typhimurium, leads to a hyperfimbriated phenotype and a concurrent loss of motility in soft agar (5). Another example of this occurs in the uropathogen *Proteus mirabilis*, where overexpression of MrpJ, a protein encoded by the last gene of the mannose-resistant/

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[∇] Published ahead of print on 18 August 2008.

Proteus-like (MR/P) fimbrial operon, inhibits both swimming and swarming motility in *P. mirabilis* (27). Also, PapX of UPEC, a homolog of MrpJ encoded at the end of the *pheV*-associated but not the *pheU*-associated *pap* gene cluster of CFT073, caused reduced motility when overexpressed in *P. mirabilis* (27).

Recently, we identified PapX as a possible reciprocal regulator of type 1 fimbria-mediated adherence and motility in CFT073 (43). A *papX* mutant of CFT073 constitutively expressing type 1 fimbriae (CFT073 *fim* L-ON Δ *papX*) exhibited increased motility compared to the parent strain. Furthermore, a *papX* mutant of wild-type CFT073 (CFT073 Δ *papX*) also exhibited increased motility compared to the wild-type strain (43). In the work presented here, we more closely examine the role of PapX in the regulation of motility and gene expression in UPEC CFT073. We conclude from these studies that PapX regulates motility by binding the promoter of *flhD*, leading to a repression of transcription of the flagellar master regulator FlhD₂C₂.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. *E. coli* CFT073 was initially isolated from the blood and urine of a patient with acute pyelonephritis (32), and its genome has been fully sequenced and annotated (47). *E. coli* strain BL21(DE3)/pLysS was used as the host strain for the transformation of plasmids containing His-tagged PapX. For overexpression of *papX*, pDRM001 (27), which contains the *papX* gene of CFT073 under the control of an IPTG (isopropyl- β -D-thiogalactopyranoside)-inducible promoter, was used. pLX3607 (27) was used as the vector control. *E. coli* were cultured on Luria-Bertani (LB) agar or in LB broth incubated at 37°C. Antibiotics were added as needed at the following concentrations: ampicillin, 100 μ g/ml; and kanamycin, 25 μ g/ml. In vitro growth curves in LB broth were generated in triplicate using a Microbiology Reader Bioscreen C (Oy Growth Curves AB, Ltd.) in 0.2-ml volumes; the optical density at 600 nm (OD₆₀₀) was recorded every 15 min for 24 h.

Motility assays. The motility of CFT073 containing the *papX* overexpression plasmid pDRM001 or its vector control pLX3607 was evaluated using soft agar plates as described previously (24, 43). Briefly, a sample of overnight culture of each strain was used to inoculate 5 ml of sterile LB broth containing ampicillin, followed by incubation at 37°C with aeration (200 rpm) to an OD₆₀₀ of 1.0 to 1.2. Cultures were standardized to an OD₆₀₀ of 1.0 and stabbed into the middle of soft agar plates by using a sterile inoculating needle. Ampicillin was added to plates for maintenance of plasmids. Care was taken to avoid touching the bottom of the plate to avoid possible spreading from twitching motility (although type IV pili have not been demonstrated in strain CFT073). Plates were incubated for 16 h at 30°C, after which the diameter of motility was measured. The results of the motility agar assay were confirmed by phase-contrast microscopy. Wet mounts of bacterial cultures, grown to an OD₆₀₀ of 0.3 to 0.4 corresponding to optimal wild-type motility (25), were viewed at \times 400 magnification using a Zeiss Axioplan microscope and examined for flagellum-directed motility.

Detection of flagellum production. Flagella were detected by Western blotting of standardized whole-cell lysates as previously described (43). Briefly, bacteria were cultured for optimal wild-type motility (25), followed by standardization to an OD₆₀₀ of 0.35. Whole-cell lysates of these cultures were electrophoresed on a 10% denaturing sodium dodecyl sulfate-polyacrylamide gel, followed by transfer 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 by using chemiluminescence according to the manufacturer's instructions (Amersham ECL Plus; GE Healthcare Life Sciences).

Indirect immunofluorescent antibody staining. Detection of H1 flagellin on the surface of CFT073 containing either the *papX* expression plasmid pDRM001 or the vector plasmid pLX3607 was performed by a sequential staining procedure as described previously (43). Briefly, bacteria, cultured for optimal motility in LB medium, were spotted onto slides, dried, and fixed in phosphate-buffered saline (PBS) with 4% formaldehyde at 4°C. Slides were incubated with a polyclonal rabbit serum raised against purified FlhC protein of CFT073. Goat anti-rabbit

immunoglobulin G (Alexa 488 conjugate; Molecular Probes) was used as the secondary antibody. All antisera were diluted in PBS containing 4% fetal bovine serum, and incubations were performed for 1 h at room temperature. Slides were rinsed three times with PBS after each incubation. Propidium iodide (Sigma) was used to stain bacterial nucleic acids in the first PBS wash after incubation with the secondary antibody. Microscopy was performed in the Center for Live Cell Imaging at the University of Michigan Medical School using an Olympus BX60 upright microscope (Olympus; Center Valley, PA) and Olympus U-MWB2, 31004, and 61002 filters. Illumination was provided by a BH2-RFL-T3 100-W high-pressure mercury lamp burner for fluorescence microscopy. All images were obtained and analyzed with an Olympus DP70 charge-coupled device color digital video camera and DP Controller/Manager software v3.02.

