The Multifunctional Protein, YdiV, Represses P Fimbriae-Mediated Adherence in Uropathogenic Escherichia coli

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Running title: P fimbrial regulation by YdiV

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Abstract

YdiV, a degenerate EAL domain protein, represses motility by interacting with FlhD to abolish FlhDC interaction with DNA. Here, we demonstrate that deletion of ydiV dysregulates coordinate control of motility and adherence, by increasing adherence of *E. coli* CFT073 to a bladder epithelial cell line by specifically increasing production of P fimbriae. Interestingly, only one of the two P fimbrial operons, *pap_2*, encoded in the genome of *E. coli* CFT073 was upregulated. This de-repression of the *pap_2* operon is abolished following deletion of either *cya* or *crp*, demonstrating cAMP-dependent activation of the P-fimbrial operon. However, the absence of YdiV does not affect the gene expression of *cya* and *crp* and loss of SdiA in the *ydiV* mutant does not affect the de-repression of the *pap_2* operon suggesting YdiV control of adherence acts in response to cAMP levels. Deletion of *ydiV* increases motility by increasing expression of *fliA*, suggesting that in *E. coli* CFT073, YdiV regulates motility by the same mechanism described previously for commensal *E. coli* strains. Furthermore, analysis of site-directed mutations found two conserved Mg$^{2+}$-binding residues of four conserved YdiV residues (E29 and Q219) that were involved in regulation of motility, while two conserved c-di-GMP-binding residues (D156 and D165) did not affect motility. None of the four conserved YdiV residues appeared to affect regulation of adherence. Therefore, we propose a model in which a degenerate EAL, YdiV, utilizes different domains to regulate motility through interaction with FlhD and adherence to epithelial cells through cAMP-dependent effects on the *pap_2* promoter.
Introduction

Uropathogenic *Escherichia coli* (UPEC) is the most common etiological agent of uncomplicated urinary tract infections. This heterogeneous group of bacteria utilizes a variety of virulence factors to colonize and ascend the urinary tract including fimbriae and flagella. Flagella are transiently utilized to ascend the urethra to the bladder, and again when the bacteria ascend the ureters to the kidneys (1). Without expression of FliC, the main subunit of the flagellum, UPEC are non-motile and cannot ascend the urinary tract (2). On the other hand, fimbriae are utilized to adhere to epithelial cells in the bladder and kidneys, allowing UPEC to colonize these tissues and withstand the sheer force of urination. *E. coli* CFT073, a prototypical pyelonephritis isolate, encodes 12 fimbrial operons in its genome (3). Two of these operons encode P fimbriae, which bind to Gal (α1-4) Gal-terminal globoceramide receptors on kidney epithelium (reviewed in) (4). While P fimbriae are thought necessary for colonization of the kidneys (5), these fimbriae can also bind to exfoliated human bladder epithelial cells (6).

P fimbriae are regulated by an epigenetic switch controlled by two methylation sites (GATC\textsuperscript{prox} and GATC\textsuperscript{dist}) in its promoter region found within the two sets of binding sites (promoter proximal sites 1, 2, and 3) and (promoter distal sites 4, 5, and 6) for the leucine-responsive regulatory protein, Lrp (7). When the promoter is in the OFF state Lrp is bound to sites 1, 2, and 3 blocking methylation of GATC\textsuperscript{prox}, but in the ON state Lrp is bound to the distal sites 4, 5, and 6. PapI, an activator of the *pap* operon induces the switch to the On state by increasing the affinity of Lrp for sites 5 and 2. However, shifting the binding of Lrp from sites 1, 2, and 3 to sites 4, 5, and 6 is not sufficient for activation of *pap* operon transcription. To fully express P fimbriae, the cAMP-Catabolite
Gene Activator Protein (CAP-cAMP) must bind to the promoter upstream of Lrp site 4 (8). Deletion of either crp (encodes CAP) or cya (encodes adenylate cyclase that synthesizes cAMP), abolishes expression of P fimbriae (8). The expression of P fimbriae is enhanced on solid agar medium as opposed to liquid cultures (9). However, the mechanism of preferential expression has not been elucidated.

The reciprocal control of flagellum-mediated motility and fimbria-mediated adherence is controlled in part by the intracellular concentration of cyclic diguanylate monophosphate (c-di-GMP), a second messenger that is regulated in bacteria by diguanylate cyclases that synthesize c-di-GMP and phosphodiesterases that degrade c-di-GMP (10). *E. coli* CFT073 carries 32 genes that encode diguanylate cyclases, phosphodiesterases, or related proteins that regulate c-di-GMP concentration and thus the balance between motility and adherence (3, 11). Each of these genes was mutated and motility and adherence phenotypes were assigned (11). One of these proteins, YdiV, has been examined in more detail because of its demonstrated link between fimbriae and flagella expression (11-15). Here we demonstrate that YdiV, a degenerative c-di-GMP phosphodiesterase known to be a repressor of flagella-mediated motility within genera of the *Enterobacteriaceae* (13, 16, 17), represses expression of P fimbriae (specifically the *pap_2* operon) in liquid medium, subsequently reducing adherence to epithelial cells through a pathway that involves cAMP. Deletion of *ydiV* increases the expression of *papA_2*, however, this de-repression is abolished following deletion of either *cya* or *crp*.

The mechanism by which YdiV regulates motility is now understood to be the same in both *Salmonella enterica* serovar Typhimurium and *E. coli* K-12. YdiV binds to
FlhD in the FlhD$_4$C$_2$ complex inhibiting its interaction with the *fliA* promoter (15, 18).

