

Coordinate Expression of Fimbriae in Uropathogenic *Escherichia coli*

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Uropathogenic *Escherichia coli* is the most common etiological agent of urinary tract infections. Bacteria can often express multiple adhesins during infection in order to favor attachment to specific niches within the urinary tract. We have recently demonstrated that type 1 fimbria, a phase-variable virulence factor involved in adherence, was the most highly expressed adhesin during urinary tract infection. Here, we examine whether the expression of type 1 fimbriae can affect the expression of other adhesins. Type 1 fimbrial phase-locked mutants of *E. coli* strain CFT073, which harbors genes for numerous adhesins, were employed in this study. CFT073-specific DNA microarray analysis of these strains demonstrates that the expression of type 1 fimbriae coordinately affects the expression of P fimbriae in an inverse manner. This represents evidence for direct communication between genes relating to pathogenesis, perhaps to aid the sequential occupation of different urinary tract tissues. While the role of type 1 fimbriae during infection has been clear, the role of P fimbriae must be further defined to assert the relevance of coordinated regulation in vivo. Therefore, we examined the ability of P fimbrial isogenic mutants, constructed in a type 1 fimbrial-negative background, to compete in the murine urinary tract over a period of 168 h. No differences in the colonization of these mutants were observed. However, comparison of these results with previous studies suggests that inversely coordinated expression of adhesin gene clusters does occur in vivo. Interestingly, the mutant that was incapable of expressing either type 1 or P fimbriae compensated by synthesizing F1C fimbriae.

Uropathogenic *Escherichia coli* (UPEC) strains cause the majority of all urinary tract infections (UTIs). Forty to 50% of women experience at least one UTI during their lifetime, leading to an estimated 8 million physician visits annually in the United States (39, 55). Recent efforts to understand the mechanisms of virulence in this important pathogen include the sequencing of UPEC (52), complete transcriptome analysis (45), signature-tagged mutagenesis (4), and differential fluorescence induction (33). These studies collectively implicate adhesins, iron acquisition systems, capsules, lipopolysaccharides, and toxins in UPEC pathogenesis.

Adherence to host tissues is often the first step towards colonization; thus, adhesins are essential for pathogenesis. The recent sequencing of UPEC strain CFT073, along with previous virulence studies, has predicted or demonstrated as many as 12 fimbrial gene clusters in this strain (5, 17, 52). Many fimbrial and afimbrial adhesins are phase variable (28, 34), including the most ubiquitous type 1 fimbriae encoded by the *fim* gene cluster. The expression of type 1 fimbriae is controlled by a promoter situated on an invertible element of DNA, also referred to as the *fim* switch (1). Bacteria are phase on, and type 1 fimbriae are expressed when the promoter faces the direction of *fimA*, which encodes the main structural subunit. When the promoter faces the opposite orientation, no type 1 fimbrial transcription occurs and bacteria are phase off. The

inversion of the *fim* switch is mediated by the recombinases FimE, which primarily promotes on to off switching, and FimB, which can switch in either direction (10, 21). We demonstrated that type 1 fimbriae were highly expressed during murine UTI (45), and molecular Koch's postulates have previously been satisfied (7). Type 1 fimbriae may be most important in the initial establishment of infections (27, 38), and we have shown that type 1 fimbrial expression is especially critical in the bladder at 24 h postinfection (13).

P fimbriae, encoded by the *pap* operon, are also subject to phase variation, although by a different mechanism. Two GATC sites in the promoter region are alternately methylated by Dam methyltransferase in phase-on versus phase-off bacteria (6). The methylation state of these GATC sites is influenced by Lrp, PapI, and PapB. *E. coli* strain CFT073 possesses two functional copies of the *pap* operon (26). The presence and expression of *pap* genes is epidemiologically linked with pyelonephritis-causing *E. coli* strains (18), but the role of P fimbriae in virulence has not been well defined. Earlier studies in our laboratory found no difference in murine urinary tract colonization or histology when deletions were made in both copies of the *pap* operon in *E. coli* CFT073 (denoted as strain UPEC76) (26) despite the presence of P fimbrial receptors in mice (19, 29). However, it has been argued that this adhesin represents a virulence factor in other UTI studies (35, 53).

It was previously observed that *E. coli* expresses mainly one fimbrial type at a time (28), so it is not surprising that examples of coordinated regulation between adhesins have been uncovered. Regulators SfaB (regulator of S fimbriae) and PapB have been shown to inhibit type 1 fimbrial expression via inhibiting

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TABLE 1. Bacterial strains used in this study

<i>E. coli</i> strain	Description	Reference
CFT073	Wild-type pyelonephritis isolate	25
UPEC76	CFT073 nalidixic acid-resistant (Nal ^r) derivative; both copies of P fimbrial operon disrupted	26
CFT073-ON	CFT073 with <i>fim</i> invertible element phase locked on; constitutive type 1 fimbrial expression	13
CFT073-OFF	CFT073 with <i>fim</i> invertible element phase locked off; no type 1 fimbrial expression	13
CFT073 <i>fim pap</i>	UPEC76 with a deletion of the type 1 fimbrial operon <i>fimA-H</i>	This study

FimB or both inhibiting FimB and increasing FimE, respectively (16, 54). These studies were carried out in vitro, and the relevance of this fimbrial “cross talk” during an infection is not known. Microarray studies comparing an *E. coli* K-12 laboratory strain with a deletion mutant of the entire *fim* operon demonstrated that antigen 43 (Ag43, encoded by *flu*) was the only adhesin that increased in expression (40, 41). However, this study has limited implications for pathogenesis since many genes specific for UPEC are not present in the K-12 chromosome (52).