Microarray analysis. CFT073, CFT073 Δ *papX*, CFT073(pDRM001), and CFT073(pLX3607) were grown in broth to an OD₆₀₀ of \sim 0.5. Triplicate cultures of each strain were treated with RNAsprotect (Qiagen) to stabilize RNA according to the manufacturer's protocol. Total RNA from bacterial samples was extracted by using the RNeasy Mini kit (Qiagen) as described by the manufacturer. RNA was treated with TURBO DNase (Ambion) and quantified by UV spectroscopy, and the integrity was verified by using a BioAnalyzer 2100 (Agilent). Subsequently, RNA was labeled, hybridized, and scanned by using Affymetrix *E. coli* Genome 2.0 GeneChips according to the manufacturer's standard prokaryotic protocol. The data were analyzed by using the robust multiarray average algorithm for normalization, background correction, and expression value calculation (17). Expression levels obtained by three independent replicates of every strain were compared by using the Affymetrix and Limma package of the Bioconductor software. The robustness of the data were further enhanced by the Bayes adjustment (44). RNA labeling, hybridization, microarray scanning, and data analysis were performed by the University of Michigan Comprehensive Cancer Center Affymetrix and Microarray Core Facility.

Comparative quantitative reverse transcription-PCR (qPCR). Total RNA from bacterial samples was extracted as described above. RNA was treated with TURBO DNase (Ambion) and subjected to agarose gel electrophoresis to verify quality and yield. cDNA was synthesized by using the SuperScript first-strand synthesis system for reverse transcription-PCR (RT-PCR) according to the manufacturer's protocol (Invitrogen). Subsequently, the RNA present in the cDNA samples was digested with RNase H (Invitrogen), and the cDNA was purified by using a QIAquick PCR purification kit (Qiagen) according to the manufacturer's specifications. PCR with primers specific to the *gapA* gene (glyceraldehyde-3-phosphate dehydrogenase) (Table 1) of CFT073 was performed on cDNA samples prepared with or without reverse transcriptase to confirm that there was no genomic DNA contamination of the RNA preparations following DNase treatment. Primers designed to amplify *flhC*, *flhD*, *flhC*, *flhA*, *yjhH*, *sat*, *lshA*, and *sfaB* (Table 1) were targeted to regions of unique sequence within each gene. For each sample, 30 ng of cDNA and 300 nM concentrations of each primer set were mixed with 12.5 μ l of 2 \times Sybr green PCR master mix (Stratagene) per well. Assays were performed in triplicate with the Stratagene Mx3000P instrument. All data were normalized to the endogenous reference gene *gapA*. Melting-curve analysis demonstrated that the accumulation of Sybr green-bound DNA was gene specific and not due to primer dimers. The data were analyzed by the 2^{- $\Delta\Delta$ CT} method (29). The choice of a baseline calibrator varied depending upon what strains were examined. The data were then transformed by log₂ to obtain a fold change difference between strains.

Construction of His-tagged PapX. A 558-bp NdeI-KpnI product containing *papX* without its stop codon was PCR amplified (Table 1) and cloned into the NdeI-KpnI sites of the His tag cloning vector pET30b(+) (Novagen). Restriction digest analysis and DNA sequencing confirmed the presence of the *papX* gene. PapX was expressed as a His-tagged fusion protein upon induction with 0.5 mM IPTG. The overexpressed PapX-His₆ was purified by using Ni-NTA resin (Qiagen) under native conditions according to the manufacturer's protocol. Imidazole was removed from the purified PapX-His₆ protein by dialysis.

Electrophoretic mobility gel shift analysis (EMSA). A digoxigenin (DIG) gel shift kit (2nd Generation; Roche) was used for protein-DNA binding assays. DNA fragments upstream of the *flhD* gene and the *gapA* gene were PCR amplified, DIG labeled, and used in the gel shift reaction with purified His-tagged PapX according to the manufacturer's instructions. The primer sequences are listed in Table 1. Protein-DNA complexes were resolved on a 5% native polyacrylamide gel in 0.5 \times TBE (Tris-borate-EDTA buffer), followed by transfer to nylon membrane. DIG-labeled DNA was detected by chemiluminescence according to the manufacturer's protocol (Roche). The *gapA* promoter was used in these assays as a negative control for PapX binding.

CBA mouse model of ascending UTI. The CBA/J mouse model of ascending UTI was used as previously described (10, 18). Briefly, 6- to 8-week-old female CBA/J mice were transurethrally inoculated with 10⁹ CFU of a 1:1 mixture of

TABLE 1. Primers used in this study

Analysis and primer	Sequence (5' to 3')
qPCR	
<i>fliC</i> F	ACAGCCTCTCGCTGATCACTCAAA
<i>fliC</i> R	GCGCTGTTAATACGCAAGCCAGAA
<i>flhD</i> F	TCCGCTATGTTTCGTCTCGGCATA
<i>flhD</i> R	ACCAGTTGATGGTTTCTGCCAGC
<i>flhC</i> F	AAACTGGCTTGTAATGGCGTCG
<i>flhC</i> R	TCAACAAACCGCACCAATGTCCAG
<i>fliA</i> F	AACGCTATGACGCCCTACAAGGAA
<i>fliA</i> R	AGTTCCTGCTCCAGTTGCCCTATT
<i>yhjH</i> F	TATTAACGGTGGTCACGCATCCCT
<i>yhjH</i> R	TGCTCTTTCACAACCTCCACGA
<i>sat</i> F	ATGGATTACAGGCTGAGTGGTGGAA
<i>sat</i> R	TTCAGTTTCAGGCTGTCCCAGAGT
<i>lrhA</i> F	AATGCTGGAATCCCAGGAAGTGGAA
<i>lrhA</i> R	ACCATATCGCGAAACGGACTTGGA
<i>sfaB/focB</i> F	TCCGGCTCCATGTCTGAAGAACA
<i>sfaB/focB</i> R	TTACGGGAATGACCACTGACCAGA
<i>gapA</i> F	CGTTAAAGGCGCTAACTTCG
<i>gapA</i> R	ACGGTGGTCATCAGACCTTC
His-tagged PapX	
PapX-His F	CATATGCGCGCTTGACACAGACA
PapX-His R	GGTACCTGAGCTGACATCATCAAGAT
EMSA	
<i>flhD</i> F	GTTGTGCGGTAAGTGTITG
<i>flhD</i> R	CCAGAATAACCAACT
<i>gapA</i> F	ACGTGACTGATTCTA
<i>gapA</i> R	CAGCTATTTGTTAGTG
<i>fliA</i> F	AACGTGAGACTGACGGCAAC
<i>fliA</i> R	GATAAACAGCCCTGCGTTA
<i>fliC</i> F	TCGACACGTAAAACGAATACC
<i>fliC</i> R	GATTCGTTATCCTATATTGC