Specifically, YdiV binds each of the four FlhD subunits of the heterohexamer, forcing the ring-like structure of the FlhD$_4$C$_2$ complex to open. Consequently, the master regulator of the flagellar expression no longer binds DNA (19). A second mechanism by which YdiV inhibits flagellar gene expression has also been suggested, in which YdiV binds and strips FlhD$_4$C$_2$ from DNA, and targets the complex for ClpXP-dependent proteolysis (14). Here we show that although YdiV regulates motility in *E. coli* CFT073 by the same mechanism as observed in *E. coli* K-12, key residues which are essential for the regulation of motility are not necessary for repression of the expression of P fimbriae. Therefore, we propose a model in which YdiV utilizes different domains to regulate motility through interaction with FlhD and adherence to epithelial cells through cAMP-dependent regulation of the *pap_2* promoter.
Materials and Methods

Construction of mutants. Deletion mutants were constructed in *E. coli* CFT073 using the lambda red recombinase system (20). Primers containing sequences homologous to the 5’ and 3’ ends of the target sequence were designed and used to amplify the resistance cassette from the template plasmid pKD3 (encoding chloramphenicol resistance). Lambda red-mediated recombination was used to replace the genes *slyB*, *sdiA*, *crp*, and *cya*, individually with these PCR products in both *E. coli* CFT073 wild-type background and an unmarked Δ*ydiV* background. Primers homologous to flanking regions of each gene were designed for confirmation of replacement.

Site-directed mutagenesis. Previously, *ydiV* was cloned into pGEN-MCS with its endogenous promoter for complementation experiments, and designated pYdiV (13). Using QuikChange Site-Directed Mutagenesis Kit (Stratagene), specific base pair changes were introduced resulting in alanine substitutions for seven polar conserved residues in YdiV according to the manufacturer’s instructions. Mutations were confirmed by sequencing. Plasmids bearing site-directed mutants are pYdiV-S11A, pYdiV-E29A, pYdiV-E55A, pYdiV-E116A, pYdiV-K125A, pYdiV-D156A, pYdiV-D165A, and pYdiV-Q219A.

Motility assays. Motility was evaluated for each mutant in soft agar plates (1% tryptone, 0.5% NaCl, and 0.25% agar) and compared to the parental wild-type strain as described previously (2). Mutants were cultured overnight in LB broth, used to inoculate 5 ml of sterile LB broth, and incubated at 37°C with aeration to an OD₆₀₀ = 1.0-1.2. Cultures were standardized to an OD₆₀₀ = 1.0 and used to stab the center of soft agar plates with an inoculating needle. Plates were incubated for 16 h at 30°C, at which time...
the diameter of motility was measured. Diameters are directly correlated with bacterial motility (21). Motility of the complemented mutants was examined similarly, but the medium and the soft agar contained ampicillin (100 µg/ml) for maintenance of the plasmid. Wild-type *E. coli* CFT073 and each mutant transformed with the empty plasmid pGEN-MCS were included as controls.

**Western Blots to detect flagella and P fimbriae.** Samples to detect FliC (flagellin), the main subunit of flagella, were prepared as described previously (13) with the following modification. Briefly, cultures were grown in LB medium to OD600 of 0.3 and 1.5 ml of culture was carefully collected by centrifugation (1,000 x g, 10 min, 4°C) to avoid shearing flagella into the supernatant. Whole cells were resuspended in 100 µl of dH20 and 10 µl was prepared for SDS-polyacrylamide gel electrophoresis. Following SDS-PAGE samples were transferred to polyvinylidene difluoride membrane (Immobilon-P; Millipore Corp.). Blots were incubated with a 1:20,000 dilution of rabbit polyclonal antiserum to H1 flagella, followed by a 1:40,000 dilution of peroxidase-conjugated goat anti-rabbit immunoglobulin G (Sigma). Blots were developed using chemiluminescence according to the manufacturer’s instructions (Amersham ECL Prime; GE Healthcare Life Sciences).

Samples for the detection of P fimbriae were prepared by diluting aerated overnight LB cultures into fresh LB media (1:100) and grown statically or inoculated onto agar plates at 37° for 24 h. Bacterial cultures were standardized to an OD600 = 1.0 and 1.0 ml of the standardized suspensions was centrifuged (6,000 x g, 10 min, 4°C) to pellet bacteria. Bacteria were lysed by resuspension in 100 µl dH20 and 6x sodium dodecyl sulfate SDS sample buffer (20 µl), and boiled for 10 min. Sample lysates (20-30
µl) were electrophoresed on a 12% denaturing SDS-polyacrylamide gel and transferred to polyvinylidene difluoride membrane (Immobilon-P; Millipore Corp.). Blots were incubated with a 1:10,000 dilution of rabbit polyclonal antiserum to PapA, followed by a 1:40,000 dilution of peroxidase-conjugated goat anti-rabbit immunoglobulin G (Sigma). Blots were developed using chemiluminescence according to the manufacturer’s instructions (Amersham ECL Prime; GE Healthcare Life Sciences).

