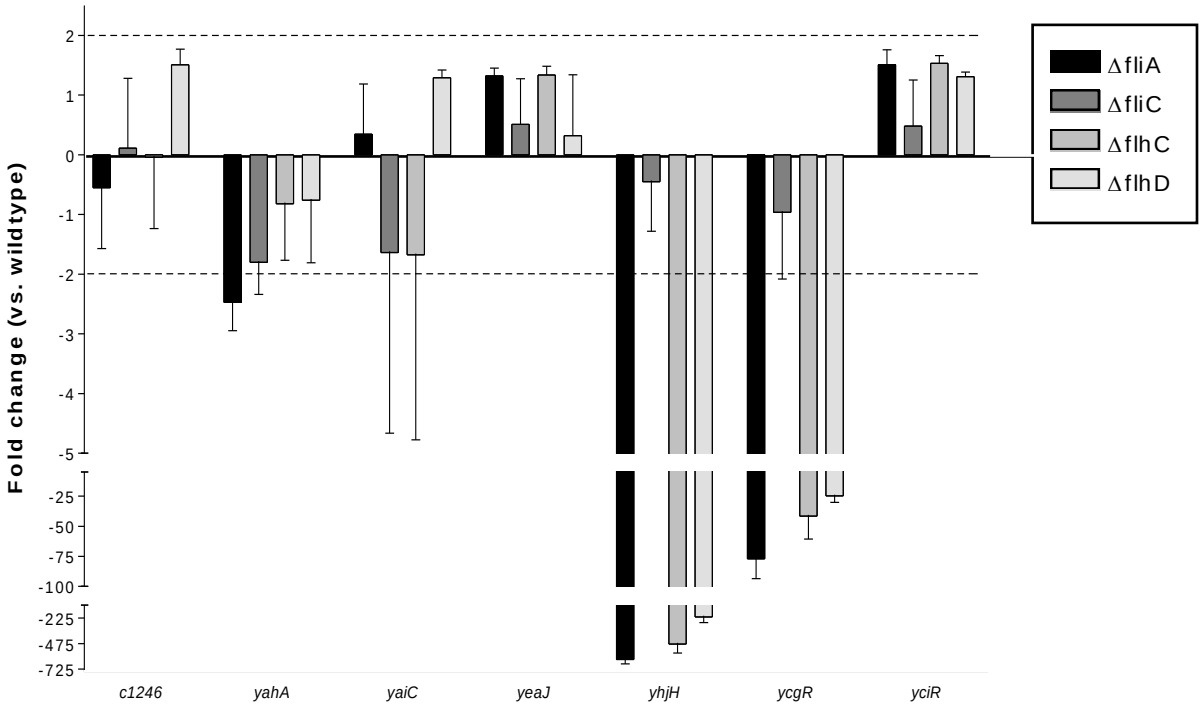
Figure 1.

A


Fold change (vs. wildtype)
2
1
0
-1
-2
-3
-4
-5
-25
-50
-75
-100
-225
-475
-725
c1246
yahA
yaiC
yeaJ
yhjH
ycgR
yciR
ΔfliA
ΔfliC
ΔflhC
ΔflhD

B

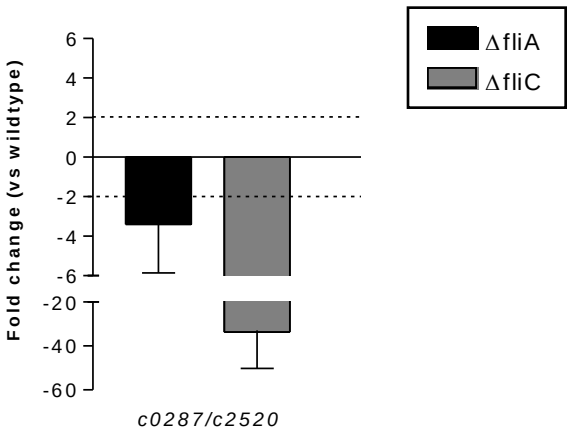
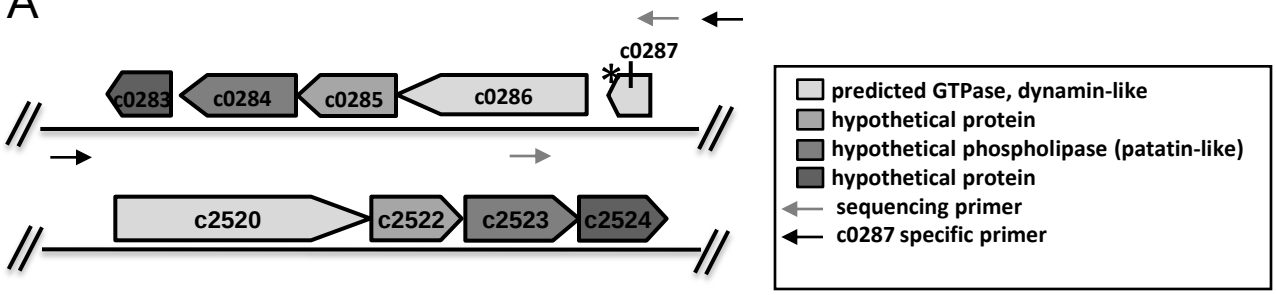

Fold change (vs wildtype)
6
4
2
0
-2
-4
-6
-20
-40
-60
c0287/c2520
ΔfliA
ΔfliC

Figure 2.

A


c0287
c0283
c0284
c0285
c0286
c2520
c2522
c2523
c2524
predicted GTPase, dynamin-like
hypothetical protein
hypothetical phospholipase (patatin-like)
hypothetical protein
sequencing primer
c0287 specific primer