wild-type CFT073 and CFT073 $\Delta papX$. At 48 h postinfection, mice were sacrificed, and the bladders and kidneys were aseptically removed, homogenized, and plated onto LB agar plates with or without kanamycin to determine the output CFU per gram of tissue.

RESULTS

PapX is transcribed with the *pap* operon. Two copies of the *pap* gene cluster (*papIBAHCDJKEFG*) are present in UPEC CFT073 (33). Interestingly, CFT073 possesses only one copy of *papX*, which is downstream of the *pheV*-associated *pap* gene cluster (9, 21, 39). The gene encoding *papX* is 263 bp downstream of the digalactoside-binding adhesin gene, *papG* (Fig. 1A). Due to the previous observation that the deletion of *papX* has no effect on P-fimbria expression in *E. coli* (31), we first examined whether *papX* is part of the *pap* operon. To determine whether PapX is indeed cotranscribed with the P-fimbrial operon, RT-PCR was performed using primers within the *papG* and *papX* genes (Fig. 1A). Reactions with primers within the *papA* gene were included to control for the integrity of the transcript (Fig. 1B). RNA was isolated from mid-log-phase broth cultures of wild-type CFT073 and CFT073 $\Delta papX$ and reverse transcribed into cDNA. The 482-bp *papG-papX* PCR product was seen in CFT073 (Fig. 1B), indicating that *papX* is transcribed with the *pap* operon and would be expressed under conditions favorable for P-fimbrial expression.

PapX represses motility and flagellum expression in UPEC CFT073. Previous work in our laboratory identified MrpJ as a

fimbria-encoded inhibitor of motility in the uropathogen *P. mirabilis* (27). In that same study, PapX was hypothesized to be a functional homolog of MrpJ and, indeed, ectopic expression of *papX* in *P. mirabilis* led to a reduction of motility (27). Recently, we identified PapX as a possible reciprocal regulator of type 1 fimbria-mediated adherence and motility in CFT073 (43). It was observed that a *papX* mutant of CFT073 constitutively expressing type 1 fimbriae (CFT073 *fim* L-ON $\Delta papX$) exhibited increased motility compared to the parent strain. Furthermore, a *papX* mutant of wild-type CFT073 (CFT073 $\Delta papX$) also exhibited increased motility compared to the wild-type strain (43). Here, we examined more closely the role of PapX in its native background of UPEC strain CFT073. In the *P. mirabilis* study (27), the expression of *papX* was elevated using pDRM001, where the *papX* gene is under the control of an IPTG-inducible promoter. We noticed early in our studies that IPTG induction of *papX* in CFT073 transformed with pDRM001 decreased the growth rate of the bacterium (Fig. 2A). This decrease in growth was not observed when bacterial cultures were not induced with IPTG (Fig. 2A). Microarray analysis indicated that, in the absence of IPTG, expression of *papX* in CFT073 containing pDRM001 is increased 11-fold compared to CFT073(pLX3607) (see Table 4). Therefore, to make sure our results were due to the actions of PapX and not because of the growth defect, all further studies were conducted without IPTG. As expected, elevated expression of *papX* in CFT073(pDRM001) led to a marked decrease in motility in soft agar (Fig. 2B and C). This repression of motility was associated with a decrease in total (Fig. 2F) and surface-expressed flagellin protein (Fig. 2D and E), indicating that overexpression of *papX* inhibits motility in CFT073 by decreasing the number of flagella synthesized. Interestingly, the impaired motility seen when *papX* is overexpressed is apparently

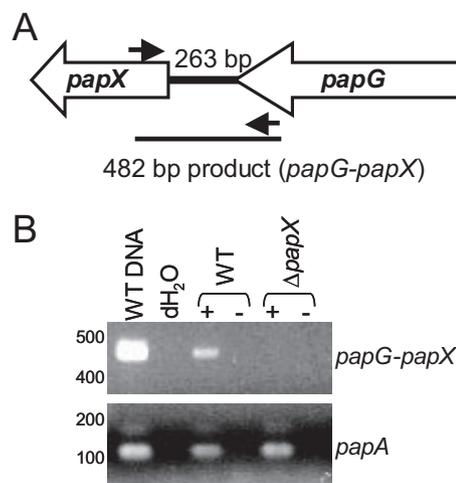


FIG. 1. PapX is transcribed as part of the *pap* operon. (A) Schematic of the *papG* and *papX* genes of CFT073. *papX* is 263 bp downstream of the *papG* allele. Arrows indicate primer sequences designed to amplify a 482-bp product (*papG-papX*). (B) RT-PCR using *papG-papX* primers (top), as well as control primers within the *papA* gene (bottom), to determine whether *papX* is transcribed with *papG*. CFT073 genomic DNA (wild-type DNA) and water are included as positive and negative controls. A "+" or "-" indicates the presence or absence of reverse transcriptase in the cDNA synthesis reaction.