**Shear preparation of fimbriae.** Overnight static cultures (50 ml) of wild-type *E. coli* CFT073 and ΔyiV were shaken horizontally for 2 min to shear fimbriae from the bacterial cell surface. Cultures were centrifuged (8,000 x g, 20 min, 4°C) to pellet intact bacterial cells. Culture supernatants containing fimbriae (as confirmed by TEM) were ultracentrifuged (40,000 x g, 1 h, 25°C). Supernatant was removed, and pellets were resuspended in 100 µl of dH2O and 20 µl 6x SDS sample buffer. Samples were boiled for 7 min and a sample (30 µl) was electrophoresed on a 15% denaturing SDS-polyacrylamide gel, and stained with Coomassie blue. For type 1 fimbriae samples were first boiled in acidified water, pH 1.8. Any polypeptide that was differentially expressed was excised and sent to the Proteomics Resource Facility at the University of Michigan for identification by LC-MS/MS sequencing.

**Adherence assays.** Cell culture and adherence assays were performed as described (22) using the immortalized bladder epithelial cell line, UM-UC-3 (ATCC #CRL-1749). Adherence was expressed as [cell-associated CFU/initial CFU/well], and each mutant was normalized to the wild-type control. All assays were performed in triplicate.
*In vivo* murine co-challenge model of ascending UTI. Six to eight week old CBA/J mice were infected transurethrally as previously described (23) with the following modification. Overnight cultures of wild-type *E. coli* CFT073 and ΔydIV were centrifuged (3500 x g, 30 min, 25°C) to collect bacteria. Bacteria were resuspended in 30 ml PBS, quantified in a spectrophotometer at 600 nm, and diluted to an OD$_{600}$ of 4.0 (~10$^9$ CFU/ml). ΔydIV was mixed 1:1 with the parental strain, and then 50 µl of this mixture (10$^8$ CFU) was transurethrally inoculated into the bladder of each mouse through a sterile 0.28-mm polyethylene catheter attached to an infusion pump (Harvard Apparatus). The inoculum was quantified on LB agar with and without kanamycin to differentiate the resistant mutant from susceptible wild-type strain. At 48 h post inoculation (hpi), mice were euthanized and target organs were removed, homogenized in 3 ml sterile PBS with a GLH homogenizer (Omini International), and dilutions were spiral plated onto LB agar with and without kanamycin to quantify bacterial load using an Autoplate 4000 spiral plater (Spiral Biotech). Competitive Index (CI) was calculated as [Mutant(Output/Input)/Wild-type(Output/Input)]. The CI was log$_{10}$ normalized, and a two-tailed Wilcoxon Signed Rank Test was conducted, where a P-value <0.05 was considered significant.

**RNA isolation, cDNA synthesis and reverse transcriptase quantitative PCR.** *E. coli* CFT073, cultured overnight in LB broth with aeration at 37°C, was quantified by spectrophotometry at OD$_{600}$ and 1 ml samples were diluted to 10$^9$ CFU/ml. Bacteria were collected by centrifugation, washed once with PBS, and resuspended in 5 ml fresh LB broth. Bacteria were cultured statically at 37°C and samples (2 ml) removed during mid-exponential phase. RNA was stabilized with 4 ml RNAprotect (Qiagen) and total
RNA was isolated using the RNeasy Mini system (Qiagen) according to the manufacturer’s instructions. Total RNA and cDNA sample concentrations were determined using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific). cDNA was synthesized from total RNA using the Superscript Double-Stranded cDNA Synthesis system (Invitrogen) according to the manufacturer’s instructions. Reverse transcriptase quantitative PCR (qPCR) was performed in an Mx300P thermalcycler (Stratagene), using 30 ng cDNA template, 0.1 μM primers, and Brilliant SYBR Green reagents (Stratagene). Data were normalized to gapA and analyzed with MxPro 4.0 software (Stratagene).

**Transmission electron microscopy.** Wild-type *E. coli* CFT073 and ΔydiV were cultured in LB broth statically overnight at 37°C. Samples were swirled gently to resuspend and 10 µl of the culture was dropped onto formvar carbon support film on TEM specimen grids (Electron Microscopy Sciences). Drops were incubated at room temperature for 5 min, and excess medium was wicked off with filter paper. Grids were washed once with 10 µl of dH2O, and stained for 2 min with 10 µl of 1% phosphotungstic acid (pH 6.8). Excess stain was removed and the grids were washed immediately with dH2O. Grids were dried under a petri dish lid on the bench top and visualized using a Philips CM-100 transmission electron microscope.
Results

YdiV reduces P fimbriae production. Previously, we reported that ΔydiV is more adherent than wild-type E. coli CFT073 to the UM-UC-3 immortalized human bladder epithelial cell line (11). Thus, we hypothesized that ΔydiV expresses more fimbriae than wild-type E. coli CFT073, or a specific fimbria that is not ordinarily expressed under these conditions. To pursue this possibility, wild-type E. coli CFT073 and ΔydiV were cultured statically overnight in LB broth at 37°C, and tested for mannose-resistant hemagglutination of human red blood cells. CFT073 ΔydiV demonstrated a four-fold increase in mannose-resistant HA titer (data not shown). In addition, fimbriae were visualized by negative staining and transmission electron microscopy. Interestingly, both the wild-type bacteria (Figure 1A) and the ΔydiV mutant (Figure 1B) appeared highly fimbriated. These fimbriae, however, were not type 1 fimbriae, based on a Western blot of whole cell lysates from wild-type E. coli CFT073 and ΔydiV cultured statically overnight in LB broth at 37°C, developed with anti-FimA (11). To determine which fimbrial type was over-expressed in ΔydiV, fimbrial shear preparations from the mutant and wild-type E. coli CFT073 were prepared, denatured, and separated on a 15% denaturing gel by SDS-PAGE and stained with Coomassie blue (Figure 1C). Two bands were found at higher density in ΔydiV than wild-type (apparent molecular size = 75 kDa and 23 kDa). The over-expressed bands were excised and subjected to mass spectrometry. The ~75 kDa protein corresponded to FlIC (actual molecular size = 65.5 kDa), and the ~23 kDa protein was PapA_2 (actual molecular size = 21.8 kDa). The latter result was consistent with the increase in mannose-resistant hemagglutination titer. One band, absent in ΔydiV but present in wild-type (apparent molecular size = 15
kDa) was identified as SlyB, a putative lipoprotein (actual molecular size = 17.0 kDa) (Figure 1C).

