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 *Escherichia 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*, present in the genome of *E. coli* CFT073 was upregulated. This derepression of the *pap_2* operon is abolished following deletion of either *cya* or *crp*, demonstrating cyclic AMP (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 derepression of the *pap_2* operon, suggesting that 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 as that described previously for commensal *E. coli* strains. Furthermore, analysis of site-directed mutations found two putative Mg²⁺-binding residues of four conserved YdiV residues (E29 and Q219) that were involved in regulation of motility and FlIC production, while two conserved c-di-GMP-binding residues (D156 and D165) only affected 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.

Uropathogenic *Escherichia coli* (UPEC) is the most common etiological agent of uncomplicated urinary tract infections (UTIs). 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 nonmotile 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 shear force of urination. *E. coli* CFT073, a prototypical pyelonephritis isolate, carries 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 reference 4). While P fimbriae are thought to be 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 methyltransfer sites (GATCprox and GATCdist) 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 GATCprox, but in the “on” state, Lrp is bound to the distal sites 4, 5, and 6. PapL, 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 fimbria, the cyclic AMP-catabolite gene activator protein (CAP-cAMP) must bind to the promoter upstream of Lrp site 4 (8). Deletion of either *crp* (which encodes CAP) or *cya* (encoding adenylate cyclase, which 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 flagellar expression (11–15). Here we demonstrate that YdiV, a degenerative c-di-GMP phosphodiesterase known to be a repressor of flagellum-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 derepression is abolished following deletion of either *cya* or *crp*.

The mechanism by which YdiV regulates motility is now un-
nderstood to be the same in *Salmonella enterica* serovar Typhimurium and in *E. coli* K-12. YdiV binds to FlhD in the FlhD-C2 complex, inhibiting its interaction with the fliA promoter (15, 18). Specifically, YdiV binds each of the four FlhD subunits of the heterohexamer, forcing the ringlike structure of the FlhD-C2 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, whereby YdiV binds and strips FlhD-C2 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 that observed in *E. coli* K-12, key residues that 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*, *slyA*, *crp*, and *cya*, individually with these PCR products in both the *E. coli* CFT073 wild-type background and an unmarked *ΔydiV* 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 (Strategene), 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 that of 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 optical density at 600 nm (OD600) of 1.0 to 1.2. Cultures were standardized to an OD600 of 1.0 and incubated at 37°C for 16 h at 30°C, at which time the diameter of motility was measured. Diameters are directly correlated with bacterial motility (21). Bacterial cultures were centrifuged (1,000 g, 10 min, 4°C) to avoid bacterial aggregation. Bacteria were lysed by resuspension in 100 μl of dH2O and 6X SDS sample buffer (20 μl) and boiled for 10 min. Sample lysates (20 to 30 μl) were electrophoresed on a 12% denaturing SDS-polyacrylamide gel and transferred to a 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 *E. coli* CFT073 and the *ΔydiV* mutant were shaken horizontally for 2 min to shear fimbriae from the bacterial cell surface. Cultures were centrifuged (8,000 × g, 20 min, 4°C) to pellet intact bacterial cells. Culture supernatants containing fimbriae (as confirmed by transmission electron microscopy [TEM]) were ultracentrifuged (40,000 × 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 liquid chromatography-tandem mass spectrometry (LC-MS/MS) sequencing.

**Adherence assays.** Cell culture and adherence assays were performed as described previously (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 *in vivo* murine coagglutination model of ascending UTI. Six- to 8-week-old CBA/J mice were infected transurethrally as previously described (23) with the following modification. Overnight cultures of *E. coli* CFT073 wild-type and *ΔydiV* strains were centrifuged (3,500 × g, 30 min, 25°C) to collect bacteria. Bacteria were resuspended in 30 ml phosphate-buffered saline (PBS), quantified in a spectrophotometer at 600 nm, and diluted to an OD600 of 4.0 (–10 CFU/ml). The *ΔydiV* mutant was mixed 1:1 with the parental strain, and then 50 μl of this mixture (104 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 the susceptible wild-type strain. At 48 h postinoculation (hpi), mice were euthanized, target organs were removed and homogenized in 3 ml sterile PBS with a GLH homogenizer (Omni International), and dilutions were spiral plated onto LB agar with and without kanamycin to quantify the bacterial load using an Autoplate 4000 spiral plater (Spiral Biotech). The competitive index (CI) was calculated as mutant/(Output/Input)/wild-type. The CI was log_{10} normalized, and a two-tailed Wilcoxon signed-rank test was conducted, where a P value of <0.05 was considered significant. Animal protocols were approved for use by the University of Michigan UCUCA (approval #08999-3).