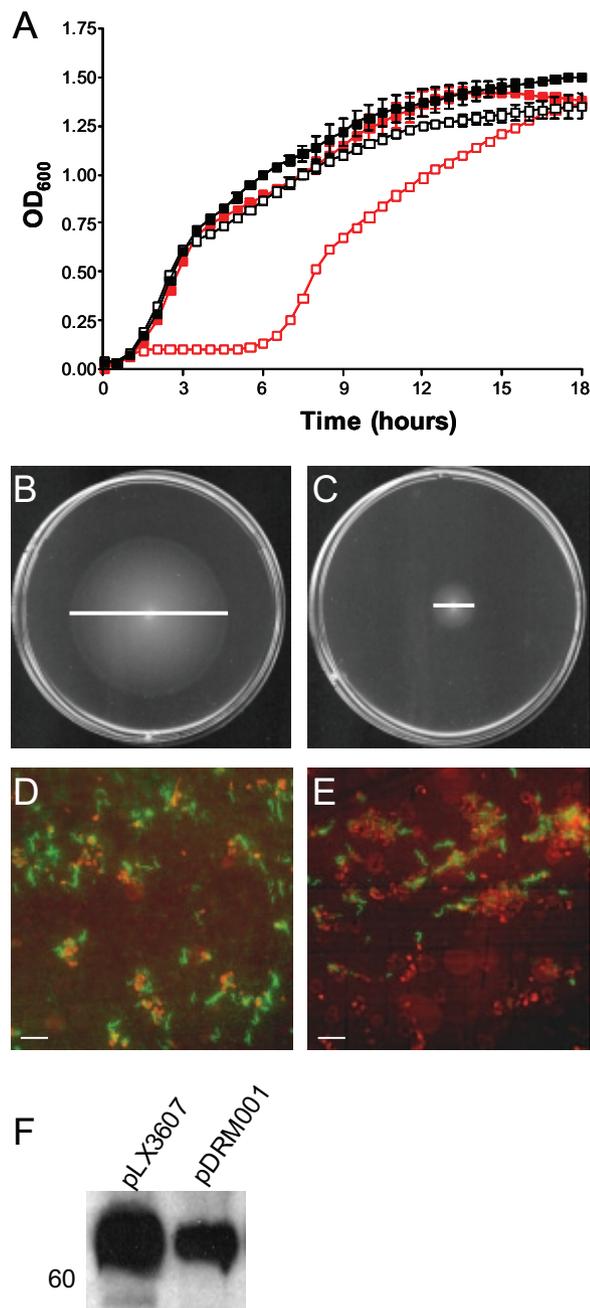


FIG. 2. Overexpression of *papX* in UPEC CFT073 represses motility and flagellar production. (A) In vitro growth curves of CFT073(pLX3607) (vector control; ■) and CFT073(pDRM001) (encodes *papX*; □) with (red) or without (black) addition of 0.5 mM IPTG. (B and C) The motilities of CFT073(pLX3607) (B) and CFT073(pDRM001) (C) in soft agar without IPTG are shown. White bars indicate the diameter of motility of each strain. (D and E) Indirect immunofluorescence analysis of flagellum expression. Suspensions of CFT073(pLX3607) (D) and CFT073(pDRM001) (E) 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, 10 μ m. (F) Western blot analysis of flagellin production. Whole-cell lysates from standardized mid-log-phase cultures were prepared, electrophoresed onto sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels, and subjected to Western blot analysis with antiserum specific to H1 flagella. The molecular mass marker is indicated on the left in kilodaltons.

TABLE 2. Flagellar and motility genes upregulated in CFT073 Δ *papX*

Gene	Annotation	Fold change in CFT073 Δ <i>papX</i> ^a
<i>flhD</i>	Flagellar transcriptional activator	1.74
<i>flhC</i>	Flagellar transcriptional activator	1.96
<i>fliL</i>	Flagellar biosynthesis protein	1.8
<i>fliE</i>	Flagellar hook-basal body complex protein	2.16
<i>flgB</i>	Flagellar basal body rod protein	1.78
<i>flgC</i>	Flagellar basal body rod protein	1.49
<i>flgE</i>	Flagellar hook protein	1.64
<i>flgF</i>	Flagellar basal body rod protein	1.79
<i>flgG</i>	Flagellar basal body rod protein	1.64
<i>flgH</i>	Flagellar L-ring protein precursor	1.79
<i>flgI</i>	Flagellar P-ring protein precursor	2.04
<i>fliA</i>	RNA polymerase sigma factor (σ^{28})	1.52
<i>flgA</i>	Flagellar basal body P-ring formation protein	1.59
<i>aer</i>	Aerotaxis receptor	2.55
<i>yhjH</i>	EAL domain-containing protein involved in flagellar function	1.97

^a Compared to expression levels in wild-type CFT073. $P < 0.005$.

not absolute (Fig. 2E) in that a small percentage of individual bacteria contain normal intact flagella. When these bacterial cultures were viewed by phase-contrast microscopy, bacteria that were motile in the population swam normally (data not shown).