Western blots were conducted to confirm that ΔydiV over-expresses PapA, the main structural subunit of P fimbria (Figure 1D). When cultured overnight in LB broth, ΔydiV demonstrated increased PapA expression as compared to both E. coli CFT073 wild-type and the complemented mutant, E. coli CFT073 ΔydiV (pYdiV). However, when cultured overnight on LB agar, no difference in PapA expression was observed. Therefore, when statically cultured in liquid medium, which is generally non-permissive for the production of P-fimbriae, ΔydiV over-expresses P-fimbriae.

YdiV represses activation of the pap_2 operon through catabolite-activating protein and adenylate cyclase. Since the PapA_2 protein is over-expressed in ΔydiV, we hypothesized that YdiV must ordinarily repress the expression of the pap_2 operon, and since CAP-cAMP is known to activate the expression of the pap operon (8), we determined whether deletion of either crp (encodes CAP) or cya (encodes adenylate cyclase) affected the adherence of ΔydiV to bladder epithelial cells. Mutation of crp or cya in ΔydiV eliminated PapA production as determined by Western blot (Figure 1E). Similarly, deletion of either crp or cya from wild-type E. coli CFT073 had no significant effect on adherence to bladder epithelial cells (Figure 2). However, when either crp or cya was also deleted in a ΔydiV background, adherence was restored to wild-type levels, as compared to ΔydiV (Figure 2). The results from the adherence assay indicate that the wild-type base-line adherence was independent from the production of P-
fimbriae, and in the absence YdiV, CAP-cAMP activates the expression of P-fimbriae, which resulted in an increase in adherence to the bladder cell line.

YdiV has previously been linked to the production of cAMP, as a double deletion of ydiV-sdiA reduces intracellular cAMP concentration in *E. coli* K-12 (24). Therefore, the effect of deletion of *sdiA*, which encodes a quorum sensing transcriptional regulator thought to activate transcription of *ydiV* (24), on adherence in both wild-type and Δ*ydiV* backgrounds was assessed. As observed with Δ*crp* and Δ*cya*, Δ*sdiA* had no effect on adherence when compared to wild-type *E. coli* CFT073 (92.7 ± 16.2% of wild-type, *P*=0.683) (Figure 2). However, a double mutant in Δ*ydiV* and Δ*sdiA* remained more adherent than wild-type to the bladder epithelial cell line (187.3 ± 16.4%, *P* = 0.013).

Since SlyB appeared to be absent in the fimbrial prep of Δ*ydiV*, a single deletion and a double mutant with Δ*ydiV* was constructed to determine if any of the phenotypes observed were due to the absence of SlyB. Deletion of *slyB* (Δ*slyB*), however, had no effect on adherence to bladder epithelial cells, demonstrating the effect of Δ*ydiV* on adherence is not an indirect effect due to loss of SlyB (Figure 2). Likewise, the double deletion (Δ*ydiV*Δ*slyB*) remained more adherent than wild-type, again demonstrating the increase adherence to the bladder cell line results solely from the absence of *ydiV*.

Δ*ydiV*, Δ*ydiV*Δ*crp*, and Δ*ydiV*Δ*cya* specifically affect expression of *papA_2*, but not *papA*. Since there are two *pap* operons encoded in the genome of *E. coli* CFT073, and the mass spectrometry data demonstrated that PapA_2 was over-expressed in Δ*ydiV*, the effect of each deletion on gene expression of each *pap* operon was determined. Briefly, RNA was extracted from overnight static LB cultures and gene
expression was assessed by qPCR using operon-specific primers. When compared to wild-type E. coli CFT073, there were no differences in papA expression for ΔydiV, Δcrp, Δcya, ΔydiVΔcrp, and ΔydiVΔcya (data not shown). However, papA_2, was 2.36-fold higher in ΔydiV (P = 0.0027) than wild type E. coli CFT073, and, as was observed in the adherence assays, deletion of either Δcrp or Δcya restored papA_2 expression to wild-type levels. Together, finding that the increased expression of Pap_2 and the increased adherence, in the absence of YdiV, can be restored to wild-type levels by mutation of either crp or cya suggests that the YdiV effect on adherence may results from a role in controlling or responding to cAMP levels in the cell.

Neither sdiA, cya, nor crp affect ydiV expression. A previous study in E. coli K-12 found that SdiA activates expression of ydiV (24). Therefore, we determined by qPCR if ydiV expression was altered in ΔsdiA compared to wild-type E. coli CFT073. ydiV expression, however, was not significantly different in ΔsdiA (0.867 ± 0.158-fold of wild-type, P = 0.487). Likewise deletion of cya or crp had no effect on ydiV expression (Δcya: 0.906 ± 0.222-fold of wild-type, P = 0.712; and Δcrp: 0.986 ± 0.209-fold, P = 0.954). Therefore, in E. coli CFT073, the expression of ydiV is not affected by CAP-cAMP or SdiA. Thus, it is likely that YdiV acts to affect cAMP or cAMP-CRP levels rather than cAMP or cAMP-CRP acting to control ydiV expression.