**RNA isolation, CDNA synthesis, and reverse transcriptase qPCR.** *E. coli* CFT073, cultured overnight in LB broth with aeration at 37°C, was quantified by spectrophotometry at OD600 and 1-ml samples were diluted to 10^6 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) were 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 thermal cycler (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 the ΔydiV mutant were cultured in LB broth statically overnight at 37°C. Samples were swirled gently to resuspend the cultures, 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 benchtop and visualized using a Philips CM-100 transmission electron microscope.

RESULTS

YdiV reduces P fimbria production. Previously, we reported that the ΔydiV mutant 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 the ΔydiV mutant expresses more fimbriae than does wild-type E. coli CFT073 or expresses a specific fimbria that is not ordinarily expressed under these conditions. To pursue this possibility, wild-type E. coli CFT073 and the ΔydiV strain were cultured statically overnight in LB broth at 37°C and tested for mannose-resistant hemagglutination (HA) of human red blood cells. CFT073 ΔydiV demonstrated a 4-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 (Fig. 1A) and the ΔydiV mutant (Fig. 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 the ΔydiV strain cultured statically overnight in LB broth at 37°C and tested for mannose-resistant hemagglutination (HA) of human red blood cells. CFT073 ΔydiV demonstrated a 4-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 (Fig. 1A) and the ΔydiV mutant (Fig. 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 the ΔydiV mutant cultured statically overnight in LB broth at 37°C, developed with anti-FimA (11). To determine which fimbrial type was overexpressed in the ΔydiV mutant, 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 (Fig. 1C). Two bands were found at higher density in the ΔydiV mutant than in the wild type (apparent molecular sizes, 75 kDa and 23 kDa). The overexpressed 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 an increase in mannose-resistant hemagglutination titer. One band, absent in the ΔydiV strain but present in the wild type (apparent molecular size, 15 kDa) was identified as SlyB, a putative lipoprotein (actual molecular size, 17.0 kDa) (Fig. 1C).

Western blot assays were conducted to confirm that the ΔydiV mutant overexpresses PapA, the main structural subunit of P fimbriae, which resulted in an increase in adherence to the bladder cell line. Western blotting (Fig. 1E). Similarly, deletion of either crp or cya from wild-type E. coli CFT073 had no significant effect on adherence to bladder epithelial cells (Fig. 2). However, when either crp or cya was also deleted in a ΔydiV background, adherence was restored to wild-type levels compared to those of the ΔydiV strain (Fig. 2). The results from the adherence assay indicate that the wild-type baseline adherence was independent from the production of P fimbriae, and in the absence of 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 and sdiA reduces the 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 ΔydiV backgrounds was assessed. As observed with Δcrp and Δcya strains, ΔsdiA had no effect on adherence compared to wild-type E. coli CFT073 (92.7% ± 16.2% of wild-type adherence, P = 0.683) (Fig. 2). However, a double mutant in ΔydiV and ΔsdiA remained more adherent than the wild type to the bladder epithelial cell line (187.3% ± 16.4%, P = 0.013).

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

FIG 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 percent wild-type adherence. Error bars indicate standard errors of the means. Gray bars have a P value of <0.05 compared to the wild type as assessed by Student’s t test. The gray bar with diagonal stripes is significantly different from both the wild-type (P = 0.0129) and the ΔydiV (P = 0.0233) strains.