B

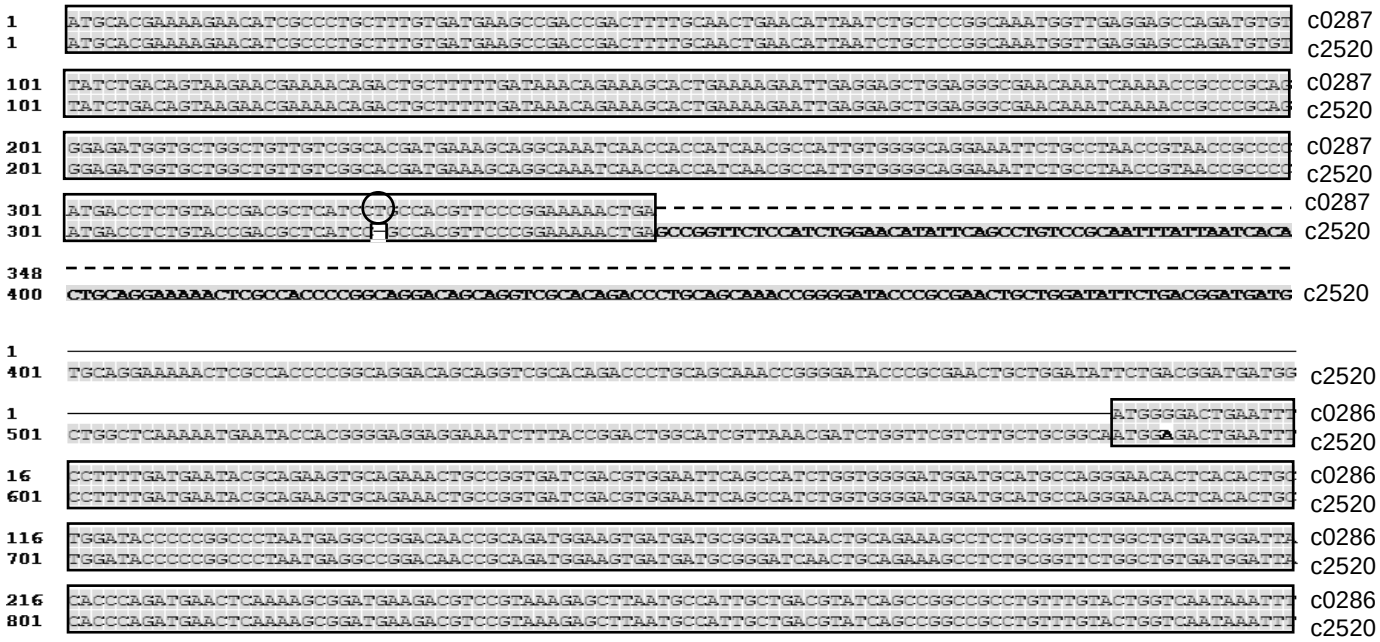

1 ATGCACGAAAAGAACATCGCCCTGCTTTGTGATGAAGCCGACCGACTTTTGCAACTGAACATTAATCTGCTCCGGCAAATGGTTGAGGAGCCAGATGTGT c0287
1 ATGCACGAAAAGAACATCGCCCTGCTTTGTGATGAAGCCGACCGACTTTTGCAACTGAACATTAATCTGCTCCGGCAAATGGTTGAGGAGCCAGATGTGT c2520
101 TATCTGACAGTAAGAACGAAAACAGACTGCTTTTTGATAAACAGAAAGCACTGAAAAGAATTGAGGAGCTGGAGGGCGAACAAATCAAAACCGCCCGCAG c0287
101 TATCTGACAGTAAGAACGAAAACAGACTGCTTTTTGATAAACAGAAAGCACTGAAAAGAATTGAGGAGCTGGAGGGCGAACAAATCAAAACCGCCCGCAG c2520
201 GGAGATGGTGCTGGCTGTTGTCGGCACGATGAAAGCAGGCAAATCAACCACCATCAACGCCATTGTGGGGCAGGAAATTCTGCCTAACCGTAACCGCCCC c0287
201 GGAGATGGTGCTGGCTGTTGTCGGCACGATGAAAGCAGGCAAATCAACCACCATCAACGCCATTGTGGGGCAGGAAATTCTGCCTAACCGTAACCGCCCC c2520
301 ATGACCTCTGTACCGACGCTCATCCTTGCCACGTTCCCGGAAAAACTGA c0287
301 ATGACCTCTGTACCGACGCTCATCCTTGCCACGTTCCCGGAAAAACTGAGCCGGTTCTCCATCTGGAACATATTCAGCCTGTCCGCAATTTATTAATCACA c2520
348
400 CTGCAGGAAAAACTCGCCACCCCGGCAGGACAGCAGGTCGCACAGACCCTGCAGCAAACCGGGGATACCCGCGAACTGCTGGATATTCTGACGGATGATG c2520
1
401 TGCAGGAAAAACTCGCCACCCCGGCAGGACAGCAGGTCGCACAGACCCTGCAGCAAACCGGGGATACCCGCGAACTGCTGGATATTCTGACGGATGATGG c2520
1 ATGGGGACTGAATTT c0286
501 CTGGCTCAAAAATGAATACCACGGGGAGGAGGAAATCTTTACCGGACTGGCATCGTTAAACGATCTGGTTCGTCTTGCTGCGGCAATGGAGACTGAATTT c2520
16 CCTTTTGATGAATACGCAGAAGTGCAGAAACTGCCGGTGATCGACGTGGAATTCAGCCATCTGGTGGGGATGGATGCATGCCAGGGAACACTCACACTGC c0286
601 CCTTTTGATGAATACGCAGAAGTGCAGAAACTGCCGGTGATCGACGTGGAATTCAGCCATCTGGTGGGGATGGATGCATGCCAGGGAACACTCACACTGC c2520
116 TGGATACCCCCGGCCCTAATGAGGCCGGACAACCGCAGATGGAAGTGATGATGCGGGATCAACTGCAGAAAGCCTCTGCGGTTCTGGCTGTGATGGATTA c0286
701 TGGATACCCCCGGCCCTAATGAGGCCGGACAACCGCAGATGGAAGTGATGATGCGGGATCAACTGCAGAAAGCCTCTGCGGTTCTGGCTGTGATGGATTA c2520
216 CACCCAGATGAACTCAAAAGCGGATGAAGACGTCCGTAAAGAGCTTAATGCCATTGCTGACGTATCAGCCGGCCGCCTGTTTGTACTGGTCAATAAATTT c0286
801 CACCCAGATGAACTCAAAAGCGGATGAAGACGTCCGTAAAGAGCTTAATGCCATTGCTGACGTATCAGCCGGCCGCCTGTTTGTACTGGTCAATAAATTT c2520

Figure 3.

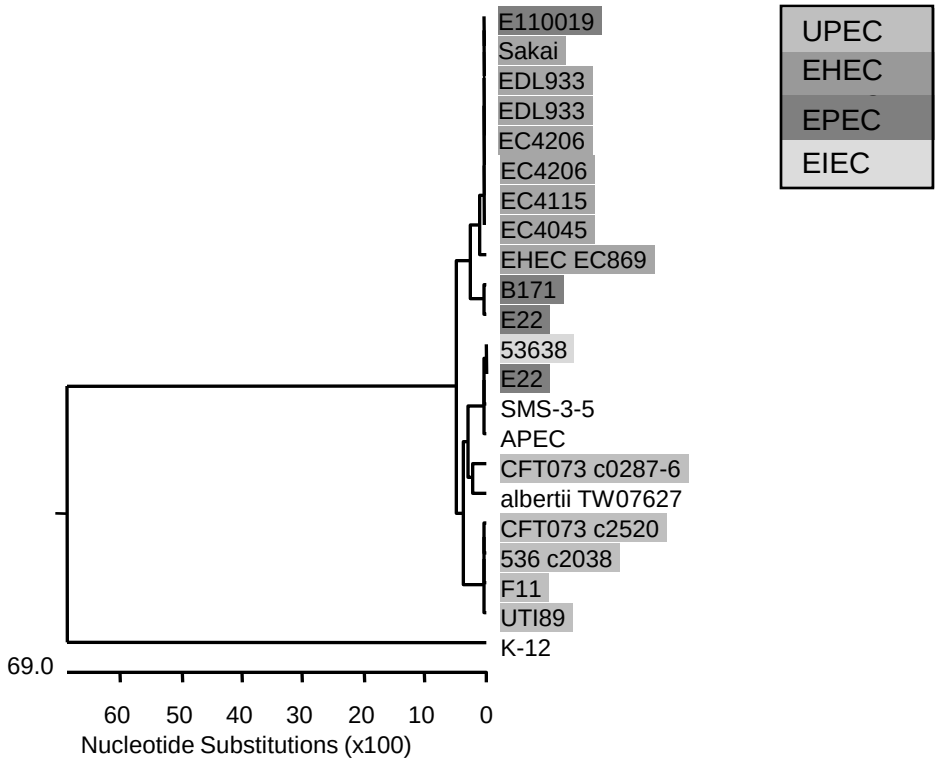
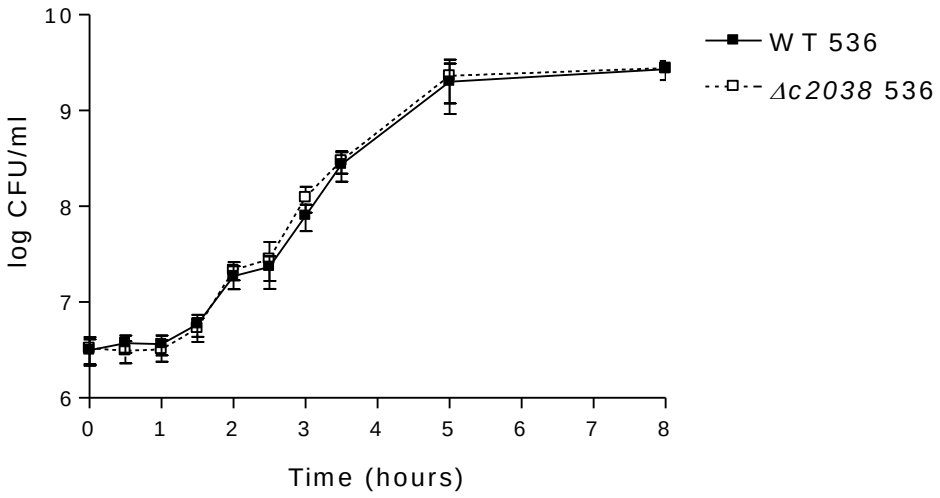