Deletion of both ydiV and sdiA decreases crp and cya expression. To determine if YdiV affects transcription of the pap_2 operon by affecting the expression of crp or cya, expression of these genes was measured by qPCR in ΔydiV, ΔsdiA, and ΔydiVΔsdiA.
Expression of *crp* was also analyzed in Δ*cya*, and Δ*ydiVΔcya*, whereas cya expression was also analyzed in Δ*crp* and Δ*ydiVΔcrp*. Expression in Δ*ydiV* of *crp* (0.733 ± 0.165-fold, *P* = 0.25) or cya (0.782 ± 0.066-fold, *P* = 0.08) was not significantly different from wild-type. Likewise, no single gene deletion tested significantly modulated *crp* or cya expression. However, the double deletion of Δ*ydiVΔsdiA* reduced expression of *crp* to 0.554 ± 0.011-fold of wild-type levels (*P* = 0.0006), and cya to 0.580 ± 0.082-fold of wild-type (*P* = 0.036). This result is consistent with the observation by Zhou *et al.* (24) that Δ*ydiVΔsdiA* reduces intracellular cAMP concentration about 2-fold in *E. coli* K-12.

**YdiV affects class II flagellar gene expression.** Several reports of the effect of YdiV on motility have demonstrated that YdiV is a repressor of flagellar biosynthesis in *Salmonella* and is a cryptic repressor of flagellar biosynthesis in *E. coli* K-12 (15, 18, 19). We recently reported, as previously observed in *Salmonella*, that deletion of *ydiV* increases motility (11). In *S. Typhimurium*, YdiV affects class II flagellar gene expression by binding to FlhD, inhibiting the master regulatory complex FlhD₄C₂ from activating the expression of FliA (15). In *E. coli* CFT073, YdiV reduces FliC expression at the protein level (Figure 3B). To determine if YdiV affects flagellar biosynthesis by controlling transcript levels, we conducted qPCR of *flhD* (class I), *fliA* (class II), and *fliC* (class III) in Δ*ydiV*, Δ*ydiV* (p*YdiV*), Δ*ydiV* (pBAD-*ydiV*) induced with 5 mM arabinose, and wild-type *E. coli* CFT073. There were no differences in expression of *flhD* between the strains, however, Δ*ydiV* expressed 83.4-fold more *fliA* transcript than wild-type (*P* = 0.0009), and when *ydiV* was over-expressed, *fliA* expression was reduced to 0.023-fold of wild-type levels (*P* = 0.0003). Consistent with the Western blot and motility data,
expression of *fliC* was increased in Δ*ydiV* (7.0-fold, \( P=0.0455 \)), and reduced in both Δ*ydiV* (pYdiV) (0.2-fold, \( P=0.0021 \)) and when YdiV was over-expressed (0.022-fold, \( P<0.0001 \)). Thus YdiV inhibits motility by reducing expression of the sigma factor FliA, thereby reducing *fliC* expression independent from transcription of the class I *flhD* promoter.

**Four site-directed mutants of YdiV affect motility.** To determine residues that may be involved in the inhibition of motility by YdiV, site-directed mutagenesis of the conserved Mg\(^{2+}\) binding residues E29A (the E in the ELI motif), E116A, and Q219A, and the conserved c-di-GMP-binding residue D165A were constructed, along with three 100% conserved polar residues (S11, K125, and D156) from an alignment of YdiV with 20 homologs from other members of the *Enterobacteriaceae* (Figure S1). As a control, E55, a residue that is not conserved, was also replaced with alanine. These constructs were used to complement Δ*ydiV* and motility assays were conducted (Figure 3A). The entire set of site-directed mutants was less motile than either wild-type (pGEN) or the Δ*ydiV* (pGEN) controls suggesting that these residues partially contribute to the function of the protein. In addition, when compared to Δ*ydiV* (pYdiV) (+), the mutant complemented with pYdiV-E29A, pYdiV-D156A, pYdiV-D165A, or pYdiV-Q219A were significantly more motile (2.17-3.66-fold more motile; \( P < 0.05 \)) (Figure 3A), while complementation of Δ*ydiV* with four other mutants, pYdiV-S11A, pYdiV-E55A, pYdiV-E116A, or pYdiV-K125A had no effect on motility (data not shown).

Western blots using antisera specific for HI flagellin (FliC) demonstrated that the mutant complemented with pYdiV-E29A or -Q219A both produced more flagellin than
ΔydiV (pYdiV) (Figure 3B). These two site-directed mutations with the largest effects on motility and FliC production, E29A and Q219A, were tested for dominant-negative phenotypes in a wild-type background. The diameter of swimming motility in the wild-type background carrying cloned YdiV [E. coli CFT073 (pYdiV)] was 5.2 ± 0.4 mm and was increased to 6.5 ± 1.4 mm by pYdiV-Q219A and 9.5 ± 1.0 mm by pYdiV-E29A. Thus, the E29A mutation, and to a lesser extent, the Q219A mutation is dominant negative to YdiV, as the mutant proteins affect the ability of YdiV inhibit motility because wild-type E. coli CFT073 bearing either pYdiV-E29A (P<0.0001) or pYdiV-Q219A (P=0.0464) was significantly more motile than wild-type E. coli CFT073 bearing pYdiV.

Interestingly, only mutations of the known Mg²⁺ binding residues within YdiV (E29 and Q219) affected the proteins ability to inhibit motility and reduce FliC production, while mutation of c-di-GMP binding residues in YdiV did not affect FliC production.