Transcriptional regulation by PapX in UPEC. We identified PapX as a regulator of motility in UPEC CFT073. To examine the molecular mechanism of action of PapX, as well as to see what other systems are affected by PapX, microarray analysis of wild-type CFT073 and CFT073 Δ *papX*, as well as the PapX overexpression strain CFT073(pDRM001) along with its vector control CFT073(pLX3607), was performed. As expected, the majority of the genes regulated by *papX* expression were associated with flagellum-mediated motility and chemotaxis. More than 50 genes are known to be involved in flagellum-directed motility and chemotaxis in UPEC CFT073. Motility genes from all three classes of the flagellar regulatory cascade, as well as the aerotaxis receptor and a protein involved in flagellar function, were upregulated in the *papX* mutant compared to the wild-type strain (Table 2), indicating that PapX acts at the level of transcription to lower the motility of a population. In accordance with this observation, a large number of known motility and chemotaxis genes were significantly downregulated when *papX* was overexpressed in CFT073 (pDRM001) (Table 3). These results were verified by qPCR, where transcription of a representative of each class of flagellar genes was analyzed by the $2^{-\Delta\Delta CT}$ method using *gapA* as the normalizing internal standard. Transcription of the class I *flhD* and *flhC* genes was upregulated in the *papX* mutant 2.5-fold and downregulated in the *papX* overexpression strain by similar levels (Fig. 3). As expected based on this result, transcription of the class II flagellar gene *fliA* and the class III gene *fliC* was also markedly affected by the absence or elevated expression of *papX*. Expression of *fliA* was upregulated 2-fold in the *papX* mutant and repressed 7-fold in CFT073(pDRM001), while *fliC* expression was increased >4-fold in the absence of *papX* and decreased ~15-fold when *papX* was overexpressed in the

TABLE 3. Flagellar and motility genes downregulated in CFT073 overexpressing *papX*

Gene	Annotation	Fold change in CFT073 (pDRM001) ^a
<i>flhD</i>	Flagellar transcriptional activator	-1.82
<i>flhC</i>	Flagellar transcriptional activator	-1.98
<i>fliH</i>	Flagellar biosynthesis protein	-2.31
<i>fliJ</i>	Flagellar biosynthesis protein	-1.79
<i>fliK</i>	Flagellar hook length control protein	-2.10
<i>fliL</i>	Flagellar biosynthesis protein	-2.53
<i>fliM</i>	Flagellar motor switch protein	-2.14
<i>fliE</i>	Flagellar hook-basal body complex protein	-1.88
<i>flhB</i>	Flagellar biosynthesis protein	-1.57
<i>flgB</i>	Flagellar basal body rod protein	-2.39
<i>flgC</i>	Flagellar basal body rod protein	-2.11
<i>flgD</i>	Flagellar basal body rod modification protein	-1.87
<i>flgE</i>	Flagellar hook protein	-2.20
<i>flgF</i>	Flagellar basal body rod protein	-1.85
<i>flgG</i>	Flagellar basal body rod protein	-2.01
<i>flgH</i>	Flagellar L-ring protein precursor	-1.82
<i>flgI</i>	Peptidoglycan hydrolase	-1.80
<i>fliA</i>	RNA polymerase sigma factor (σ^{28})	-2.27
<i>fliZ</i>	Predicted regulator of FliA activity	-2.00
<i>flgA</i>	Flagellar basal body P-ring formation protein	-1.91
<i>flgM</i>	Negative regulator of flagellar synthesis (anti- σ^{28})	-2.08
<i>flgK</i>	Flagellar hook-associated protein 1	-1.91
<i>flgL</i>	Flagellar hook-associated protein 3	-2.04
<i>fliD</i>	Flagellar hook-associated protein 2	-3.89
<i>fliS</i>	Flagellar protein FliS	-2.41
<i>fliC</i>	Flagellin	-2.20
<i>motA</i>	Chemotaxis protein	-2.50
<i>motB</i>	Chemotaxis protein	-2.87
<i>cheA</i>	Chemotaxis protein	-3.43
<i>cheW</i>	Chemotaxis protein	-3.01
<i>tar</i>	Methyl-accepting chemotaxis protein II	-2.57
<i>tsr</i>	Methyl-accepting chemotaxis protein I	-2.89
<i>cheR</i>	Chemotaxis protein methyltransferase	-1.87
<i>cheB</i>	Protein-glutamate methylesterase	-1.92
<i>cheY</i>	Chemotaxis protein	-3.05
<i>cheZ</i>	Chemotaxis protein	-2.60
<i>aer</i>	Aerotaxis receptor	-2.46
<i>yhjH</i>	EAL domain containing protein involved in flagellar function	-3.46

^a pDRM001 encodes *papX*. Values are given compared to the expression levels in the vector control CFT073(pLX3607). *P* < 0.005.

wild-type strain (Fig. 3). Also, transcription of *yhjH*, an FlhD₂C₂-dependent gene involved in flagellar function (22), was upregulated threefold in the *papX* mutant (Fig. 3A). Taken together, our microarray and qPCR results suggest that PapX most likely represses the motility of UPEC by inhibiting transcription of the master regulator FlhD₂C₂. The transcription of more motility genes was significantly decreased when *papX* was overexpressed than the number of motility genes increased in CFT073 Δ *papX*. This is likely due to greater change in expression of *papX* in CFT073(pDRM001) (11-fold increase) versus the 2-fold decrease in *papX* in CFT073 Δ *papX* (Table 4).