E110019
Sakai
EDL933
EDL933
EC4206
EC4206
EC4115
EC4045
EHEC EC869
B171
E22
53638
E22
SMS-3-5
APEC
CFT073 c0287-6
albertii TW07627
CFT073 c2520
536 c2038
F11
UTI89
K-12
UPEC
EHEC
EPEC
EIEC
69.0
60
50
40
30
20
10
0
Nucleotide Substitutions (x100)

Figure 4.

A


10
9
8
7
6
log CFU/ml
0
1
2
3
4
5
6
7
8
Time (hours)
WT 536
Δc2038 536

B

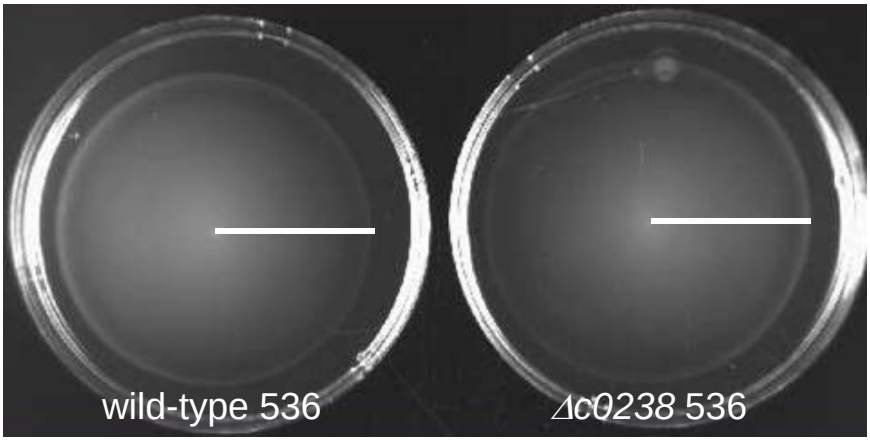

wild-type 536
Δc0238 536

Figure 5.

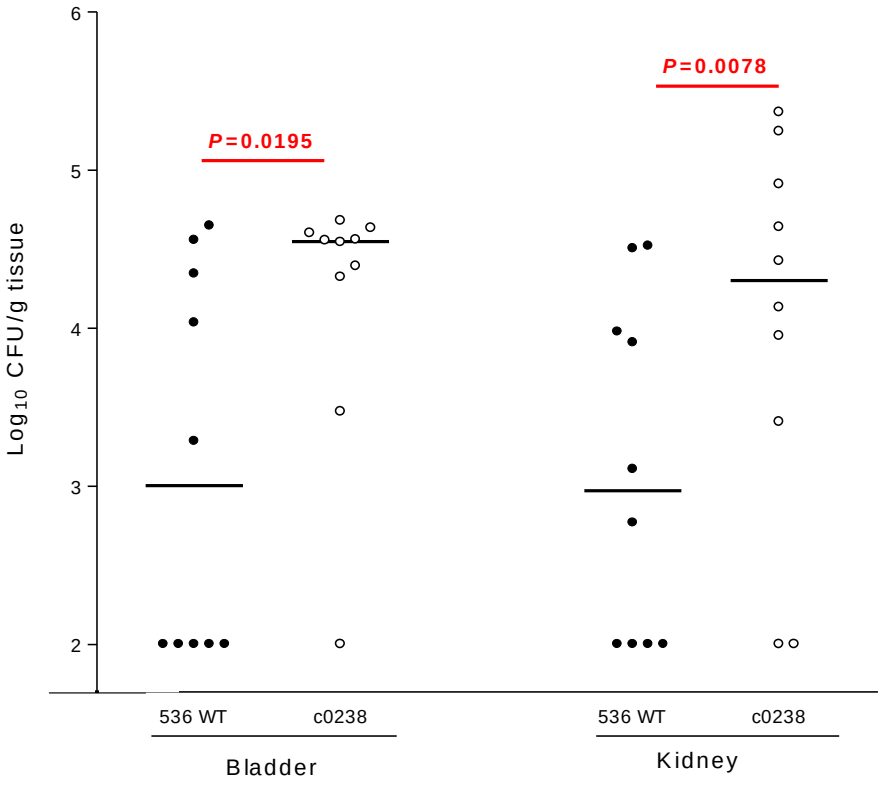
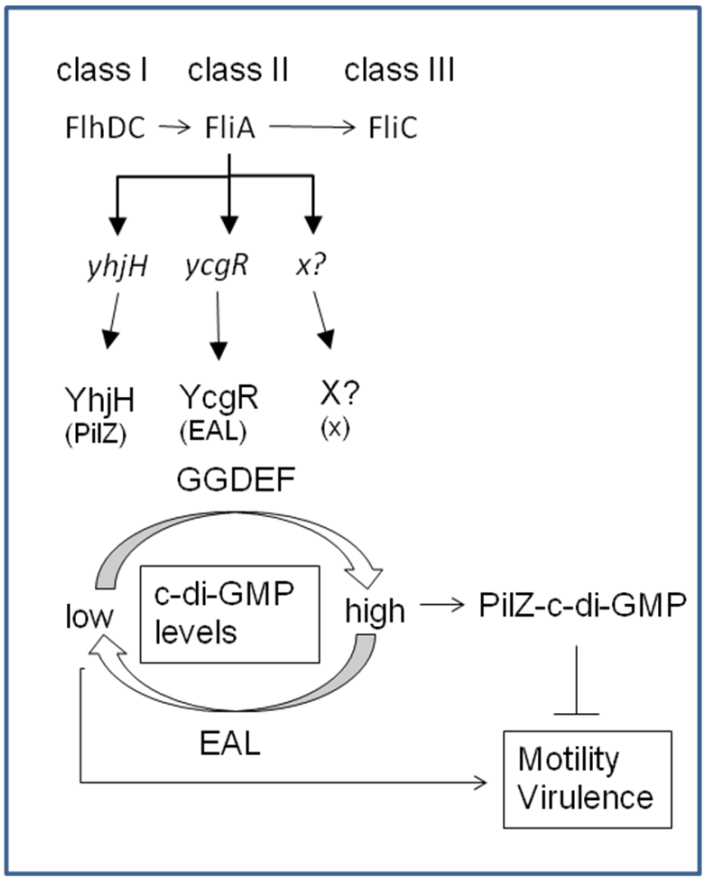

6
5
4
3
2
Log10 CFU/g tissue
P=0.0195
P=0.0078
536 WT
c0238
536 WT
c0238
Bladder
Kidney

Figure 6.



Adapted from Claret *et al.*

The flagellar sigma factor FliA transcriptome links motility to c-di-GMP signaling and to a putative GTPase in uropathogenic *Escherichia coli.*

Thesis research performed and original manuscript written by Stefanie A. Deeds.