Neither sdiA, cya, nor crp affects 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 the ΔsdiA mutant compared to wild-type E. coli CFT073. ydiV expression, however, was not significantly different in the ΔsdiA strain ([0.867 ± 0.158]-fold wild-type levels, P = 0.487). Likewise, deletion of cya or crp had no effect on ydiV expression ([0.906 ± 0.222]-fold wild-type levels, P = 0.712; and [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 CFT073 ΔydiV, ΔsdiA, and ΔydiV ΔsdiA. Expression of crp was also analyzed in CFT073 Δcya and ΔydiV Δcya, whereas cya expression was also analyzed in CFT073 Δcrp and ΔydiV Δcrp. Expression in CFT073 ΔydiV of crp ([0.733 ± 0.165]-fold, P = 0.25) or cya ([0.782 ± 0.066]-fold, P =
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 FlhDC2 from activating the expression of FliA (15). In *E. coli* CFT073, YdiV reduces FliC expression at the protein level (Fig. 3B). To determine if YdiV affects flagellar biosynthesis by controlling transcript levels, we conducted qPCR of *fliD* (class I), *fliA* (class II), and *fliC* (class III) in CFT073 ΔydiV, Δ*ydiV* (pYdiV), Δ*ydiV* (pBAD-ydiV) induced with 5 mM arabinose, and wild-type *E. coli* CFT073. There were no differences in expression of *fliD* between the strains; however, the Δ*ydiV* strain expressed 83.4-fold more *fliA* transcript than did the wild type (*P* = 0.0009), and when *ydiV* was overexpressed, *fliA* expression was reduced to 0.023-fold wild-type levels (*P* = 0.0003). Consistent with the Western blot and motility data, expression of *fliC* was increased in the Δ*ydiV* mutant (7.0-fold, *P* = 0.0455) and reduced both in CFT073 Δ*ydiV* (pYdiV) (0.2-fold, *P* = 0.0021) and when YdiV was overexpressed (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 Mg2⁺-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* (see Fig. S1 in the supplemental material). As a control, E55, a residue that is not conserved, was also replaced with alanine. These constructs were used to complement the Δ*ydiV* mutant, and motility assays were conducted (Fig. 3A). The entire set of site-directed mutants was less motile than either wild-type (pGEN) or the CFT073 Δ*ydiV* (pGEN) controls, suggesting that these residues partially contribute to the function of the protein. In addition, compared to CFT073 Δ*ydiV* (pYdiV) (+), the mutants complemented with pYdiV-E29A, pYdiV-D156A, pYdiV-D165A, or pYdiV-Q219A were significantly more motile (2.17- to 3.66-fold more motile; *P* < 0.05) (Fig. 3A), while complementation of CFT073 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 H1 flagellin (FliC) demonstrated that the mutants complemented with pYdiV-E29A or Q219A both produced more flagellin than CFT073 Δ*ydiV* (pYdiV) (Fig. 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 are dominant negative to YdiV, as the mutant proteins affect the ability of YdiV to 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 the Δ*ydiV* mutant with pYdiV restored adherence to bladder epithelial cells (15% ± 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 Dulbecco’s modified Eagle medium (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). The Δ*ydiV* mutant had significantly increased motility compared to wild-type *E. coli* CFT073 in soft DMEM agar, to 23.7 ± 2.0 mm (*P* < 0.0001), and CFT073 Δ*ydiV*Δ*ydiV* had 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 effect than the c-di-GMP residues, as also suggested by Western blotting results and soft agar motility assays (Fig. 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 is involved in the effect of *YdiV* on motility. Motility is abolished in the Δ*crt* strain, as previously observed (25), due to the absence of FlhD, in both the wild-type *E. coli* CFT073 and the Δ*ydiV* backgrounds (Fig. 4A). While not exhibiting as severe of a phenotype, the Δcya mutant (37.7 ± 3.7 mm) was less motile than the wild type (47.0 ± 1.4 mm, *P* = 0.0094) but had no significant effect in the Δ*ydiV* background (Δ*ydiV* strain, 79.4 ± 1.4 mm; Δ*ydiV*Δ*crt* strain, 76.0 ± 1.0 mm; *P* = 0.071 [Fig. 4A]). Finding that the hypermotility caused by the absence of *YdiV* is dependent on *crt* but is independent of adenylate cyclase suggests that cAMP is not required for CAP activation of *flhD* or that *cya* is dispensable for cAMP production in the *ydiV* mutant bacteria.

SlyB is encoded by an operon downstream of *slyA*. In a previous study, the Δ*slyA* mutation was found to confer increased motility in *E. coli* CFT073, similar to what was observed with Δ*ydiV* (13). Therefore, we hypothesized that the Δ*slyB* mutant would be more motile than the wild type. However, Δ*slyB* had no effect on motility. Furthermore, the Δ*ydiV*Δ*slyB* strain is not significantly different from the Δ*ydiV* strain, 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, the ΔsdiA mutant had no motility defect, and the Δ*ydiV*Δ*sdiA* mutant was not significantly different in motility from the Δ*ydiV* mutant, 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 hypermotility 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 the Δ*ydiV* mutant. As expected, addition of glucose, which would decrease cAMP levels by decreasing adenylate cyclase activity, had no effect on the motility of the Δ*ydiV* mutant, which swam with a diameter of 78.1 ± 1.3 mm in the absence and 79.7 ± 1.5 mm in the presence of excess glucose (*P* = 0.442) (Fig. 4B). As expected, motility was significantly reduced in the wild type, by 8.6 ± 3.5 mm (*P* = 0.026), since glucose would limit CAP-cAMP activation of the *flhD* promoter. Furthermore, while the Δ*cya* mutant responds to addition of glucose similarly to wild-type *E. coli* CFT073, the Δ*ydiV*Δ*cya* mutant is glucose insensitive. Interestingly, Δ*sdiA*, which has no effect on motility in regular soft agar, also confers glucose insensitivity. Similar to what is observed in regular soft agar plates, motility in the Δ*ydiV*Δ*sdiA* strain is not significantly different from that in the Δ*ydiV* strain and is also glucose insensitive (Fig. 4B). Together, these results support the hypothesis that *cya* is dispensable for cAMP production in the *ydiV* mutant bacteria and demonstrate that CAP is required for CAP activation of *flhD* when *YdiV* is present.