Our previous study employed DNA microarrays specific for *E. coli* CFT073 to examine gene expression of this strain during murine urinary tract infection (45). We demonstrated that while type 1 fimbrial expression was significantly upregulated in vivo during UTI, P fimbrial expression was downregulated, suggestive of coordinated regulation. In this report, we directly examine whether type 1 fimbriation affects the expression of other adhesin genes in a coordinated manner by employing both CFT073-specific microarrays and phase-locked mutants of this strain. We found that the expression of type 1 fimbriae coordinately affected the expression of P fimbriae in an inverse manner. To assert that this regulation has a role during infection, we must first determine whether P fimbriae are important to UPEC pathogenesis. Thus, we also investigated whether P fimbriae play a role in infection by in vivo competition of P-fimbriated and P fimbrial-negative isogenic mutants constructed in a type 1 fimbrial-negative background. Effects on the expression of F1C fimbriae were also documented.

MATERIALS AND METHODS

Bacterial strains and culture conditions. Uropathogenic *E. coli* strain CFT073 was isolated at the University of Maryland Medical Center from the blood and urine of a patient with acute pyelonephritis (25). All bacterial strains used in this study are listed in Table 1. Except when noted, bacteria were grown at 37°C in Luria broth (LB) with aeration or on Luria agar for 16 h with 15 µg/ml nalidixic acid, 50 µg/ml kanamycin, or 100 µg/ml ampicillin as appropriate. For RNA preparation, *E. coli* CFT073 was grown statically at 37°C in 100 ml of LB until the optical density at 600 nm (OD₆₀₀) reached 0.65. Bacteria were immediately treated with RNprotect bacterial reagent (QIAGEN) to stabilize RNA according to the manufacturer’s instructions and harvested by centrifugation (8 min, 7,500 × g, 25°C), and the bacterial pellet was frozen at –20°C until RNA extraction.

RNA isolation and cDNA synthesis. RNA from bacterial samples was extracted using the RNeasy Mini kit with 1-h on-column DNase digestion (QIAGEN) according to the RNeasy Mini handbook. A total of 10 µg of RNA was mixed with 750 ng random hexamers (Invitrogen) for each cDNA synthesis reaction according to a previously described protocol (37). SuperScript II reverse transcriptase (1,500 U; Invitrogen) was added to these reaction mixtures, along with First Strand buffer, dithiothreitol, and deoxyribonucleotides at concentrations recommended by the manufacturer (Invitrogen). The reaction mixtures were incubated at 25°C for 10 min, 37°C for 60 min, 42°C for 60 min, and 70°C for 10 min. Following RNaseH (Invitrogen) and RNaseA (Ambion) digestion, cDNA was purified using a QIAquick PCR purification kit (QIAGEN) according to the QIAquick Spin handbook.

Microarrays and hybridization. The *E. coli* CFT073-specific DNA microarray (NimbleGen Systems, Inc.) includes 5,611 open reading frames (ORFs) and stable RNAs from version 17 of the compiled CFT073 genome sequence. Each ORF is represented on the glass slide by 17 unique “probe pairs” of 24-mer in situ-synthesized oligonucleotides. Each pair consists of a sequence perfectly matched to the ORF, and another adjacent sequence harbors two mismatched bases for the determination of background and cross-hybridization. For each microarray, 5 µg cDNA was fragmented using 2.0 U of RQ1 DNaseI (Promega) partial digest for 7.5 min at 37°C and then labeled with biotin-N6-ddATP (Perkin-Elmer Life Sciences) using terminal transferase (Roche) as described previously (37). Labeled cDNA samples were hybridized individually to the CFT073-specific microarray according to the NimbleGen standard operating procedure (NimbleGen Systems, Inc.). Following washes and labeling with a streptavidin-Cy3 complex according to the NimbleGen procedure, microarrays were scanned at 5-µm resolution using a GenePix 4000b scanner.

Microarray data and statistical analysis. Microarray data were extracted using NimbleScan (NimbleGen) and an algorithm (courtesy of Y. Qiu, University of Wisconsin School of Medicine) applied to obtain a single measurement of signal intensity for each ORF. Data were normalized and converted to estimates of transcript abundance using the total signal intensity to allow comparison of individual microarrays (2). Changes (*n*-fold) of an ORF between UPEC strains were calculated by transformation of the following ratio: log₂ [(CFT073-ON signal intensity)/(CFT073-OFF signal intensity)]. Only changes (*n*-fold) of at least ±2 were considered significant in this report. Thus, ORFs characterized as “upregulated” (change [*n*-fold], ≥2) or “downregulated” (change [*n*-fold], ≤2) in *E. coli* CFT073-ON are relative to those in CFT073-OFF.

Quantitative real-time reverse transcription-PCR (qRT-PCR). Primers designed to amplify *papA_2* were targeted to regions of unique sequence based on the alignment of *papA* and *papA_2*. Primers for each gene are listed in Table 2. A total of 30 ng of cDNA and 300 nM (final concentration) of each primer were mixed with 12.5 µl 2× SYBR green PCR master mix (ABI). Assays were performed in quadruplicate with the ABI Prism model 7900 instrument. All data were normalized to the endogenous reference gene *gapA* (encoding glyceraldehyde 3-phosphate dehydrogenase), and melting curve analysis demonstrated that the accumulation of SYBR green-bound DNA was gene specific. Data were analyzed by the 2^{–ΔΔCT} method (22) using CFT073-OFF as the baseline “calibrator” strain. The data were transformed by log₂ to obtain a change (*n*-fold) difference between strains.

Construction of CFT073 *fim pap*. *E. coli* UPEC76 (lacking P fimbriae) acted as the parent strain in which *fimABCDEFGHI* was deleted using the λ Red recombination system according to Datsenko and Wanner (8). Primers (60-mer) were designed to PCR amplify the kanamycin resistance gene from the plasmid template pKD4 (8). These primers included 40-nucleotide extensions of homology to the intergenic region between the *fim* invertible element and *fimA* (upstream primer, CFT073 NCBI accession number NC_004431, bases 5′ 5137744 to 5137783), and homology to the intergenic region between *fimH* and *gntP* (downstream primer, bases 5′ 5144500 to 5144461). Red-mediated recombination replaced the *E. coli* UPEC76 *fimABCDEFGHI* chromosomal sequence with this resulting PCR product. After kanamycin selection, the mutation was confirmed by PCR. Helper plasmid pCP20 was then used to eliminate the kanamycin

TABLE 2. qRT-PCR primers

Primer	Sequence 5′→3′
GapA ForCATCGTTTCCAACGCATCCT
GapA RevACCTTCGATGATGCCGAAGTT
PapA_2 ForGTGCCTGCAGAAAATGCAGAT
PapA_2 RevCCCGTTTTCCACTCGAATCA

resistance gene. The resulting strain was designated CFT073 *fim pap*, and PCR was again used to confirm the mutant genotype.