PapX appears to regulate more than just motility in UPEC CFT073. In our microarray analysis, significant changes were seen in a number of nonmotility associated genes (Table 4). In CFT073 Δ *papX*, transcription of six hypothetical genes—*ybcL*, *ynda*, *ydjR*, c3188, c3189, and c3190—was upregulated compared to the wild-type strain. Another hypothetical gene, c3568, appears to be positively regulated by PapX, in that its

transcription was decreased in CFT073 Δ *papX*. Also, the *sfaB/focB* gene, which encodes a regulatory protein needed for F1C fimbriae production in *E. coli* (28), was upregulated in the *papX* mutant (Table 4 and Fig. 3A), suggesting PapX may regulate the expression of other *E. coli* fimbriae. Two of the genes identified in the *papX* mutant microarray were downregulated when *papX* expression was elevated: *ynda* and *ydjR* (Table 4). In addition, *ibpA*, which encodes a protein involved in the stress response to heat shock (2), was downregulated when *papX* was overexpressed in CFT073, compared to CFT073 containing the vector alone (Table 4). Transcription of *sat*, which encodes a secreted autotransporter toxin of CFT073, was upregulated ~2-fold when *papX* was overexpressed in CFT073 (Table 4 and Fig. 3B), suggesting that PapX may positively regulate expression of this virulence factor.

PapX directly binds the *flhD* promoter. Our microarray and qPCR results indicated that PapX acts to repress motility and flagellum synthesis functions at the transcriptional level directly on or upstream of the *flhDC* regulon. To examine whether PapX binds to the regulatory regions of the *flhDC* operon, gel mobility shift assays were performed with a 500-bp

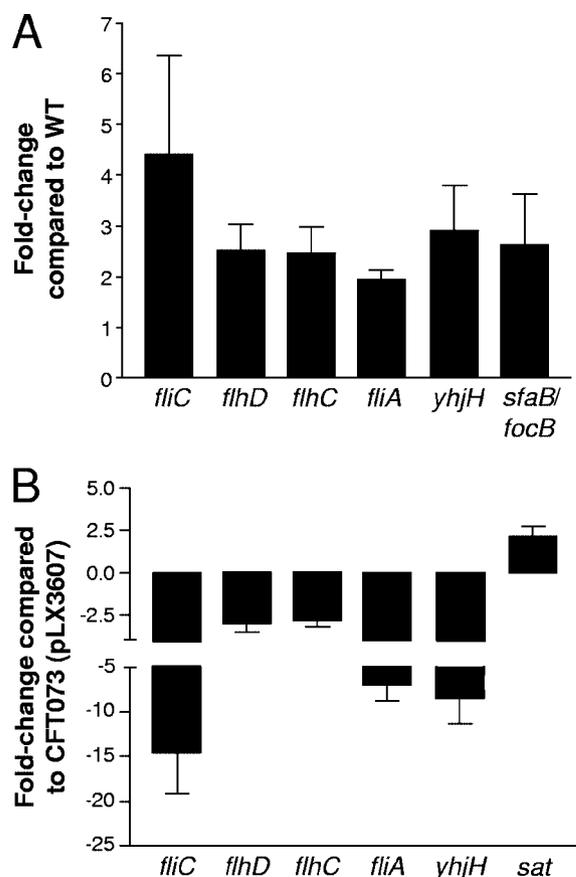


FIG. 3. qPCR analysis of expression of selected UPEC genes identified during microarray analysis. Black bars represent the average fold changes (*n* = 3) in gene expression. (A) qPCR results for CFT073 Δ *papX*. Changes were calculated using wild-type CFT073 as the relative measure of comparison. (B) qPCR results for CFT073(pDRM001) encoding *papX*. Changes were calculated using CFT073 carrying the vector plasmid pLX3607 alone as the relative measure of comparison. Error bars represent the standard errors of the means.

TABLE 4. Non-motility-associated genes differentially expressed in CFT073 $\Delta papX$ and CFT073 overexpressing $papX$

Gene	Annotation	Fold change ^a in:	
		CFT073 $\Delta papX^b$	CFT073 (pDRM001) ^c
<i>papX</i>	PapX protein	-2.07	11.00
<i>ibpA</i>	Heat shock protein A		-2.62
<i>sat</i>	Secreted autotransporter toxin		2.01
<i>sfaB/focB</i>	Putative F1C and S fimbrial switch regulatory protein	2.73	
<i>ybcL</i>	Putative kinase inhibitor	1.50	
<i>ymdA</i>	Hypothetical protein	3.32	-1.74
<i>ydjR</i>	Hypothetical protein	1.55	-1.75
c3568	Hypothetical protein	-1.56	
c3190	Hypothetical protein	2.66	
c3189	Hypothetical protein	1.75	
c3188	Hypothetical protein	1.74	

^a $P < 0.005$.

^b Values are compared to the expression levels in wild-type CFT073.

^c pDRM001 encodes *papX*. Values are compared to expression levels in the vector control CFT073(pLX3607).

fragment that contains the promoter region of *flhD*. PapX was overexpressed and purified as a His₆-tagged fusion protein. Purified PapX-His₆ bound the promoter region of the *flhD* operon in a dose-dependent and sequence-specific manner (Fig. 4). A 300-bp fragment containing the promoter of *gapA*, which encodes glyceraldehyde-3-phosphate dehydrogenase, was included as a negative control for this experiment. PapX also did not bind the promoter of *fliA* or *fliC* (data not shown), suggesting that the effect of PapX on expression of the class II and III flagellar genes is due to its repression of transcription of the master regulator FlhD₂C₂ and not due to specific actions of PapX on the class II and III promoters.