**The residues of YdiV important for motility have no effect on adherence.** Complementation of ΔydiV with pYdiV restored adherence to bladder epithelial cells (135 ± 21% of wild-type, P= 0.123) to wild-type levels. We therefore examined the effect of the site-directed mutants of YdiV on adherence to the bladder epithelial cell line, UM-UC-3 to determine if the residues found to be involved in regulation of motility are also necessary to regulate P-fimbrial adherence to host cells. Surprisingly, all of the site-directed mutants of YdiV (S11A, E29A, E55A, E116A, K125A, D156A, D165A, and Q219A) complemented adherence (data not shown). Thus, YdiV may function through different mechanisms to affect adherence and motility.
Motility assays in soft DMEM agar were conducted to determine whether the medium accounted for the differential effect of the site-directed mutants on motility and adherence to epithelial cells. In soft DMEM agar, wild-type *E. coli* CFT073 displayed reduced motility (12.9 ± 2.8 mm). Δ*ydiV* significantly increased motility compared to wild-type *E. coli* CFT073 in soft DMEM agar to 23.7 ± 2.0 mm (*P*<0.0001), and Δ*ydiV* (pYdiV) reduced motility further than the wild-type strain (2.9 ± 0.6 mm, *P*=0.0019). As observed in regular motility agar, substitution of E29 (*P*=0.0163), D156 (*P*=0.0202), D165 (*P*=0.0162), and Q219 (*P*=0.0154) with alanine significantly increased motility. Thus, the change in medium does not account for the differential effects of the site-directed mutants of YdiV on sessility and motility. Intriguingly, the Mg$^{2+}$-binding residues appeared to have a greater affect than the c-di-GMP residues as also suggested by western results and soft agar motility (Figure 3A).

**The effect of YdiV on motility does not occur via SlyB, CAP-cAMP, or SdiA.** Since CAP-cAMP has been implicated in the regulation of motility by activation of the *flhD* operon (25), we determined if either CAP or Cya are involved in the effect of YdiV on motility. Motility is abolished in Δ*crp*, as previously observed (25), due to the absence of FlhD, in both the wild-type *E. coli* CFT073 and Δ*ydiV* backgrounds (Figure 4A). While not as severe of a phenotype, Δ*cya* (37.7 ± 3.7 mm) was less motile than wild-type, (47.0 ±1.4 mm, *P*= 0.0094), but had no significant effect in the Δ*ydiV* background (Δ*ydiV*: 79.4 ± 1.4 mm, Δ*ydiVΔcya*: 76.0 ± 1.0 mm, *P*= 0.071, Figure 4A). Finding that the hyper-motility caused by the absence of YdiV is dependent on *crp* but is
independent of adenylate cyclase suggests that cAMP is not required for CAP activation of flhD or that cya is dispensible for cAMP production in the ydiV mutant bacteria.

SlyB is encoded in an operon downstream of slyA. In a previous study, ΔslyA was found to have increased motility in E. coli CFT073, similar to what was observed in ΔydiV (13). Therefore, we hypothesized that ΔslyB would be more motile than wild-type. However, ΔslyB had no effect on motility. Furthermore, ΔydiVΔslyB is not significantly different from ΔydiV, demonstrating that SlyB is not involved in the regulation of motility.

Deletion of sdiA was shown to increase motility in enterohemorrhagic E. coli O157:H7 (26). However, in E. coli CFT073, ΔsdiA had no motility defect, and ΔydiVΔsdiA was not significantly different in motility from ΔydiV, demonstrating that SdiA is not involved in the regulation of motility in E. coli CFT073.

YdiV affects motility downstream of glucose import and adenylate cyclase. Since glucose was reported to reduce ydiV expression (24), and we found that crp but not cya is required for hyper-motility in ydiV mutant bacteria, we hypothesized that addition of 0.8% glucose to motility medium would reduce the expression of ydiV in wild-type E. coli CFT073, thus increasing motility as observed in ΔydiV. As expected, addition of glucose, which would decrease cAMP levels by decreasing adenylate cyclase activity, had no effect on the motility of ΔydiV, which swam with a diameter of 78.1 ± 1.3 mm in the absence and 79.7 ± 1.5 mm the presence of excess glucose (P = 0.442) (Figure 4B). As expected, motility was significantly reduced in wild-type by 8.6 ± 3.5 mm (P = 0.026) since glucose would limit CAP-cAMP activation of the flhD promoter. Furthermore, while Δcya responds to addition of glucose similarly to wild-type E. coli
CFT073, ΔydiVΔcya is glucose-insensitive. Interestingly, ΔsdiA, which has no effect on
motility in regular soft agar, is also glucose-insensitive. Similar to what is observed in
regular soft agar plates, motility in ΔydiVΔsdiA is not significantly different from ΔydiV,
and is also glucose-insensitive (Figure 4B). Together, these results support the
hypothesis that cya is dispensible for cAMP production in the ydiV mutant bacteria and
demonstrate that cAMP is required for CAP activation of flhD when YdiV is present.