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ABSTRACT

FliA, the flagellar sigma factor, is critical for the motility of *Escherichia coli*. The role of FliA in controlling the transcription of class III flagellar genes is well characterized. Some studies have suggested that FliA may play a regulatory role on other virulence factors in addition to motility. To examine this further, in this study we compared the global transcriptional profiles of wild type and a *fliA* mutant of uropathogenic *E. coli* (UPEC) strain CFT073 using microarray analyses. We identified a novel set of genes that may be under the direct or indirect control of FliA. Among the 56 genes that were significantly differentially expressed were two genes of the c-di-GMP signaling pathway (*yhjH* and *yaiC*). Expression of two hypothetical genes annotated as putative GTPases were also reduced in the *fliA* mutant (c0287 and c2520). We confirmed that the expression of all of these genes was decreased in a *ΔfliA* background using qRT-PCR. Mutation of the homologous GTPase in UPEC strain 536 had no effect on motility or growth, but surprisingly resulted in enhanced colonization of the urinary tract in a mouse model (bladder P=0.0195 and kidney P=0.0078).

INTRODUCTION

More than 80% of uncomplicated urinary tract infections are caused by the Gram-negative bacterium *Escherichia coli* (Mobley 1996). Infections occur through the ascending route in which bacteria enter the urinary tract via the urethra, colonize the bladder, and ascend the ureters to reach the kidneys (Bacheller and Bernstein 1997). Strains of pathogenic *E. coli* have acquired virulence genes that can distinguish them from commensal strains. Virulence genes are often located on pathogenicity islands and include adhesins, toxins, serum resistance factors, and iron acquisition systems (Lloyd *et al.* 2007, Johnson 1991). Many of these genes contribute to successful colonization of the urinary tract by uropathogenic *E. coli* (UPEC). Although much is known about the contribution of established virulence factors and motility in pathogenesis, the genome sequences of pyelonephritis strain CFT073 and other UPEC strains have revealed that much of the UPEC genome encodes genes with unknown function, some of which are candidate virulence determinants that may play roles in UTI pathogenesis.

In addition to possessing a unique set of virulence genes, UPEC takes advantage of motility to ascend the urinary tract (Lane *et al.* 2007), specifically from the bladder to the kidneys via the ureters. Motility is tightly regulated by a three tiered transcriptional cascade of flagellar genes. The class I genes are *flhD* and *flhC*, which encode the heterotetrameric transcription factor FlhDC. FlhDC in turn positively regulates the transcription of class II genes which include structural and assembly proteins for the basal body and hook of the flagellum, as well as *fliA*.

FliA is the flagellar sigma factor, σ^{28}. FliA up-regulates the transcription of class III genes that encode the filament, FliM (anti-σ^{28} factor), motor proteins, and two chemotaxis operons (Kalir *et al.* 2001, Macnab 1991, and Chilcott and Hughes 2000). When *fliA* is mutated the resulting non-motile strain of *E. coli* is less effective at colonizing the urinary tract (Lane *et al.* 2005). Thus, up-regulation of motility by FliA enhances the fitness of UPEC during infection.

Recent studies have found that, in addition to the known flagellar genes, FliA is responsible for regulating other genes, some of which contribute to virulence. In *Salmonella enterica* serovar Typhi, *fliA* mutants failed to enter cultured epithelial cells, suggesting that flagellar regulatory proteins may influence invasion (Eichelberg and Galan 2000). They found that FliA up-regulates genes associated with the SPI-1 encoded type III secretion (T3SS). In contrast, FliA down-regulates plasmid-encoded virulence genes, including the T3SS via the transcriptional activator VirF, in *Yersinia enterocolitica* and down-regulate pectate lyase activity in the plant pathogen *Dickeya dadantii* (Horne and Pruss 2006, Jahn *et al.* 2008). In addition, a study by Carrillo *et al.* found that the flagellar regulatory system plays an important role in *Campylobacter jejuni* pathogenesis. In this study, they found that *fliA* mutants had decreased invasion and adhesion, which was partially restored by overexpression of the phosphodiesterase A (PDEA) *yhjH* and *yahA*, which are involved in breaking down the bacterial secondary messenger 3', 5'-cyclic diguanylic acid (c-di-GMP) (Carrillo *et al.*). Thus, FliA may play a role in c-di-GMP-dependent control. These studies provide evidence for an overlap between regulatory control of flagellar and non-flagellar gene expression. However, our current understanding of the regulatory effects of FliA on non-flagellar and potentially virulent genes in UPEC is limited.

In this study, we examined the global transcriptional profile of FliA in pyelonephritis strain CFT073 using microarray analysis to compare the transcriptome of wild-type CFT073 to that of the non-motile *ΔfliA* CFT073. We observed that regulatory proteins of the c-di-GMP signaling pathway and a novel putative GTPase are down-regulated in a *ΔfliA* background. Little is known

about the putative GTPase and its role in UPEC. We believe that, if functional, this GTPase may play a role in the virulence pathogenic *E. coli*.

RESULTS

Transcriptional regulation by FliA E. coli CFT073.

To better understand the global regulatory effect of FliA we used microarrays to analyze the transcriptome of *ΔfliA vs.* wild-type *E. coli* CFT073*.* RNA was isolated from *ΔfliA* and wild-type CFT073 cell cultures grown to late-exponential phase in rich medium. Based on the number of differentially expressed genes, there appears to be an extensive physiological response in *fliA* mutant cells. In total, 785 genes were differential expressed. Fifty-six of these genes were considered significantly different, with a fold-change of two or greater from wild-type expression.

Nineteen genes were found significantly up-regulated, while 37 genes were found significantly down-regulated in a *ΔfliA* background as compared to wild-type CFT073 (Table 1). As expected, the expression of a number of class III flagellar genes was highly reduced in the *ΔfliA* mutant. Specifically, *cheBYZ, motBAcheAW*, *fliC*,and *fliA* itself were among the top 35 most down-regulated genes in *ΔfliA* vs. wild-type. These results provide validation of our microarray.

Because we were interested in identifying novel genes positively regulated by FliA, we focused on those genes that were down-regulated in a *ΔfliA* background (Table 1). In addition to flagellar and motility related genes, expression of a variety of non-flagellar and hypothetical genes was reduced. Among those genes that we found most interesting because of their potential to be

related to virulence were those from the c-di-GMP signaling pathway and hypothetical proteins annotated as putative GTPases.

Microarray analysis revealed that two genes from the c-di-GMP signaling pathway, *yhjH* and *ycgR*, were significantly down-regulated in the Δ*fliA* mutant. Transcription of *yhjH* and *ycgR* were 3.26 and 2.47 down-fold, respectively (Table 2). In *E.* coli, YhjH is an EAL domain protein and YcgR is a PilZ protein, both of which play a role in regulating levels of the secondary messenger c-di-GMP. Two more genes from this pathway appeared to have reduced expression in the *ΔfliA* microarray, although they were not statistically significant (*yaiC* and *yciR*). As validation of the microarray, we confirmed that *yhjH* and *ycgR* were significantly down-regulated in the *ΔfliA* mutant using qRT-PCR (Figure 1A).

A large number of hypothetical proteins were also down-regulated in the *fliA* mutant. Of particular interest were the genes c0283 – c0287. The probes that recognize c0287 and c0285 also recognize c2520 and c2522 respectively, as these two loci are nearly identical. c2520 and c0287 are both annotated as hypothetical proteins related to the dynamin family of the Ras-like superfamily of GTPases. We confirmed that c0287/c2520 were down-regulated in the *ΔfliA* mutant vs. wild-type CFT073 via qRT-PCR (Figure 1B). Because of their interesting annotation and unknown connection to motility and virulence, we chose to focus on these genes throughout the rest of our study.