Role of PapX in virulence in the mouse model of ascending UTI. It has previously been shown that flagellar mutants of UPEC are outcompeted by the wild-type strain during experimental cochallenge of mice (24), indicating that flagella contribute to colonization of the urinary tract. Also, during ascension of UPEC to the upper urinary tract, flagella are expressed (23). Our *papX* mutant, CFT073 $\Delta papX$, exhibits increased motility compared to wild-type CFT073 (43). Therefore, we hypothesized that the increased motility of CFT073 $\Delta papX$ would allow it to better colonize the kidneys during UTIs of

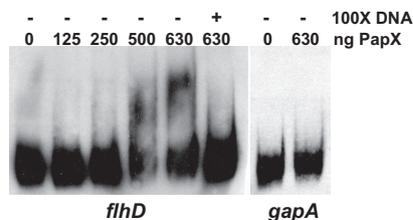


FIG. 4. Binding of PapX to the promoter of *flhD* as revealed by EMSA. All lanes contained 0.2 ng of either a DIG-labeled 500-bp DNA fragment corresponding to the *flhD* promoter (left panel) or a DIG-labeled 300-bp DNA fragment containing the *gapA* promoter (right panel). Increasing amounts of purified PapX protein were mixed with the DIG-labeled probes as indicated. A 100-fold excess of unlabeled *flhD* promoter DNA was included (left panel) as a control to illustrate the specificity of the PapX-*flhD* binding reaction.

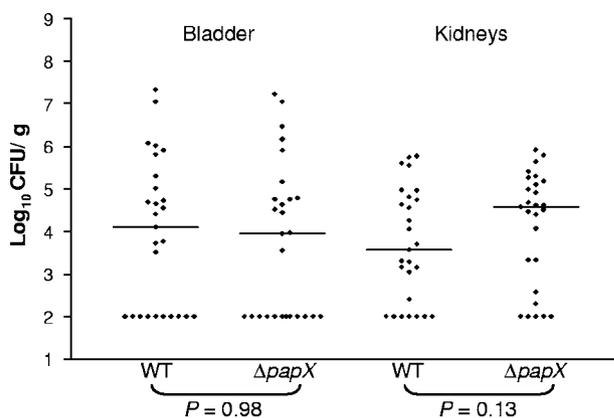


FIG. 5. Cochallenges of mice with UPEC CFT073 and CFT073 $\Delta papX$. In three separate experiments, a total of 30 mice were transurethraly inoculated with a 1:1 mixture of the wild type and the mutant. At 48 h postinfection, mice were sacrificed and bacterial counts in the bladder and kidneys were determined. Each data point is represents the log₁₀ CFU per gram of tissue collected per mouse. Bars indicate the median log₁₀ CFU per gram of tissue collected for each group. The limit of detection of this assay is 10² CFU/g tissue. Statistical differences between the median number of log₁₀ CFU per ml or per gram tissue for UPEC CFT073 and its $\Delta papX$ mutant were determined by using the Wilcoxon rank sum test. P values are indicated below the graph.

mice. We examined this by competitive coculture experiments in which mice were infected transurethraly with a 1:1 mixture of wild-type and the *papX* mutant. After 48 h, the CFU/gram of bladder and kidney tissue were determined (Fig. 5). No statistical significance was seen in the numbers of wild-type CFT073 and CFT073 $\Delta papX$ recovered in the bladder, indicating that the increased motility of CFT073 $\Delta papX$ does not enhance colonization of the bladder after 48 h of infection. Although this was also true for the kidney, there was a clear trend of increased colonization of the kidney of the $\Delta papX$ mutant (Fig. 5). This increased recovery of the *papX* mutant was not seen when the wild type and mutant were passaged serially in vitro (data not shown). Taken together, these data suggest that the loss of PapX (or its effect on the expression of other genes) may increase the fitness of UPEC during kidney infection.

DISCUSSION

In the present study, we define the mechanism by which PapX regulates motility in UPEC. *papX* is expressed as the 12th and last gene of the *pheV*-associated *pap* gene cluster. When P fimbriae are expressed, PapX is synthesized and binds to the *flhDC* promoter, the master regulator of flagellar synthesis, chemotaxis, and motility. When *flhDC* transcription is repressed, class II and III motility genes are in turn downregulated and flagella synthesis is markedly repressed. Only a small percentage of these bacteria express flagella. When phase variation results in no transcription of the *pap* operon, no *papX* is transcribed and FlhD₂C₂ drives normal levels of gene expression, resulting in full motility. Therefore, phase variation of P fimbriae, and thus *papX* expression, mediates reciprocal control of adherence and motility in UPEC CFT073.

Previous work in our laboratory implicated PapX as a possible repressor of motility in UPEC (27, 43). Indeed, PapX was

shown to repress the motility of the uropathogen *P. mirabilis* (27), and a *papX* mutant of UPEC strain CFT073 exhibited increased motility compared to the wild-type strain (43). PapX is a P-fimbrial gene product, in that it is transcribed with the *papG* allele of the *pheV*-associated *pap* gene cluster. Our data also build upon the earlier observation that PapX represses motility in UPEC (43). Microarray and qPCR revealed that PapX acts at the transcriptional level to repress motility, and gel shift analysis indicated that PapX binds to the promoter of *flhD*.

Two copies of the *pap* gene cluster are present in CFT073, *pap* and *pap_2* (33), and each are contained on pathogenicity islands, PAI-CFT073-*pheV* and PAI-CFT073-*pheU*, respectively (47). Interestingly, UPEC clinical isolates containing multiple P-fimbrial operons are more likely to be associated with pyelonephritis (14, 38). The *papX* gene is only associated with the first *pap* gene cluster in UPEC CFT073 (9, 21, 39). We show here that *papX* is transcribed as part of the *pap* operon, indicating that PapX would be synthesized in conditions favorable for P-fimbria expression. This is interesting in light of the observation that phase variation of the two *pap* operons differs depending upon environmental conditions (13). During growth in human urine, for example, the first *pap* operon is turned on at a higher rate than that of the *pap_2* operon, while the *pap_2* operon is turned off at a higher rate than the *pap* operon in that same growth medium (13). Expression of the flagellin gene is highly downregulated during growth in human urine (3). These data indicate that it is possible this occurs as a result of the preferential expression of the *pap* operon and not the *pap_2* operon during growth in this medium; that is, elevated expression of *papX* could be responsible for the decreased motility phenotype.