Hemagglutination assay. A 3% (vol/vol) solution of guinea pig erythrocytes (Cambrex Bio Science Walkersville, Inc.) with or without 50 mM mannose was used to determine type 1 fimbrial mannose-sensitive hemagglutination. Approximately 1×10^9 CFU of bacteria, either from broth or from agar plates resuspended in phosphate-buffered saline (PBS), was serially diluted twofold in round-bottom 96-well microtiter plates. An equal volume of erythrocyte solution was mixed with the bacterial suspension. A diffuse mat of cells across the bottom of the well indicated positive hemagglutination.

Gal-Gal-coated latex bead agglutination. Latex beads coated with α -Gal(1-4) β -Gal (Chembiomed, Ltd.) were used to determine the presence of P fimbriae by latex agglutination. Approximately 1×10^9 CFU of bacteria, cultured either in broth or from agar plates resuspended in PBS, in a total volume of 10 μ l, was mixed with 25 μ l PBS and 2 μ l latex beads in a round-bottom 96-well microtiter plate. A granular settling of latex beads on the bottom of the well indicated positive latex agglutination.

Isolation of fimbriae and N-terminal sequencing. Fimbriae were isolated from 15 ml static (48 h) and exponential (harvested when OD₆₀₀ reached 0.5) Luria broth cultures of *E. coli* strains CFT073, UPEC76, and CFT073 *fim pap*. Fimbriae were detached from the bacterial cells by blending bacterial cultures in a commercial blender (Waring) for 5 min at half speed. After centrifugation (3,000 \times g, 12 min, 4°C), supernatants (15 ml) were concentrated using 50,000 molecular weight cutoff Centriprep filters (Millipore) to a volume of 2 ml. Protein was precipitated with 20% trichloroacetic acid for 30 min on ice, collected by centrifugation at maximum speed for 15 min, and washed with acetone. Dried pellets were then resuspended in an equal volume of 2 \times Laemmli sample buffer and boiled for 5 min. Samples were loaded onto a 5% stacking and a 15% sodium dodecyl sulfate (SDS)-polyacrylamide gel. After electrophoresis in SDS running buffer, the gel was stained with Coomassie blue (0.25% [wt/vol] Coomassie brilliant blue, 10% [vol/vol] glacial acetic acid, 45% [vol/vol] methanol) for 30 min at room temperature and then destained overnight (5% glacial acetic acid, 25% methanol). For N-terminal sequencing, proteins were transferred onto an Immobilon P membrane (Millipore) for 1 h (~100 V, 4°C) in a transfer chamber containing transfer buffer (25 mM Trizma base, 192 mM glycine, 20% methanol). The membrane was then stained with Coomassie blue for 5 min at room temperature and destained overnight (5% glacial acetic acid, 25% methanol). The band of interest was cut from the membrane and sent to the Protein Structure Facility of the University of Michigan for N-terminal sequencing, determined by Edman degradation.

Western blot analysis. Whole-cell bacterial samples in SDS sample buffer (50 μ l) were acid dissociated by adding 1 μ l 1 N HCl and boiling 5 min; the solution was neutralized with 1 μ l 1 N NaOH. Samples were electrophoresed under denaturing conditions on a 15% SDS-polyacrylamide gel and transferred to a polyvinylidene difluoride membrane (Immobilon-P; Millipore). The blot was first incubated with a 1:5,000 dilution of murine antiserum against FimH (courtesy of S. Langermann) and then immunoglobulin G-peroxidase-labeled anti-murine antibody and developed using chemiluminescence according to the manufacturer's instructions (ECL Plus western blotting kit; Amersham).

Transmission electron microscopy. A 5- μ l volume of bacteria (grown in LB to 1×10^8 CFU) was dropped onto a copper grid coated with a Formvar/carbon support film (Electron Microscopy Sciences). After 2 min, the majority of the culture was removed by touching a Kimwipe to the droplet. About 5 μ l of 1% phosphotungstic acid solution was then dropped onto the grid until covered. After 30 sec, the solution was removed by again touching a Kimwipe to the droplet. Samples were viewed with the JEOL JEM 1200 EX II microscope at the University of Maryland Dental School Biomedical Sciences Electron Microscopy Facility.

Coinoculation in the murine model of ascending UTI. Forty female CBA/J mice were transurethrally inoculated as previously described (14, 17) using a sterile 0.28-mm-diameter polyethylene catheter connected to a Harvard infusion pump. The inoculum contained Luria agar-grown *E. coli* CFT073-OFF and CFT073 *fim pap* resuspended in PBS and then mixed together in a 1:1 ratio. A total of 50 μ l of this bacterial suspension containing 1.55×10^9 CFU was delivered to each mouse. At each time point (4, 24, 48, 72, and 168 h postinfection), urine samples were collected prior to the sacrifice of eight mice. The bladder and kidneys were removed, weighed, and homogenized in PBS. Samples were quantitatively cultured using a spiral plater (Spiral System Instruments, Inc.) on Luria agar, containing nalidixic acid as appropriate, to determine the CFU/ml of urine or gram of tissue for each strain. The Wilcoxon matched-pairs test of nonparametric data was used to compare the median colonization levels for each strain.