Overall, our microarray analysis revealed significant changes in a number of non-motility associated genes (Table 1). These data suggest that FliA is playing a role in regulating more than just class III flagellar genes in UPEC CFT073.

CFT073 has two copies of a putative GTPase gene cluster.

Aligning the two loci revealed that there was 100% identity at the nucleotide sequence level between c0287 and the first 348 nucleotides of c2520 (Figure 2B) and 93.9% identity between c0286 and the last 1811 nucleotides of c2520. Among the differences between c0287 and c2520 is a single thymine insertion in the c0287 sequence after bas pair 348 that causes a possible frame shift mutation.

To discover if the presence of the thymine nucleotide, and thus the resulting frame shift, was a sequencing error, we submitted c0287 – c0286 for sequencing. Because of the great homology to the *c2520* region, we first had to amplify c0287-c0286 specifically by designing primers flanking outside of c0287 – c0283, where the sequence differs from the c2520 region. We used the c0287-c0283 product and primers flanking the region around the thymine for sequencing. Our results indicate that the extra thymine is not present in CFT073, and is thus misannotated as two separate genes (Figure 2B). Henceforth, we will refer to c0287-c0286 as one gene, c0287. When this thymine is no longer in the sequence, the identity between c0287 and c2520 is 95.4% at the nucleotide level and is 93.5% at the amino acid level. The high sequence identity suggests that there may have been a duplication of one copy of the locus which reinserted elsewhere in the genome.

The first gene of each of the two loci, c2520 and c0287, is annotated as a member of the Ras-like GTPase superfamily and are encoded by the genomic island-CFT073-*cobU* and the pathogenicity island-CFT073-*aspV*, respectively (Lloyd *et al.* 2007). The Ras superfamily is divided into at least four families in eukaryotes, which play roles in gene expression, cytoskeleton reorganization, vesicle trafficking, nucleocytoplasmic transport and microtubule organization. Members of the Ras superfamily have a GTP binding site and two switch regions. Our Ras-like GTPase is related to the dynamin family of GTPases. The second gene of each loci, c2522 and c0285, is a hypothetical protein with no annotated domains. The third gene of

the loci, c2523 and c0284, is annotated as a patatin-like phospholipase. The patatin superfamily consists of various patatin glycoproteins from plants and is known for catalyzing the cleavage of fatty acids from membrane lipids.

Because of the homology between these two gene regions and the proximity of the genes within each region, we speculated that the genes may be transcribed in an operon. To uncover the nature of these genes, we performed RT-PCR on the cDNA collected for the c0287/c2520 qRT-PCR . If the RT-PCR amplifies the region between the genes of the cDNA, then a product would be revealed on the agarose gel, confirming the genes are transcribed as one operon. However, because of the homology between c0284 and c2523*,* the primers used could amplify both regions. Using these primers we performed RT-PCR on the wild-type cDNA. When visualized on an agarose gel, the control genomic DNA showed a band indicating amplification of this region, but the cDNA template did not yield a product. Thus, it does not appear that either of the GTPase gene clusters, c0287-c0284 or c2520-c2523, are transcribed as one transcript (data not shown).

CFT073 has two copies of a genes locus that contains a dynamin-like GTPase (Figure 2A). However, little is understood about the role a bacterial dynamin-like protein may have and the prevalence of functional GTPases among bacterial species.

The putative GTPase is pathogen-specific.

To determine how conserved the GTPase-like gene is among various strains of *E.* coli, we performed BLAST analysis using c0287 and c2520 amino acid sequences. It appears that the putative GTPase is not present in the commensal strain K-12 *E. coli*, but homologs were found among *E. coli* pathogenic strains. Other *E. coli* strains contain proteins with greater than 90%

identity to c2520, and the greatest identity was seen in UPEC strains F11 and UTI89. Other bacterial species, such as *Burkholderia vietnamiensis, Enterobacter cancerogenus, Citrobacter koseri, Shigella flexneri*, and *Shigella sonnei*, show 30-40% identity at the amino acid level. BLAST revealed only 34% identity with a conserved protein with a nucleoside triphosphate hydrolase domain in *E. coli* K-12 (non-pathogenic strain). We used MegAlign protein alignment ClustalW to create a phylogenic tree of c2520 homologs of various pathogenic strains of *E. coli* (Figure 3). This revealed that the putative GTPase is conserved among *E. coli* pathogens. The enterohemorrhagic *E. coli* strains, along with most of the enteropathogenic *E. coli* strains, cluster together on one branch of the phylogenetic tree, while UPEC clusters on a separate branch. Interestingly, most of the strains had one copy of this GTPase gene, while EDL933, E22, CFT073 each appear to have two copies. These data lead us to believe that these genes may be pathogen-specific.

Expression of candidate putative GTPase genes.

In vitro expression of the putative GTPase was analyzed by qRT-PCR in WT CFT073, *ΔfliA* CFT073, and *ΔfliC* CFT073. Because of the high sequence identity between c0287 and c2520, primers were designed that recognized both genes, similar to the probes used in the microarray. The results indicate that expression is down-regulated in both *ΔfliA* CFT073 and *ΔfliC* CFT073 as compared to WT CFT073 (Figure 1B). This validated the expression seen in the *ΔfliA* CFT073 vs. WT CFT073 microarray. Interestingly, *ΔfliA* CFT073 showed 3.8-fold less expression of c0287 and c2520 than WT CFT073 while *ΔfliC* CFT073 had about ten times as much reduced expression at 35-fold. This indicates that the observed decreased expression levels may not be a direct effect of limited FliA in the cell, but rather an indirect response to limited motility.

In vitro *characterization of putative GTPase mutant.*

To identify a phenotype associated with the GTPase genes, we used the lambda red recombinase system to construct a deletion mutation in c0287. Unfortunately, we were unable to successfully make a mutation in the second copy, c2520, in CFT073. This suggests that the growth conditions may be too stringent for the *c2520* mutant to grow or that this copy of the gene is more stable than c0287. The ∆c0287 single mutant had no apparent phenotype in motility or growth, but this may be due to the fact that the second copy is still intact.

To examine the function of this gene in a similar well recognized pyelonephritis strain (Brzuszkiewicz *et al.* 2006), we made a mutation in UPEC strain 536, which only has one copy of the putative GTPase, c2038. c2038 has over 99% identity to c2520 of CFT073 at the nucleotide sequence level. To check for growth defects in 536 ∆c2038, we cultured cells in LB for 8 hours and periodically measured OD_{600} and counted CFUs/ml of 536 ∆c2038 vs. WT 536 (Figure 4A). No growth defect was seen in 536 ∆c2038, indicating that the putative GTPase does not effect replication of 536 cells in rich medium culture. We also looked for a motility phenotype in 536 ∆c2038 by stabbing cells into soft agar plates and observing cells under a microscope (Figure 4B). The 536 c2038 mutant had no apparent defect in motility.

Role of putative GTPase in virulence in the mouse model of ascending UTI.

To study the role of the GTPases *in vivo*, we used a mouse model of ascending urinary tract infection. We examined this by competitive co-challenge experiments in which mice were transurethrally inoculated with a 1:1 mixture (10^8 CFU/mouse) of wild-type 536 and the c2038 mutant. After 48 hours of infection, mice were sacrificed and harvested for bladder and kidney. CFU/gram of tissue were determined (Figure 5). Statistical significance using the Wilcoxon matched-pairs test was observed in the numbers of wild-type 536 and 536 *∆c2038* recovered from bladder (P=0.0195) and kidney (P=0.0078). Surprisingly, the c2038 536 mutant outcompeted wild-type 536. These data indicate that loss of the putative GTPase enhances the

fitness of UPEC during bladder and kidney infection. It will be necessary to complement the mutation with cloned c2038 to determine whether CFU/g tissue would be restored to wild-type levels.