The Δ*ydiV* mutant overproduces an extracellular matrix under both aerated and static culture conditions. Similarly, mutation of *ydiV* increases biofilm formation in CFT073 (Fig. 5A). As seen with motility, this increase in biofilm is insensitive to glucose, suggesting that *YdiV* acts downstream from cAMP-CRP. In the absence of CAP (Δ*crt*), biofilm formation is abrogated in wild-type bacteria and in the *ydiV* mutant (Fig. 5A). Mutation of adenylate cyclase (Δ*cya*) 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 (Fig. 5A). In both aerated mid-expo-

![FIG 4](http://jb.asm.org) 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 and the Δ*ydiV*, ΔslyB, Δ*crt*, Δ*cya*, Δ*sdiA*, Δ*ydiV*Δ*slyB*, Δ*ydiV*Δ*crt*, Δ*ydiV*Δ*cya*, and Δ*ydiV*Δ*sdiA* mutants, cultured at 30°C in soft LB agar plates for 16 h. Data are averages of three independent experiments performed in triplicate. Error bars indicate standard errors of the means. Gray bars have a *P* value of <0.05 compared to the 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 the Δ*cya* mutant, but the Δ*ydiV*, Δ*sdiA*, Δ*ydiV*Δ*cya*, and Δ*ydiV*Δ*sdiA* strains are glucose insensitive. Data are averages of three independent experiments performed in triplicate. Error bars indicate standard errors of the means. *, *P* value < 0.05 as assessed by Student’s *t* test.
in a strain of E. coli 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, the ΔydiV mutant is significantly more motile than the wild-type strain (references 11 and 13 and this study). In E. coli K-12, one study has proposed that ydiV expression is inhibited posttranslationally 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 and FlhC production to the greatest extent in E. coli CFT073 were in residues conserved within the EAL domain that are putatively required for Mg\(^{2+}\) binding (E29 and Q219) (27), while c-di-GMP-binding residues (D156 and D165) affected FlhC production and motility to a lesser extent. 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 F fimbrial operons present in the CFT073 genome (28), as the ΔydiV mutant (n = 10 mice). When in direct competition with wild-type CFT073, the ΔydiV mutant was less fit in the kidneys of mice (8.7-fold, P = 0.0166). There was no significant difference from wild-type levels in bladder colonization (P = 0.8469). Therefore, YdiV contributes to successful in vivo colonization of the upper urinary tract.

**DISCUSSION**

YdiV, a versatile protein involved in repression of both motility and sessility, contributes to the control of motility and P fimbria-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 (Fig. 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, the ΔydiV mutant is significantly more motile than the wild-type strain (references 11 and 13 and this study). In E. coli K-12, one study has proposed that ydiV expression is inhibited posttranslationally 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 and FlhC production to the greatest extent in E. coli CFT073 were in residues conserved within the EAL domain that are putatively required for Mg\(^{2+}\) binding (E29 and Q219) (27), while c-di-GMP-binding residues (D156 and D165) affected FlhC production and motility to a lesser extent. 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 F fimbrial operons present in the CFT073 genome (28), as the ΔydiV mutant

**FIG 5** The ΔydiV mutant produces an extracellular matrix. (A) Biofilm formation in M9 minimal medium with glucose as the sole carbon source. Transmission electron microscopy of negatively stained CFT073 ΔydiV (B) and ΔydiV (pYdiV) (C). Bacteria were cultured for 3 h with aeration at 37°C in LB. Bar, 1.0 μm.
has increased levels of *papA_2* gene expression and PapA_2 protein compared to wild-type *E. coli* CFT073. Furthermore, deletion of either *crp* or *cya* restores the Δ*ydiV* 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 (Fig. 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* does not affect *ydiV* gene expression. However, the double deletion Δ*ydiV ΔsdiA* significantly reduced the expression of *cya*, the gene encoding adenylate cyclase, and *crp*, the gene encoding CAP, 2-fold. Thus, YdiV and SdiA are involved in activation of gene expression of CAP and the enzyme that synthesizes cAMP (Fig. 6), which is consistent with the observation by Zhou et al. (24) that Δ*ydiV ΔsdiA* 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 the Δ*ydiV ΔsdiA* mutant, while hyperadherent compared to wild-type *E. coli* CFT073, is less adherent than the Δ*ydiV* mutant, 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 (Fig. 6). While present in both pathogenic and commensal strains of *E. coli*, this protein has apparently taken on additional 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.

**ACKNOWLEDGMENT**

This work was supported by Public Health Service grants AI059722 and DK094777 from the National Institutes of Health.

**REFERENCES**