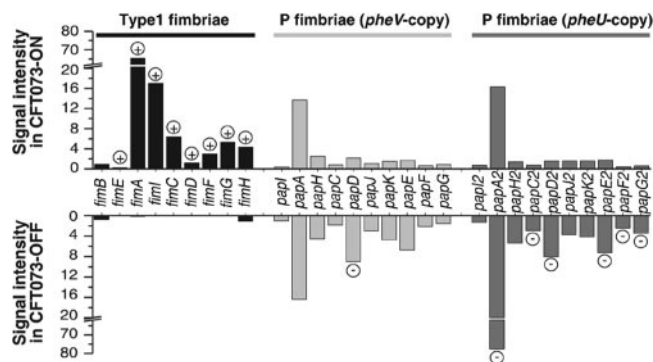


FIG. 1. Microarray analysis of the *fim* and *pap* gene clusters in *E. coli* CFT073 type 1 fimbrial phase-locked mutants. The signal intensity, corresponding to the relative expression of a gene, is shown for genes encoding type 1 fimbriae (*fim*) and both copies of P fimbriae (*pap*). Genes upregulated (⊕) or downregulated (⊖) in *E. coli* CFT073-ON relative to CFT073-OFF are indicated.

RESULTS

Microarray analysis of *E. coli* CFT073-OFF and CFT073-ON. *E. coli* strain CFT073-specific DNA microarrays were used to examine the genome-wide transcriptional responses to type 1 fimbriation. We employed isogenic mutants of *E. coli* CFT073 in which the type 1 fimbrial invertible element was phase locked either on or off (Table 1) (13). The mutant designated CFT073-ON thus constitutively produces type 1 fimbriae, and CFT073-OFF is always negative for type 1 fimbrial production. The expression level for each ORF in the genome was determined for both mutants as described in Materials and Methods. To maintain consistency, we always compared the expression of CFT073-ON to that of CFT073-OFF. Thus, “upregulation” indicates an ORF was at least twofold higher in expression when type 1 fimbriae were constitutively expressed relative to when they were not expressed. Conversely, “downregulation” indicates an ORF was at least twofold lower in expression when type 1 fimbriae were constitutively expressed relative to when they were not expressed. Because expression levels have been log₂-transformed, these reported twofold changes correspond to a fourfold difference in raw expression values.

To determine whether any adhesins exhibited inverse coordinate regulation with type 1 fimbriae, genes downregulated when type 1 fimbriae were phase on were examined. Of the 12 fimbrial gene clusters demonstrated or predicted for *E. coli* CFT073 and antigen 43 and curli adhesins, only *pap* genes encoding P fimbriae were consistently downregulated (Fig. 1). The *pheU*-associated copy of the *pap* gene cluster in the genome, shown previously by microarray as the dominant copy expressed in Luria broth (45), appeared more strongly regulated, as *papA*₂, *papC*₂, *papD*₂, *papE*₂, *papF*₂, and *papG*₂ were all downregulated about twofold. *papD* of the *pheV*-associated copy of the *pap* gene cluster was also downregulated. Most other *pap* genes displayed a strong trend towards downregulation. Antigen 43 (Ag43, encoded by *flu*), which had previously been shown by microarray to be upregulated when *fimBCDEFGH* were deleted in *E. coli* K-12 (40), was not differentially regulated here in this pathogenic strain.

To determine whether any adhesins exhibited coordinated

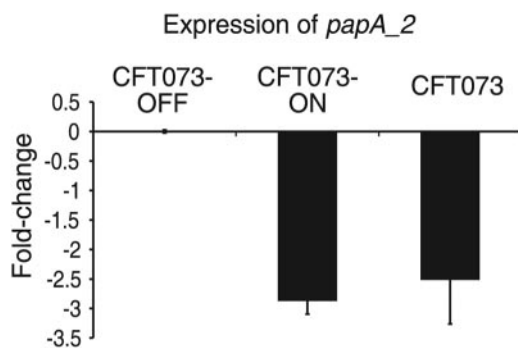


FIG. 2. qRT-PCR analysis of *papA_2* expression in *E. coli* CFT073 and type 1 fimbrial phase-locked mutants. The black bars represent the change (*n*-fold) in gene expression of *papA_2* between *E. coli* CFT073-OFF, CFT073-ON, and wild-type CFT073. Changes (*n*-fold) were calculated using CFT073-OFF as the relative measure of comparison.

regulation with type 1 fimbriae, genes upregulated when type 1 fimbriae were phase on were examined. As expected, type 1 fimbrial genes *fimABCDEFGH* were upregulated robustly from twofold to ninefold in *E. coli* CFT073-ON relative to CFT073-OFF. *fimE* (encoding the recombinase responsible for switching the invertible element into primarily the phase-off orientation) was upregulated twofold (signal intensity in CFT073-ON was 0.23, signal intensity in CFT073-OFF was 0.04, and change [*n*-fold] after log transformation was 2.4; this is consistent with others' measurements) (20, 46), while the expression of *fimB* (which switches the invertible element in both directions) remained unchanged (Fig. 1). Examination of other differentially regulated genes did not reveal any other genes of interest.

qRT-PCR verification of microarrays. qRT-PCR was used to independently verify our main finding that levels of *pap_2* transcript decreased when type 1 fimbriae were phase on. *papA_2* transcript expression was analyzed by the $2^{-\Delta\Delta CT}$ method (22) using *gapA* as the normalizing internal standard. To maintain consistency with the microarray studies, *E. coli* CFT073-OFF was used as the baseline "calibrator" strain to which CFT073-ON and the wild type were compared. qRT-PCR analysis demonstrated that *papA_2* expression was 2.86-fold lower in CFT073-ON than that in CFT073-OFF (P , <0.0001) (Fig. 2). This value was slightly greater than the 2.25-fold downregulation determined by microarray analysis. Additionally, *papA_2* expression in wild-type CFT073 was 2.50-fold lower than that in CFT073-OFF (P = 0.0072). Thus, the wild type and CFT073-ON, which both express type 1 fimbriae, had statistically similar patterns of *papA_2* expression (P = 0.28).