DISCUSSION

To explore previously uncharacterized regulatory pathways in *E. coli* CFT073, microarray analysis was used to examine the transcriptome of non-motile strain *ΔfliA* as compared to wild-type CFT073. As expected, when *fliA* transcription is repressed, class III motility genes are, in turn, down-regulated (Table 1). Microarray and qPCR indicated that non-flagellar genes are also being regulated by FliA. In this study, we found that several genes of the GMP signaling pathway and two gene clusters containing a dynamin-like GTPase were down-regulated in a *ΔfliA* background. Mutation of a homologous GTPase in pyleonephritis strain 536 did not show a growth or motility defect. However, wild-type 536 was out-competed by this mutant during experimental co-challenge of mice. Although its function remains unknown, this putative GTPase appears to be regulated by FliA or motility and the loss of it increases UPEC fitness in the urinary tract.

Our microarray and qRT-PCR analysis indicate that FliA regulates *ycgR* and *yhjH*, genes that encode proteins that play a role in the GMP signaling pathway. These data confirm observations made in adherent-invasive *E. coli* and *Chlamydia trachomatis* (Claret *et al.* 2008 and Shen *et al.* 2006).The bacterial GMP signaling pathway controls cellular levels of c-di-GMP (Figure 6). The second messenger c-di-GMP regulates many critical processes of bacterial survival, and thus it is tightly controlled. c-di-GMP is synthesized by diguanylate cyclase (DGC) and degraded by phosphodiesterase A (PDEA) (as reviewed by Tamayo *et al.*2007). A common feature for activity of DGCs and PDEAs are GGDEF and EAL amino acid domains, respectively. In

addition, c-di-GMP levels are affected by proteins containing a characteristic PilZ domain, which are able to bind c-di-GMP and mediate downstream physiological processes. YcgR contains the EAL domain of a PDEA, while HyjH contains a PilZ domain.

Studies have shown that c-di-GMP inhibits bacterial motility in *Salmonella* Typhimurium, *V. cholerae*, *P. aeruginosa*, and commensal *E. coli* (Simm 2004 and Beyhan *et al.* 2006*)*. Thus, regulation of c-di-GMP has implications for motility-based fitness during infection.

fliA mutants of a Crohns-disease associated adherent-invasive *E. coli* (AIEC) displayed decreased adhesion and invasion of intestinal epithelial cells (Claret *et al.* 2007). This decreased pathogenicity was attributed to a decrease in type 1 pili synthesis, and was partially restored by overexpression of the PDEAs *yhjH* and *yahA*. Invasion and adhesion were unaffected by overexpression or mutation of the DGC *yaiC* or mutation of the PilZ domain protein YcgR. Our microarray and qRT-PCR data suggest that FliA may also regulate *yhjH* and *ycgR* (Table 1 and Figure 1A) in UPEC CFT073, but unlike AIEC, there has been no evidence for FliA control of pili synthesis. It is possible that in UPEC CFT073, the role of FliA in c-di-GMP-dependent control may be unique and lead to a specific physiological response that enhances fitness or pathogenicity in the host urinary tract. More studies are needed to elucidate this possible mode of regulation.

In our microarray analysis we identified a previously uncharacterized gene cluster that contained a putative GTPase. Two copies of this gene cluster are present in CFT073, c0287-c0283 and c2520-c2524. The high sequence identity between these loci suggests that there was a duplication of this region. Interestingly, both c2520 and c0287 were previously identified as UPEC specific genes in a study analyzing ten UPEC and four fecal/commensal strains of *E.* coli (Lloyd *et al.* 2007). These data indicated that c2520 and c0287 may play a role during infection.

It was suggested in a previous paper that *E. coli* contained genes similar to the bacterial dynamin-like protein (BDLP) of *Nosdoc punctiforme* (Low and Lowe 2006). Dynamin is a class of GTPase in eukaryotes known for its role in vesicle formation. BLAST analysis revealed that c2520 had 47% identity with the amino acid sequence 71 to 114 of the dynamin-like GTPase in *N. punctiforme*. In this region is the conserved threonine 103 which is known to be critical for catalysis in GTPases, suggesting that the GTPases in UPEC CFT073 may also be functional. In the study of Low and Lowe, analysis of the BDLP revealed that it could self-assemble and tubulate liposomes. Further, the BDLP primarily localized to the membrane and may insert into the membrane to promote membrane curvature. The authors of this study suggested that the bacterial dynamin-like GTPase of *N. punctiforme* might play a role in cell wall morphology and shape.

In this study, we describe the novel dynamin-like GTPase c2038 in pyleonephritic strain 536 *E. coli*. We tested motility and colonization in the ∆c2038 background. Motility was unaffected and surprisingly, the mutant was found at levels significantly higher than wild-type in both the bladder and kidney. The unexpected results raised the question of how the loss of this putative GTPase could have an effect on survival of CFT073 in the urinary tract.

There is the possibility that the GTPases seen in pathogenic *E. coli* play a role in cell morphology like the BDLP of *N. punctiforme*; however, another possibility is that the genes play a role in signaling. In a study by Roesch *et al.*, a D-serine metabolism mutant in CFT073 that significantly outcompeted wild-type CFT073 in the murine model of ascending UTI. In a later study from the same lab, they identified several virulence genes that are upregulated in the mutant and responsible for this hypercolonization (Haugen *et al.* 2007). Our mutant may be involved in an analogous pathway that attributes for the hypercolonization. This pathway would link motility regulation to virulence *in vivo*. It would be interesting to perform microarray analysis to identify if virulence genes are up-regulated in 536 ∆c2038.

Another possibility is that it is energy expensive to synthesize the GTPase, so without a functional copy of the gene, 536 can utilize its energy in a manner which allows it to more efficiently colonize the urinary tract. Eliminating this energy cost could lead to enhanced fitness and survival in the urinary tract. Alternatively, the dynamin-like GTPase genes of UPEC may be functionally similar to the BDLP of *N. punctiforme*. We do not yet understand how disrupted cell morphology could contribute to the hypervirulence seen in the *c2038* 536 mutant. It is possible that, like eukaryotic dynamin, the GTPase plays a role in vesicle formation and thus may play a role in nutrient acquisition or toxin transportation. Further analysis of *c0287* and *c2520* should reveal whether or not these genes are functional GTPases, determine if these genes are directly regulated by FliA, and uncover the role they are playing in pathogenic *E. coli*.

EXPERIMENTAL TECHNIQUES

Bacterial strains and culture conditions.

UPEC strain CFT073 was isolated from blood and urine of a patient with acute pyelonephritis (Mobley *et al.* 1990). Single colonies were used to inoculate overnight liquid cultures of Luria-Bertani broth (LB) and incubated at 37°C for 18 h with aeration (200rpm). For RNA preparation, wild type and mutant strains of CFT073 were grown at 37°C with aeration (200rpm) in 150 ml or 50 ml of LB until the optical density at 600 nm (OD_{600}) reached 0.65 ± 0.05. At this time cultures were immediately prepared for RNA extraction. For RNA preparation, a 500 µl sample of bacterial culture was stabilized in 1 ml of RNAprotect bacterial reagent (QIAGEN). The RNAprotect treated-samples were then centrifuged and the supernatant was removed. Pellets were stored overnight at -20°C.

RNA isolation and cDNA synthesis.

RNA was extracted from bacterial samples using the RNeasy Mini Kit (QIAGEN) according to the manufacturer's handbook. RNA was TURBO DNase (Ambion) treated and agarose gel electrophoresis was used to verify yield of RNA samples. Following the manufacturer's protocol for the SuperScript First-Strand Synthesis (Invitrogen) system cDNA was synthesized using reverse transcriptase PCR. To remove present RNA, cDNA samples were digested with RNase H (Invitrogen) and then purified using the QIAquick Gel Extraction kit (QIAGEN) following the QIAquick Spin handbook for PCR purification.