ΔydiV overproduces an extracellular matrix both in aerated and static culture
conditions. Similarly, mutation of ydiV increases biofilm formation in CFT073 (Figure
5A). As seen with motility, this increase in biofilm is insensitive to glucose, suggesting
YdiV acts downstream from cAMP-CRP. In the absence of CAP (Δcrp), biofilm
formation is abrogated in wild-type bacteria and in the ydiV mutant (Figure 5A).
Mutation of adenylate cyclase (Δcya) did not affect biofilm formation in CFT073 or in the
ydiV mutant. Interestingly, mutation of sdiA in the ydiV mutant increased biofilm
formation above what was observed with the ydiV single mutant (Figure 5A). In both
aerated mid-exponential phase cultures and overnight static cultures, ΔydiV was
observed under transmission electron microscopy to produce an extracellular matrix
(representative micrograph shown in Figure 5B) not observed in the complemented
mutant (representative micrograph shown in Figure 5C). This matrix is not curli or
cellulose, as ΔydiV does not express these polymers (11).

Deletion of ydiV reduces colonization of the upper urinary tract. Since ydiV is
involved in regulating several phenotypes related to virulence, we determined the effect
of deletion of this gene on uropathogenesis *in vivo* in the mouse model of ascending UTI (*n* = 10 mice). When in direct competition with wild-type CFT073, Δ*ydiV* was less fit in the kidneys of mice (8.7-fold, *P* = 0.0166). There was no significant difference from wild-type in bladder colonization (*P* = 0.8469). Therefore, *YdiV* contributes to successful *in vivo* colonization of the upper urinary tract.
YdiV, a versatile protein involved in repression of both motility and sessility, contributes to the control of motility and P-fimbrial mediated adherence in *E. coli* CFT073. Here, we demonstrate that in *E. coli* CFT073, a prototypical pyelonephritis isolate, YdiV uniquely inhibits adherence to uroepithelial cells by suppressing expression of P fimbriae (Figure 6). Furthermore, we demonstrate that site-directed mutations in YdiV that affect the inhibition of motility do not affect the inhibition of adherence phenotypes, suggesting that different domains of the protein are utilized in regulation of these competing phenomena.

The mechanism by which YdiV inhibits motility in *S. Typhimurium* and *E. coli* K-12 has been well established; YdiV binds to FlhD causing a decrease in *fliA* transcription (15, 16, 18). In *E. coli* CFT073, YdiV similarly inhibits transcription of *fliA* and *fliC* without affecting *flhD* expression, causing a reduction in motility by reducing expression of flagella. These findings are consistent with other models, suggesting that YdiV inhibits motility by the same mechanism elucidated for *E. coli* K-12 and *Salmonella*. However, there are strain differences between *E. coli* K-12 and CFT073. In one study of *E. coli* K-12, deletion of *ydiV* does not affect motility, since this strain does not naturally express *ydiV* (18), whereas in *E. coli* CFT073, Δ*ydiV* is significantly more motile than the wild-type strain ((11, 13) and this study). In *E. coli* K-12, one study has proposed that *ydiV* expression is inhibited post-transcriptionally by the translational start site being occluded due to the formation of a secondary structure in the mRNA (18). However, by alignment of the intergenic region between *ydiV* and the closest gene upstream in the *E. coli* K-12 and CFT073 genomes, we found only one base pair difference between the two strains.
Although predicted to be a degenerate phosphodiesterase, three of the four site-directed mutations in YdiV that affected motility to the greatest extent in *E. coli* CFT073 were in residues conserved within the EAL domain that are required for Mg\(^{2+}\)-binding (E29 and Q219) (27), while c-di-GMP-binding residues (D156 and D165) did not affect motility. However, D156 and D165 both flank the hydrophobic region necessary for interaction with FlhD (19), and thus may be involved in direct interaction or stabilization of the interacting α-helix. E29 and Q219 appear to be located in the β-sheets that make up the TIM-barrel-like central core of YdiV (19), and therefore, substitution of these residues with alanine possibly changes the structural integrity of the protein, causing it to be less able to bind FlhD. However, the same residues are not important for inhibiting adherence to bladder epithelial cells demonstrating that a different domain of YdiV from that required to modulate motility is involved in this phenotype.

YdiV affects adherence to bladder epithelial cells by repressing fimbriae expressed on the cell surface in liquid culture. Specifically, YdiV, represses the expression of one of the two P fimbrial operons present in the CFT073 genome (28), as ΔydiV has increased levels of papA_2 gene expression and PapA_2 protein as compared to wild-type *E. coli* CFT073. Furthermore, deletion of either *crp* or *cya* restores the ΔydiV mutant to wild-type levels of both adherence and papA_2 gene expression, and since CAP-cAMP is known to be a direct activator of the pap operon, these data confirm that YdiV represses P fimbrial expression (Figure 6). While in *E. coli* K-12 YdiV has not been implicated in regulation of adhesins, in *S. Typhimurium*, YdiV has been suggested to regulate CsgD, and therefore, curli biosynthesis (17), again...
demonstrating that pathogenic Enterobacteriaceae have evolved to utilize a common protein, YdiV, for regulation of virulence factors.

Although SdiA has previously been described as an activator of ydiV gene expression in E. coli K-12 (24), deletion of sdiA had no effect on ydiV transcript levels. Similarly, deletion of crp and cya do not affect ydiV gene expression. However, the double deletion, ΔydiVΔsdiA, significantly reduced the expression of cya, the gene encoding adenylate cyclase, and crp, the gene encoding CAP by 2-fold. Thus, YdiV and SdiA are involved in activation of gene expression of CAP and the enzyme that synthesizes cAMP (Figure 6), which is consistent with the observation by Zhou et al. (24) that ΔydiVΔsdiA reduced intracellular cAMP concentration about 2-fold in E. coli K-12. Furthermore, the reduction in crp and cya expression is consistent with the observation that ΔydiVΔsdiA, while hyper-adherent compared to wild-type E. coli CFT073, is less adherent than ΔydiV, just as though the level of CAP and cAMP was reduced.