Construction, genotype, and phenotype of *E. coli* CFT073 *fim pap*. A mutant of *E. coli* CFT073 was constructed to further dissect the role of P fimbriae virulence in the absence of type 1 fimbriae. *E. coli* UPEC76 (Δ DEFG of the *pheV*-associated *pap* operon; Δ EFG of the *pheU*-associated *pap* operon) (Table 1) (26) acted as the parent strain in which *fimABCDEFGH* was deleted using the λ Red recombinase system (8) as described in Materials and Methods. The resulting strain was designated CFT073 *fim pap*. This mutation of *fimABC*

DEFGH was confirmed by PCR and did not disrupt the type 1 fimbrial invertible element sequence (data not shown).

The growth rates of *E. coli* CFT073 and CFT073 *fim pap*, cultured independently in Luria broth at 37°C with aeration, were not significantly different (data not shown). To mimic the in vivo murine urinary tract coinoculation, CFT073 *fim pap* and CFT073-OFF were grown together in vitro in coculture. Starting with an inoculum of a 1:1 ratio of these strains, both strains were recovered in similar amounts after 4 days of daily passage into fresh medium (average counts of 1.6×10^9 CFU/ml for CFT073-OFF and 5.5×10^8 for CFT073 *fim pap*) (experiments set up in triplicate, then repeated a second time using a fresh 1:1 inoculum mixture).

E. coli CFT073 *fim pap* was phenotypically characterized during in vitro growth in broth, which favors type 1 fimbrial expression (30), and on a solid agar surface, which typically inhibits type 1 fimbrial expression (12) and favors P fimbrial expression (Table 3). This mutant was negative for type 1 fimbriae, as demonstrated by the lack of mannose-sensitive hemagglutination (MSHA) of guinea pig erythrocytes. CFT073-ON, which expressed type 1 fimbriae regardless of growth condition or medium, and CFT073-OFF, which never expressed type 1 fimbriae, were included as positive and negative controls, respectively. This mutant was also negative for P fimbriae as demonstrated by a lack of agglutination of latex beads coated with the specific P fimbrial α -Gal(1-4) β -Gal receptor (47). UPEC76 was included here as a negative control. Additionally, Western blot analysis confirmed the absence of the type 1 fimbrial tip adhesin FimH in this mutant (Table 3).

To determine whether the deletion of both type 1 fimbriae and P fimbriae rendered this strain entirely afimbriate, in vitro grown *E. coli* CFT073 *fim pap* was examined by transmission electron microscopy. It remained highly fimbriated despite the loss of two of its most-well-characterized fimbriae (Fig. 3A). This strain was most often observed in large clusters of fimbriated bacteria (Fig. 3B).

Coinoculation of *E. coli* CFT073 *fim pap* and CFT073-OFF in the murine model of ascending UTI. *E. coli* CFT073 *fim pap* was employed to further dissect the role of P fimbriae, in the absence of type 1 fimbriae, during urinary tract infection. We had previously shown no difference in murine urinary tract colonization between UPEC76 and CFT073 (26) and hypothesized that a role for P fimbriae may have been masked by the

TABLE 3. Type 1 fimbrial and P fimbrial phenotypes

Strain	Absence or presence of protein				
	Cultured in broth			Cultured on agar	
	FimH ^a	MSHA ^b	Gal-Gal ^c	MSHA	Gal-Gal
CFT073	+	++	+	-	+++
UPEC76	ND	++	-	-	-
CFT073-ON	+	+++	+	+++	+++
CFT073-OFF	-	-	+	-	+++
CFT073 <i>fim pap</i>	-	-	-	-	-

^a Absence (-) or presence (+) of type 1 fimbrial tip adhesin FimH by Western blot analysis. ND, not done.

^b Absence (-) or relative present amount (+, ++, +++) of MSHA indicates type 1 fimbrial phenotype.

^c Absence (-) or relative present amount (+, ++, +++) α -Gal(1-4) β -Gal-coated latex bead agglutination (Gal-Gal) indicates P fimbrial phenotype.

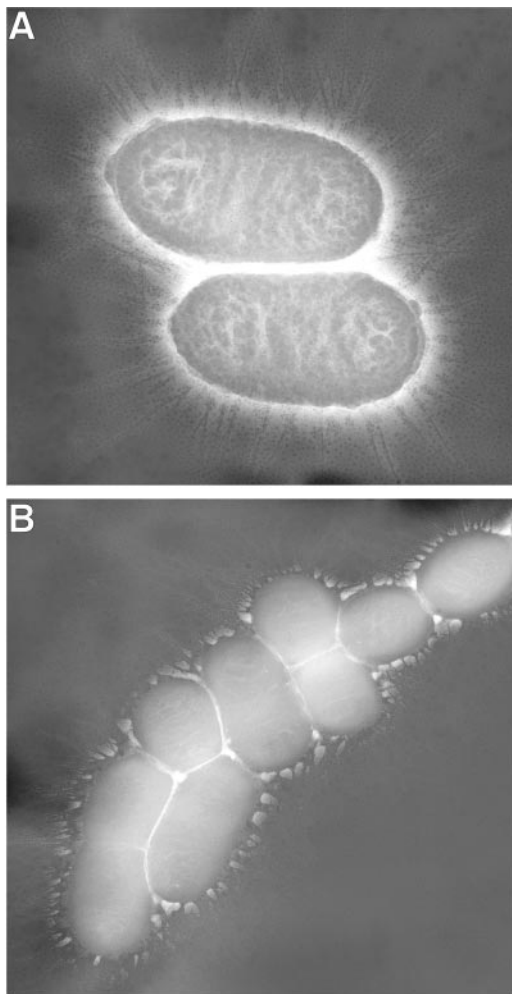


FIG. 3. Transmission electron microscopy of *E. coli* CFT073 *fim pap*. A. *E. coli* CFT073 *fim pap*, a mutant which does not express type 1 or P fimbriae, remains capable of other fimbrial production. B. This strain was most often observed in large clusters of fimbriated bacteria when grown in vitro.

overwhelming expression of type 1 fimbriae we observed in vivo (45). CBA/J mice were transurethraly inoculated with a suspension containing 1.55×10^9 CFU of *E. coli* CFT073 *fim pap* and CFT073-OFF mixed in a 1:1 ratio. At 4, 24, 48, 72, and 168 h postinfection, urine samples, bladders, and kidneys were quantitatively cultured to determine the CFU/ml of urine or gram of tissue for each strain. The Wilcoxon matched-pairs test of nonparametric data was used to compare the median colonization levels between strains.