Another adhesin implicated in UPEC pathogenesis in kidney tissue is the F1C fimbria, which is expressed by 14 to 30% of all uropathogenic strains of *E. coli* (37, 42). F1C fimbria, encoded by the *foc* gene cluster, is highly homologous to S fimbria (46). The *focC* and *focD* genes encode a chaperone and usher protein, *focA* encodes the major fimbrial subunit, and *focF* and *focG* encode minor fimbrial subunits; *focH* encodes the adhesin (40). In UPEC CFT073, a putative *papX* homolog is encoded by *focX*, a 552-bp gene that is located 1,200 bp downstream of the stop codon of *focG* (47). It remains to be seen whether *focX* is a product of the *foc* operon. Interestingly, however, the length of the *focX* gene is identical to that of *papX*, and only 11 nucleotides differ between the two genes, which translate into six amino acid differences between the proteins. Preliminary data indicate that FocX is indeed a homolog of PapX in that overexpression of *focX* in CFT073 represses motility and flagellin production (A. N. Simms and H. L. Mobley, unpublished data). Studies to characterize FocX and its role in motility and fimbrial regulation are under way in our laboratory.

PapX was initially identified as a functional homolog of the *P. mirabilis* repressor of motility MrpJ (27). Recently, the genome of *P. mirabilis* strain HI4320 has been sequenced and annotated (36). Genes encoding 17 distinct fimbriae were identified and, interestingly, 10 of these fimbrial operons are associated with an MrpJ homolog (35). In contrast, sequencing of the genome of UPEC CFT073 revealed the presence of 12 distinct fimbrial gene clusters (47). Currently, only PapX and

FocX have been identified as possible regulators of motility associated with fimbrial gene clusters in UPEC CFT073. Indeed, it would be interesting to assess the motility phenotype of a *papX focX* double mutant.

Nevertheless, cross talk between surface components is a common theme in UPEC. Reciprocal regulation is important in regulating fimbriae expression and motility, as well as regulation between different fimbrial types. UPEC strains express mainly one fimbrial type at a time, despite containing multiple fimbrial gene clusters (34). Genes encoded within the P (*papB*) (50), S (*sfab*) (15), and F1C (*focB*) (28) fimbria gene clusters encode products that repress transcription and subsequent expression of type 1 fimbriae. Preliminary data in our laboratory also indicate that PapX may play a role in P-fimbria-mediated inhibition of type 1 fimbria expression in that overexpression of *papX* in CFT073 leads to a fourfold decrease in transcription of the major fimbrial subunit gene, *fimA* (unpublished data). Inverse regulation has been demonstrated between type 1 and P fimbriae in UPEC CFT073 since constitutive expression of type 1 fimbriae in UPEC CFT073 leads to a marked decrease in transcription of both *pap* operons, indicating that type 1 fimbriae can also repress the expression of P fimbriae (45).

Interestingly, when CFT073 was engineered to no longer express type 1 or P fimbriae, F1C fimbriae, encoded by the *foc* operon, were expressed by the bacteria instead (45). In the present study, deletion of PapX led to an increase in transcription of *sfab/focB* in CFT073 (Table 3 and Fig. 3A). FocB stimulates expression of F1C fimbriae in that no expression of the major F1C fimbrial subunit FocA was seen in a *focB* mutant (28). Therefore, it is possible that, in addition to repressing motility, PapX may also repress expression of F1C fimbriae in CFT073. Another adhesin potentially expressed by UPEC CFT073 are curli, amyloid fibers expressed on the surface of the bacteria that are important in adhesion, invasion, and biofilm formation (reviewed in reference 4). Two divergently transcribed operons encode the genes necessary for curli assembly and formation (11). One contains the *csgDEFG* genes, which are essential for curli assembly (11). The *csgBA* operon encodes the major structural subunit CsgA and the nucleator protein CsgB (11, 12). The *csgC* gene is also part of the *csgBA* operon; however, no transcript has been detected, and this gene appears not to be involved in curli synthesis (6, 11). Interestingly, in UPEC CFT073, directly downstream of the *csgC* gene is the *ymdA* gene. In our study, the *ymdA* gene was shown to be negatively regulated by PapX, as transcription of *ymdA* was increased in the *papX* mutant and conversely decreased when *papX* was overexpressed (Table 3). The proximity of this gene to the curli biosynthesis operon suggests that *ymdA* may be involved somehow in the expression of curli through an unknown mechanism and, if so, PapX may then regulate curli expression.

In summary, PapX is a P-fimbrial-encoded inhibitor of motility in UPEC CFT073. We propose that the mechanism by which this occurs is through direct binding of PapX to the promoter of the master flagellar regulator, FlhD₂C₂. In the urinary tract, motility appears to be required primarily to ascend the ureters to the kidney (23). Once in the kidney, UPEC characteristically binds to kidney epithelium via P fimbriae. When P fimbriae are expressed, PapX represses motility, thus switching to the adherent state.

ACKNOWLEDGMENTS

This study was supported in part by Public Health Service grant AI059722 from the National Institutes of Health.

We thank Sara Smith for her assistance in the mouse experiments and James W. MacDonald for statistical analysis of our Affymetrix GeneChip data.

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Editor: A. J. Bäumlér