YdiV, a protein encoded by all E. coli strains tested thus far, is a repressor of flagellar motility and P fimbrial expression in the uropathogenic strain E. coli CFT073 (Figure 6). While present in both pathogenic and commensal strains of E. coli, this protein has apparently taken on extra regulatory roles in the pathogenic strain, as the regulation of P fimbriae demonstrates. Future studies must be conducted to determine if YdiV interacts directly with the pap operon promoter or, as in the case of the regulation of motility, YdiV acts as an anti-transcription factor binding to regulatory proteins necessary for the expression of P fimbriae. Alternatively or in addition, our findings
suggest that degenerate EAL domain proteins like YdiV may specifically function through an effect on cAMP levels rather than from c-di-GMP.
Acknowledgments. This work was supported by Public Health Service grants AI059722 and DK094777 from the National Institutes of Health.
References


Figure Legends

Figure 1. ΔydiV over-expresses P fimbriae under static culture conditions.  A-B. Transmission electron micrographs of (A) negatively stained wild-type *E. coli* CFT073 and (B) ΔydiV. Images are taken at 36,000 × magnification from static overnight LB broth cultures incubated at 37°C. Bar = 500 nm. C) Comparison of proteins (black arrows) found in fimbrial shear preps from wild-type and ΔydiV demonstrate that FliC and PapA_2 are over-expressed and SlyB is not expressed in ΔydiV. D) Western blot confirmed PapA is over-expressed in ΔydiV only when cultured statically in broth compared to wild-type *E. coli* CFT073 and the complemented mutant. E) When cultured on agar plates, there is no difference in PapA expression between the mutant, wild-type *E. coli* CFT073, or the complemented mutant. Deletion of *crp* or *cya* abrogates P-fimbrial production in ΔydiV.

Figure 2. Deletion of *crp* or *cya* in a ΔydiV background restores wild-type adherence to cultured immortalized bladder epithelial cells. Data represent the adherence (CFU adherent bacteria/CFU inoculum) of each mutant strain to cultured UM-UC-3 epithelial cells normalized to wild-type *E. coli* CFT073. Data are the averages of three assays conducted in triplicate and are expressed as % wild type adherence. Error bars indicate standard error of the mean. Gray bars have a *P*-value <0.05 when compared to wild-type as assessed by Student’s *t*-test. The gray bar with diagonal stripes is significantly different from both wild-type (*P* = 0.0129) and ΔydiV (*P* = 0.0233).
Figure 3. Analysis of conserved YdiV residues E29, D156, D165, and Q219 effect on motility and flagellin production. A) Swimming motility diameter of wild-type *E. coli* CFT073, Δ*ydiV*, Δ*ydiV* complemented with wild-type protein (pYdiV), and site-directed mutants (E29A, D156A, D165A, and Q219A) cultured at 30°C in soft LB agar plates for 16h. Data are averages of three independent experiments performed in triplicate. Error bars indicate standard error of the mean. For all data shown, $P$-value <0.05 as assessed by Student’s *t*-test. B) Western blot of flagellin (FliC) from *E. coli* CFT073 wild-type (WT) Δ*ydiV*, and Δ*ydiV* complemented with wild-type protein (pYdiV), or site directed mutants (E29A, D156A, D165A, and Q219A). Whole cell lysates were subjected to SDS-PAGE and Western blot using rabbit polyclonal antiserum to H1 flagella. FliC indicates the predicted electrophoretic mobility of flagellin. All cultures were diluted to an OD$_{600}$=0.30 before the samples were boiled to ensure equal loading of protein.

Figure 4. YdiV affects motility independent of glucose at a step in flagellar biosynthesis downstream from CAP activation of *flhDC*. A) Swimming motility diameter of wild-type *E. coli* CFT073, Δ*ydiV*, Δ*slyB*, Δ*crp*, Δ*cya*, Δ*sdiA*, Δ*ydiVΔslyB*, Δ*ydiVΔcrp*, Δ*ydiVΔcya*, and Δ*ydiVΔsdiA*, cultured at 30°C in soft LB agar plates for 16h. Data are averages of three independent experiments performed in triplicate. Error bars indicate standard error of the mean. Gray bars have a $P$-value <0.05 when compared to wild-type as assessed by Student’s *t*-test. B) Addition of excess glucose (0.8%) to motility agar decreases swimming motility in wild-type *E. coli* CFT073 and Δ*cya*, but Δ*ydiV*, Δ*sdiA*, Δ*ydiVΔcya*, and Δ*ydiVΔsdiA* are glucose-insensitive. Data are averages.
of three independent experiments performed in triplicate. Error bars indicate standard
error of the mean. *P-value <0.05 as assessed by Student’s t-test.

Figure 5. ΔydiV produces an extracellular matrix. A) Biofilm formation in M9
minimal medium with glucose as sole carbon source. Transmission electron
microscopy of negatively stained CFT073, B) ΔydiV, and C) ΔydiV (pYdiV). Bacteria
were cultured for 3 h with aeration at 37°C in LB. Black bar equals 1.0 μm.

Figure 6. Model for the cAMP-dependent YdiV adherence regulatory network. YdiV
inhibits expression of the pap_2 operon. Together with SdiA, YdiV activates
transcription of cya and crp. Cya produces cAMP which binds to CAP (the product of
crp) and CAP-cAMP activate transcription of the pap_2 operon.