The results from these coinoculation studies surprisingly showed no difference in colonization between *E. coli* CFT073 *fim pap* and CFT073-OFF in the urine, bladders, or kidneys at any time during infection (Fig. 4) (*P* values ranged from 0.219 to 0.999). The urine collected from infected mice demonstrated that both strains colonized equally well at 4 h postinfection, with median counts of about 5×10^5 CFU/ml. The counts steadily decreased at 24 and 48 h postinfection. At 72 h postinfection, the median count of CFT073 *fim pap* was over 3 logs higher than that of CFT073-OFF, but the *P* value (0.813)

indicates that this difference was not significant. Median CFU/g bladder counts demonstrated a similar pattern of colonization. Initial counts at 4 h postinfection were about 5×10^5 CFU/g for each strain; but, thereafter, the counts decreased to levels near the lower limit of detection (10^2 CFU/g) during the later stages of infection. The pattern of colonization in the kidneys was slightly different, where median counts of both strains remained at about 10^3 to 10^5 CFU/g throughout the infection.

Isolation and identification of fimbriae expressed in *E. coli* CFT073 *fim pap*. We noted an interesting observation during the in vitro coculture experiments. Between 2 to 4 days of daily passage, there was occasional pellicle formation found along with a particulate precipitate in several (but not all) culture tubes. Once present, the pellicle and precipitate continued to form in each subsequent passage. This phenomenon, which was duplicated in the replicate experiment, was never observed during passage of wild-type CFT073, UPEC76, or CFT073-OFF in our laboratory. This may indicate the phase-variable expression of some other adhesin that is not normally expressed when type 1 fimbriae and P fimbriae are present. Antigen 43 (Ag43, encoded by two copies of *flu*) is a phase-variable, nonfimbrial, autoaggregative surface protein that has been shown to produce thick precipitate, but not a pellicle, in *E. coli* K-12 (15).

To identify fimbriae that were expressed when type 1 and P fimbriae are not produced, shear preps containing surface appendages (fimbriae and flagella) were isolated from both static and exponential cultures of wild-type strain CFT073, UPEC76, and CFT073 *fim pap* and analyzed by SDS-polyacrylamide gel electrophoresis (Fig. 5). No difference was observed between the protein profiles of the fimbrial preparations from exponentially growing cultures of the three strains. On the other hand, we observed the presence of a ~10 kDa polypeptide in the fimbrial preparation from the static culture of CFT073 *fim pap*, which was absent in the static cultures of wild-type CFT073 and UPEC76. The N-terminal sequence of the polypeptide was determined to be VTTVNGGTVH (Fig. 5). A BLASTP analysis of the N-terminal sequence of this polypeptide revealed 100% identity with the amino acid residues 25 to 34 of FocA, the major fimbrial subunit of F1C fimbriae. This finding is consistent with the processing of the 24-amino acid signal peptide during secretion and assembly of the fimbriae.

DISCUSSION

This study provides the first evidence that the expression of type 1 fimbriae coordinately regulated the expression of other fimbriae in uropathogenic *E. coli*. Microarray analysis suggested that P fimbrial expression was downregulated when type 1 fimbriae were constitutively expressed in *E. coli* CFT073, specifically in the *pheU*-associated copy of the *pap* operon. Recall that we analyzed the microarray gene expression results of CFT073-ON relative to CFT073-OFF; if we examine CFT073-OFF relative to CFT073-ON, we can likewise state that P fimbrial expression was upregulated when type 1 fimbriae were not expressed. We verified these results by qRT-PCR of *papA*₂. This study also demonstrated that wild-type CFT073 and CFT073-ON had similarly lower levels of *papA*₂