Microarray and statistical analysis.

RNA samples from three independent replicate cultures of CFT073 and CFT073 Δ*fliA* were obtained as described above. The integrity of the RNA samples were tested using the BioAnalyzer 2100 (Agilent). RNA was labeled, hybridized, and scanned using Affymetrix *E. coli* Genome 2.0 GeneChips following the manufacturer's prokaryotic protocol, as performed by the University of Michigan Comprehensive Cancer Center Affymetrix and Microarray Core Facility. The data were analyzed by the UMCCC Microarray Core.

Quantitative reverse transcription-PCR (qRT-PCR) and statistical analysis.

Primers designed to amplify *yahA*, *yaiC*, *c1246*, *ycgR, yciR, yeaJ, yhjH*, *c0287/c2520* and *gapA* were targeted to unique regions within each respective gene of *E. coli* CFT073. Two nanograms of cDNA and 300 nM of each primer were mixed with 12.5 μl of 2x SYBER Green PCR Master Mix (Stratagene) per well. Assays were performed in triplicate with the Stratagene Mx3000P instrument. All data were normalized to the endogenous reference gene *gapA*. Melting curves analysis demonstrated that the accumulation of SYBR Green-bound DNA was not due to primer dimers or genomic DNA contamination. Data were analyzed by the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001).

Construction of CFT073 deletion mutants.

Deletion mutants of flagellar genes were generated using the lambda red recombinase system (Datsenko and Wanner 2000). Primers homologous to sequences within the 5' and 3' ends of the target genes were designed and used to replace the genes with a nonpolar kanamycin resistance cassette derived from the template plasmid pKD4 (Table 3) (Datsenko and Wanner 2000). Less than 1% of the targeted gene sequence remained after homologous recombination. Kanamycin (25 μg/ml) was used for selection of all mutant strains. Primers that flank the target gene sequence were designed (Table 3) to determine whether the kanamycin

resistance cassette recombined within the target site. Both wild-type and mutant gene sequences were amplified with each set of primers using *Taq* DNA polymerase (New England Biolabs) or Phusion Hot Start High-Fidelity DNA polymerase (New England Biolabs). Each amplified PCR product was electrophoresed on a 1.0% agarose gel and visualized, along with a 1 kb + DNA ladder (Invitrogen) to determine the sizes of the DNA product.

Motility assays.

Motility was evaluated using soft-agar plates (1% tryptone, 0.5% NaCl, 0.25% agar), which were prepared on the day of use. A 200 μl sample of overnight culture of wild-type *E. coli* 536 and 536 *Δc2038* was used to inoculate 20 ml of sterile LB broth in a 125-ml Erlenmeyer flask and incubated at 37°C with aeration (200 rpm) to an optical density at 600 nm (OD_{600}) of ~1.00. The cultures were stabbed into the middle of the soft-agar plates using an inoculating needle. Plates were incubated at 25°C for 2 h then at 30°C for approximately 16 h.

In vitro growth curves.

To verify that the independent growth of the putative-GTPase mutant was comparable to that of wild-type 536, an overnight culture of each strain was standardized to an OD_{600} of ~0.3 and diluted separately 1:100 in 50 ml LB in a 250-ml flask. The cultures were incubated at 37°C and aerated (200 rpm). The growth of the wild type and of each mutant was monitored approximately every 30 min for ~ 3.5 h and at ~ 5 and 8 h (when growth reached stationary phase). Growth was assessed by measuring OD_{600} and CFU/ml. At each time point, dilutions were spiral plated using an Autoplate 4000 (Spiral Biotech) onto plain LB plates and LB plates containing 25 μg/ml of kanamycin to determine the wild-type and mutant CFU/ml.

Microscopy.

Cell structure was determined by viewing wet mounts of overnight bacterial cultures by bright field microscopy at x400 magnification. Microscopy was performed by using a Zeiss Axioplan microscope with a Zeiss AxioCam MRm camera attached.

CBA mouse model of ascending UTI.

The CBA/J mouse model of ascending UTI was used as previously described (Hagberg *et al.* 1983 and Johnson *et al.* 1987). 6- to 8-week-old female CBA/J mice were transurethrally inoculated with 10^8 CFU of a 1:1 mixture of wild-type 536 and 536 Δc2038 in a 50 μl volume using a sterile polyethylene catheter connected to an infusion pump (Harvard Apparatus). Overnight cultures standardized to an OD_{600} of 4.0 were used for transurethral inoculation. At 48 hours postinfection, mice were sacrificed, and the bladders and kidneys were removed and homogenized. The homogenized tissue was plated onto LB agar plates with or without kanamycin to determine the CFU per gram of tissue. Wild-type counts were obtained by subtracting the CFU/ml of the LB plates with kanamycin from the CFU/ml of the plain LB plates. All spiral plates were read using a Q-Count machine and software (Spiral Biotech) to determine CFU/ml. Statistical analysis was performed using Wilcoxon matched-pairs signed-ranked test, a nonparametric paired t-test. The competitive indices for co-challenge data were calculated as CFU mutant/CFU wild-type.

TABLE 1. Top 35 most down-regulated genes in *ΔfliA* microarray

Gene name	c number	Description	Fold change	P-value
fliC	c2338	flagellar biosynthesis protein	-4.46	<0.001
fliA	c2337	flagellar biosynthesis sigma factor FliA,RNA polymerase, sigma 28 (sigma F) factor	-4.38	<0.001
c0286	c0286	hypothetical protein	-4.06	<0.001
tsr	c5430	Methyl-accepting chemotaxis protein I,methyl-accepting chemotaxis protein I, serine sensor receptor	-3.49	<0.001
tar	c2301	Methyl-accepting chemotaxis protein II	-3.41	<0.001
c0282	c0282	hypothetical protein	-3.32	<0.001
yhjH	c4336	Hypothetical protein yhjH,EAL domain containing protein involved in flagellar function	-3.26	<0.001
c0283	c0283	hypothetical protein	-3.22	<0.001
c0287,c2520	c0287,c2520	hypothetical protein	-3.18	<0.001
c0284	c0284	hypothetical protein	-3	<0.001
c2300	c2300	hypothetical protein	-2.83	<0.001
cheW	c2302	Chemotaxis protein cheW,purine-binding chemotaxis protein	-2.69	<0.001
c0281	c0281	hypothetical protein	-2.66	<0.001
cheY	c2297	Chemotaxis protein cheY,chemotaxis regulator transmitting signal to flagellar motor component	-2.63	<0.001
c0285,c2522	c0285,c2522	hypothetical protein	-2.59	<0.001
cheA	c2303	Chemotaxis protein cheA,fused chemotactic sensory histidine kinase in two-component regulatory sy	-2.47	<0.001
ycgR	c1644	Hypothetical protein ycgR,protein involved in flagellar function	-2.47	<0.001
c0869	c0869	hypothetical protein	-2.45	<0.001
motA	c2305	flagellar motor protein,proton conductor component of flagella motor	-2.39	<0.001
c4571	c4571	Hypothetical protein yfjX	-2.39	<0.001
motB	c2304	flagellar motor protein,protein that enables flagellar motor rotation	-2.24	<0.001
yjiY	c5429	Hypothetical protein yjiY,predicted inner membrane protein	-2.23	<0.001
c0567	c0567	hypothetical protein	-2.21	<0.001
glpC	c2784	Anaerobic glycerol-3-phosphate dehydrogenase subunit C,sn-glycerol-3-phosphate dehydrogenase (a	-2.19	<0.001
c3220	c3220	hypothetical protein	-2.18	<0.001
cheB	c2298	chemotaxis-specific methylesterase,fused chemotaxis regulator: protein-glutamate methylesterase ir	-2.16	<0.001
cheZ	c2296	Chemotaxis protein cheZ,chemotaxis regulator, protein phosphatase for CheY	-2.15	<0.001
c1442	c1442	Unknown protein encoded within prophage	-2.13	<0.001
aer	c3825	Aerotaxis receptor,fused signal transducer for aerotaxis sensory component/methyl accepting chemot	-2.08	<0.001
ydjR	c2141	Hypothetical protein ydjR	-2.03	<0.001
c3336	c3336	hypothetical protein	-2.03	0.001
c4563	c4563	hypothetical protein	-2.01	<0.001
c4554,c4556	c4554,c4556	hypothetical protein	-2	<0.001
c4353	c4353	hypothetical protein	-1.93	<0.001

[a] Compared to expression levels in wild-type CFT073. $P < 0.001$.