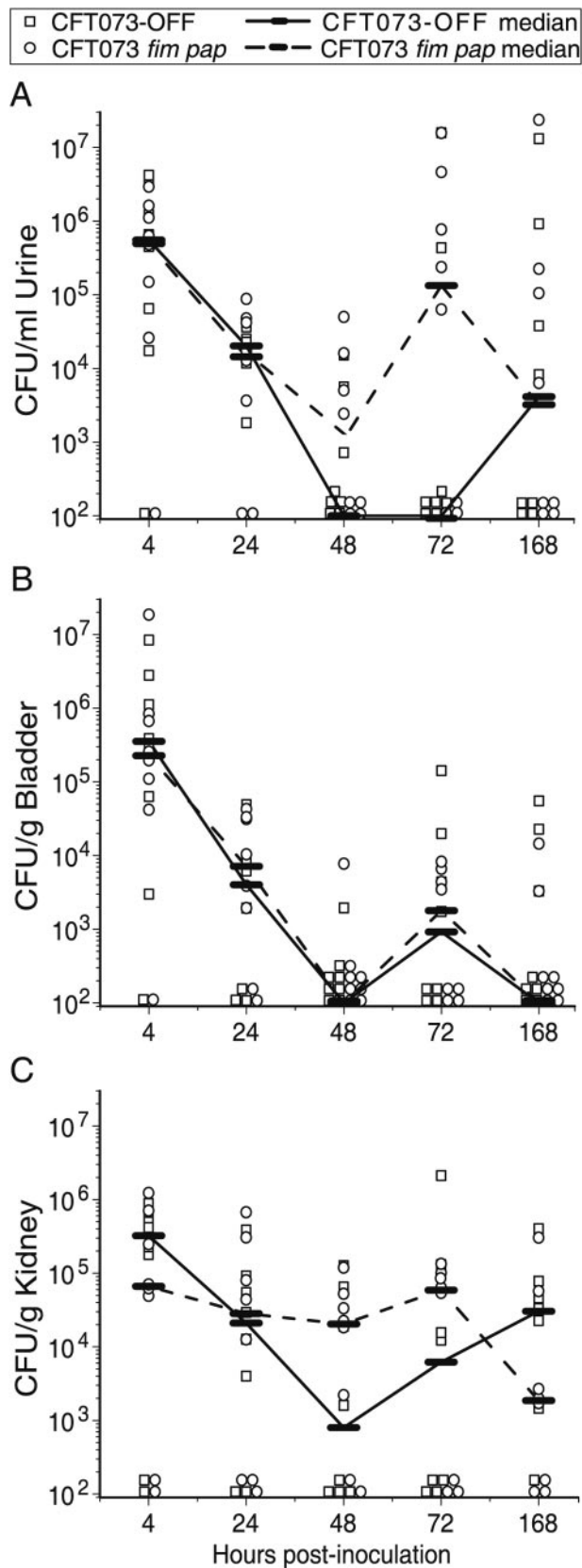


FIG. 4. Coinoculation of *E. coli* CFT073 *fim pap* and CFT073-OFF in the murine model of ascending UTI. *E. coli* CFT073 *fim pap* and CFT073-OFF were inoculated together into the murine urinary tract.

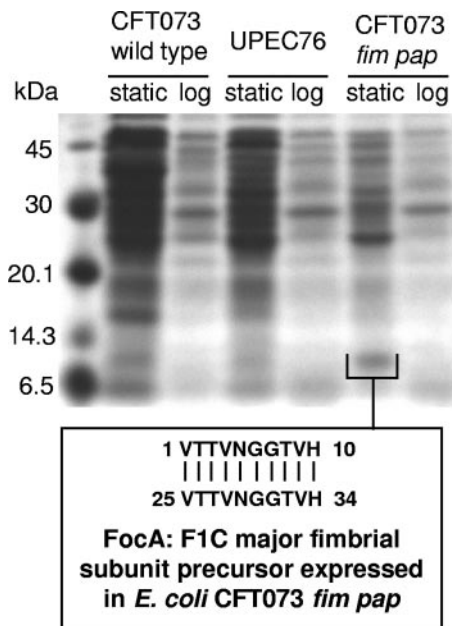


FIG. 5. F1C fimbriae expressed in static cultures of *E. coli* CFT073 *fim pap*. SDS-polyacrylamide gel electrophoresis was used to analyze the protein profiles of fimbrial preparations from concentrated static and aerated (exponential phase) Luria broth cultures of *E. coli* strain CFT073, UPEC76, and CFT073 *fim pap*. The protein ladder is shown on the left (kDa). The result of BLASTP analysis of the N-terminal sequence of the protein of small size (~10 kDa) observed in the fimbrial preparation from a static culture of CFT073 *fim pap* is shown below the gel. This protein was absent in the fimbrial preparations from static cultures of wild-type CFT073 and UPEC76.

expression in comparison to that of CFT073-OFF. This indicates that any level of type 1 fimbrial expression may repress P fimbrial expression, and P fimbrial expression is maximized when type 1 fimbriae are completely phase off. It was previously discovered that cross talk occurred from P fimbriae to type 1 fimbriae via PapB (54), but our data now provide direct evidence that regulation also occurs in the opposite direction. Phase variation and, thus, presumably coordinated regulation of fimbriae occur within a single bacterium. Although microarray analysis measures a population of bacteria, we circumvent this problem by analyzing gene expression in homogenous populations, namely, type 1 fimbrial phase-locked mutants of *E. coli* CFT073. This is in contrast to other documented environmental means of regulation. For example, Roesch and colleagues have elegantly demonstrated that uropathogenic *E. coli* respond to D-serine levels present in urine and modulate flagella expression (36).

At present, the mechanism by which type 1 fimbrial expression alters P fimbrial expression remains unclear. It is interest-

At the indicated times postinoculation, mice ($n = 8$) were sacrificed and the level of colonization was determined for each strain in the urine (A), in the bladder (B), and in the kidneys (C). Individual counts of strain CFT073-OFF are represented by squares (□), and individual counts of strain CFT073 *fim pap* are represented by circles (○). Bars, representing the median counts, are connected by a solid line in CFT073-OFF, and a dotted line in CFT073 *fim pap*.

ing to note that our phase-locked mutants were constructed by changing 7 out of 9 base pairs of the left inverted repeat flanking the *fim* invertible element, leading to the abolition of phase variation (13). This locking of the promoter does not disrupt the sequence of *fimB* or *fimE*, and each is transcribed from a separate promoter (31, 43). However, we cannot discount that FimB or FimE may act as a regulator on the *pap* operon in a situation analogous to the actions of PapB. Indeed, we demonstrated that the expression of *fimE* was slightly higher in CFT073-ON compared to that in CFT073-OFF (in other words, lower expression in CFT073-OFF) and therefore warrants further investigation of FimE as a potential regulator. Indeed, elevated *fimE* transcription in phase-on bacteria has been observed previously (20, 46).

This inverse expression between *fim* and *pap* gene clusters is consistent with our previous studies. Prior to the current study, we used the same CFT073-specific microarrays to examine the transcriptome of this strain growing in vivo during murine urinary tract infection (45). We demonstrated that while type 1 fimbrial expression was significantly upregulated in vivo during UTI, P fimbrial expression was downregulated. In addition, we found that type 1 fimbriae were extremely highly expressed during infection (*fimA* was the fourth-highest-expressed gene overall), in contrast to the low or lack of expression of the other 11 fimbrial gene clusters predicted in *E. coli* CFT073, including either copy of the *pap* operon. These data were consistent with coordinated regulation by type 1 fimbriae in vivo.