TABLE 3. Primers used in this study

Analysis and primer	sequence (5' to 3')
Sequencing	
c0287/c2520 seq F	GAATTGAGGAGCTGGAGGGCGAAC
c0287/c2520 seq R	CAGAATATCCAGCAGTTCGCGGG
qPCR	
yahA F	TCTGCAAGAGTTCGGTACAACGGT
yahA R	ATCAGCAGCTTGAGTAGCAGTCGT
yaiC F	AATGGTGGCTCTCCCTTCCCATTA
yaiC R	CCTGCGTTTATGTTCCGCCAGTTT
c1246 F	TGTCCGGGATGTTTGAACGGGTAA
c1246 R	TATGACAGGCAACAGAGTCTGCCA
yeaJ F	CCGCAAGTGTCATTGTGCTGACAT
yeaJ R	TAGCGCAGATAGGCACTCATTGCT
yhjH F	TATTAACGGTGGTCACGCATCCCT
yhjH R	TGCTCTTTCACAACCTCCATCCGA
ycgR F	CATCAATCACTTTGCGCTCGCTGA
ycgR R	ACAGCAAAGCCTGCCGAATTACAC
yciR F	TCGAGAATCACCCAACGGCCTAAA
yciR R	TGGAAGCACTAGTACGTTGGCAGT
c0287/c2520 F	GAGCTGGAGGGCGAACAAATCAAA
c0287/c2520 R	CACAATGGCGTTGATGGTGGTTGA
hlyC F	TTCTTGGGCATGTATCCTGGCTCT
hlyC R	GCATATTGGTTAGCCCGTATTGCAGG
Mutant making	
fliA H1P1	GTGAATTCACTATACCGCTGAAGGTGTAATGGATAAACATGGGAATTAGCCATGGTCC
fliA H2P2	ACTTACCCAGTTTAGTGCGTAACCGTTTAATAGCCTGGCTGTGTAGGCTGGAGCTGCTTC
c0287/c2520 H1P1[a]	ATGCACGAAAAGAACATCGCCCTGCTTTGTGATGAAGCCG
c0287/c2520 H2P2[a]	TGTCAGTAATGAGCTCAGGAACCCTTTCATTTAATGCCTG
c0287/c2520 (2) H1P1	CATTAATCTGCTCCGGCAAATGGTTGAGGAGCCAGATGTGATGGGAATTAGCCATGGTCC
c0287/c2520 (2) H2P2	GAGCTCAGGAACCCTTTCATTTAATGCCTGAAGTCGCCTGGGTGTAGGCTGGAGCTGCTTC
c0287/c2520 (3) H1P1	CAGAAAGCACTGAAAAGAATTGAGGAGCTGGAGGGCGAAC
c0287/c2520 H2P2 internal	GGACGGGCTGGTTAATATCCTGCTCAATGGAGCCTGCAGGTGTAGGCTGGAGCTGCTTC
Genetic screening	
c0287 gen F[a]	CTAGGCTCTTCAGGGATGGGTTGG
c0287 gen R[a]	GAGACTGTCAAATATTTTCCTGGA
c2520 gen F	GTCAGGACAGTATCTACTTGAGCG
c2520 gen R	CCCCTCACCTGTCAATTCACTCAC
c0287 gen F(2)	CCTGAGCGATAGCGTTCCTGAAC
c0287 gen R(2)	AATCAATCTTAACCATCGTCCGGG
c2520 gen F(2)	GTTGTCAGAAAGCTGGTTTGGACGC
c2520 gen R(2)	GGCACGTGCAATAACGCTGACC
c2520 gen F(3)	CGTTGTTGAAAGCAGGAGCGG
c0287/c2520 gen R internal	AATAGCCTCTCCACCCAAGCGG

[a] Indicates primers that were also used to mutate and screen 536 *c0238.*

FIGURE LEGENDS

Figure 1. FliA regulates non-flagellar associated genes in UPEC CFT073.

A. GMP signaling proteins. Relative transcript levels for c1246, yahA, yaiC, yeaJ, yhjH, ycgR, and yciR as assayed by quantitative RT-PCR are shown for CFT073 fliA, fliC, flhC, and flhD mutants.

B. Putative GTPases. Relative transcript levels for c0287/c2520 as assayed by quantitative RT-PCR are shown for CFT073 fliA and fliC mutants. All data points are the average of three independent biological samples. Error bars represent the standard deviations between replicates.

Figure 2. UPEC CFT073 has two copies of the putative GTPase.

A. Structure of the two homologous GTPase gene clusters in CFT073. Approximate location of primers used to amplify the cluster specifically are shown. Asterisks marks thymine insertion that was sequenced.

B. c0287 alignment with the beginning of c2520. An annotated thymine insertion in c0287 causes a frame shift. c0286 aligns with c2520 beginning at base pair 585. Sequencing data show that this thymine insertion is an error indicating that c0287 and c0286 are one gene.

Figure 3. Pathogenic E. coli contain homologs of the dynamin-like putative GTPases in CFT073. Alignment of the amino acid sequences of dynamin-like GTPases in various strains of E. coli with c2520 of CFT073 using MegAlign (Lasergene).

Figure 4. Putative GTPase mutant in UPEC 536 has no growth or motility defect.

A. Representative growth curves of wild-type 536 (closed square) and the c0238 mutant (open square) grown in LB. Aliquots were taken and dilutions plated on LB agar to determine CFU/ ml.

Data represent averages of three independent cultures. Error bars represent the standard deviation.

B. Motility assay. Wild-type and c0238 mutant were spotted onto semisolid agar and incubate at 30°C for 16 h. White bars label the radius of motility.

Figure 5. Co-challenge of mice with UPEC 536 and 536 ∆c0283 reveals hypervirulence of mutant. A total of ten mice were transurethrally inoculated with a 1:1 mixture of the wild-type and the mutant. At 48 h postinfection, mice were sacrificed and bacterial counts in the bladder and kidneys were determined. Each data point represents the $\log_{10}$ CFU per gram of tissue collected per mouse. Bars indicate the median $\log_{10}$ CFU per gram of tissue collected. The limit of detection for this assay is 10^2 CFU/g tissue. Statistical differences between the median number of $\log_{10}$ CFU per gram tissue for UPEC 536 and the c0283 mutant were determined by Wilcoxon matched-pairs test. *P* values are indicated above the data points.

Figure 6. Model of c-di-GMP regulation. FliA regulates *ycgR* and *yhjH*, which encode an EAL and PilZ protein, respectively. EAL and GGDEF domain proteins are responsible for the synthesis and degradation of c-di-GMP. PilZ domain proteins bind c-di-GMP and effect down stream physiology. High levels of c-di-GMP have been implicated in the inhibition of motility and virulence. Figure adapted from Claret *et al.* 2007.

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