In another study, we observed differing temporal regulation of the type 1 fimbrial invertible element between strains in vivo during murine UTI (12). Cystitis isolates were mostly phase on throughout infection, and pyelonephritis isolates tended to be phase on only early during infection (24 h postinfection) and primarily phase off thereafter. In our study by Gunther et al. assessing the virulence of the *E. coli* CFT073 phase-locked mutants, we also demonstrated that type 1 fimbriae were most important in early infection (24 h postinfection) due to the decreased colonization of CFT073-OFF compared to that of the wild type at that time point (13). Thereafter CFT073-OFF was able to recover to the same level as that of the wild type, indicating that type 1 fimbriae were not as critical late in the infection. We had suggested that the subsequent expression of another fimbrial type later during infection, possibly P fimbriae, may explain this phenomenon. These studies not only again assert the importance that coordinated regulation may occur in vivo, but also provide us with a time course that indicates P fimbriae are most important after the initial infection has been established (that is, after 24 h).

Microarray data typically reflect genes expressed under a specific growth condition and time point (in this study, static in vitro growth at 37°C in LB until OD₆₀₀ reached 0.65). Different fimbrial expression patterns are known to depend on environmental and growth conditions such as temperature (9, 31), media (9), and pH (42). However, since the strains used in this study are unable to phase vary with respect to type 1 fimbrial expression, we observe the inverse regulation of type 1 fimbriae on P fimbriae. Our previous studies described above (12, 13) also suggest that there may be environmental cues (36) or different niches found within the urinary tract that promote coordinated fimbrial phase variation in vivo.

To investigate these suggestions that coordinated switching from type 1 fimbrial to P fimbrial expression occurs in vivo, we needed to definitively determine that P fimbriae are indeed necessary for full virulence. Earlier studies in our laboratory found that the P fimbria-negative *E. coli* CFT073 mutant (UPEC76) displayed no difference in murine urinary tract colonization or histology at 7 days postinfection (26). In light of new data that suggest type 1 fimbriation plays a dominant role in UTIs (45), we hypothesized that more subtle contributions of P fimbriae to virulence may be revealed by examination of a P fimbria-deficient mutant in a *fim*-negative background. Surprisingly, we found that *E. coli* CFT073 *fim pap* colonized the murine urinary tract as well as CFT073-OFF in a competition experiment, and the level of colonization overall was low. This may indicate that type 1 fimbriae are essential for initial adherence and the establishment of infection. However, we gain much insight into the role of P fimbriae during infection when we again compare these results to our previous study by Gunther et al. (13). In that study, despite CFT073-OFF colonizing at levels below detectable limits at 24 h postinfection, this strain was able to recover to a wild-type median level of 10⁴ CFU/g of bladder tissue for the duration of infection. In contrast, in the current study, we saw that the colonization level of CFT073 *fim pap* remained below the level of detection at 48 and 168 h postinfection in the bladder, increasing only temporarily at 72 h to 10³ CFU/g. An interesting future study would entail construction of a strain displaying constitutive P fimbrial expression; presumably in this strain, *fim* gene expression would be reduced and one would anticipate a concomitant decrease in virulence.

There remains a possibility that other adhesins are coordinately regulated with type 1 fimbriae or P fimbriae. At least in vitro we have demonstrated by transmission electron microscopy that some other fimbrial type is produced in the *E. coli* CFT073 *fim pap* mutant. In addition, the pellicle formation and particulate settling seen during in vitro passage of CFT073 *fim pap* also suggests the expression of a phase-variable aggregative adhesin not normally observed under standard culturing conditions. This mutant deficient in two of the most notable fimbrial gene clusters also provides us with another useful tool to examine coordinate fimbrial regulation in future studies.

Indeed, we found that the mutant deficient in both type 1 and P fimbrial expression produced another fimbrial type which was not produced by wild-type strain CFT073 or UPEC76 during growth in aerated or static culture conditions. These fimbriae were identified by N-terminal sequencing as F1C fimbriae. As members of the chaperone-usheer family, F1C fimbriae are structurally related to type 1 fimbriae and also share similar genetic organizations. However, comparison of the amino acid sequence reveals that F1C fimbriae are more closely related to S fimbriae (50). F1C fimbriae are expressed by 14 to 38% of all uropathogenic strains of *E. coli* (24, 32, 44, 49). In addition, the kidney has been reported as the target tissue of F1C-expressing *E. coli* by using in vitro models and strictly biochemical approaches (23, 51). Recently, using a functional assay, Backhed et al. demonstrated that binding of F1C-fimbriated *E. coli* to human renal epithelial cells induces interleukin-8 production, suggesting a role for F1C-mediated attachment in mucosal defense against bacterial infections (3). Altogether, these data suggest that F1C fimbriae may contrib-

ute to the colonization and persistence of uropathogenic *E. coli* in the urinary tract. This could explain the observation that the *E. coli* CFT073 *fim pap* mutant colonized the urinary tract as well as CFT073-OFF did in a competition experiment. Lastly, expression of F1C fimbriae may represent a compensatory mechanism in response to the inability to express type 1 and P fimbriae in the CFT073 *fim pap* mutant.

The urinary tract is an example of a diverse environment. A successful pathogen should possess adhesins specific for an assortment of niches, such as the bladder, ureters, and kidneys. At the same time, many adhesins stimulate an inflammatory immune response (11, 27, 48) and pose a great energy burden to the bacterium. Thus, phase variation and coordinated regulation between adhesins represent a balanced response in favor of bacterial pathogenesis. We demonstrate here that expression of type 1 fimbriae is inversely coordinated with the expression of P fimbriae. We propose a scenario where type 1 fimbriae are important for early colonization and urinary tract pathogenesis, and P fimbriae are employed later as bacteria may ascend to establish kidney infection. Striving to understand the molecular mechanisms behind interadhesin regulation will provide insights into the spatial and temporal events required for bacterial pathogenesis.

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