The Flagellar Sigma Factor FliA Regulates Adhesion and Invasion of Crohn Disease-associated Escherichia coli via a Cyclic Dimeric GMP-dependent Pathway

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The invasion of intestinal epithelial cells by the Crohn disease-associated adherent-invasive Escherichia coli (AIEC) strain LF82 depends on surface appendages, such as type 1 pili and flagella. The absence of flagella in the AIEC strain LF82 results in a concomitant loss of type 1 pili. Here, we show that flagellar regulators, transcriptional activator FlhD2C2, and sigma factor FliA are involved in the coordination of flagellar and type 1 pili synthesis. In the deletion mutants lacking these regulators, type 1 pili synthesis, adhesion, and invasion were severely decreased. FliA expressed alone in trans was sufficient to restore these defects in both the LF82ΔflhD and LF82ΔfliA mutants. We related the loss of type 1 pili to the decreased expression of the FliA-dependent yhjH gene in the LF82ΔfliA mutant. YhjH is an EAL domain phosphodiesterase involved in degradation of the bacterial second messenger cyclic dimeric GMP (c-di-GMP). Increased expression of either yhjH or an alternative c-di-GMP phosphodiesterase, yahA, partially restored type 1 pili synthesis, adhesion, and invasion in the LF82ΔfliA mutant. Deletion of the GGDEF domain diguanylate cyclase gene, yaiC, involved in c-di-GMP synthesis in the LF82ΔfliA mutant also partially restored these defects, whereas overexpression of the c-di-GMP receptor YcgR had the opposite effect. These findings show that in the AIEC strain LF82, FliA is a key regulatory component linking flagellar and type 1 pili synthesis and that its effect on type 1 pili is mediated, at least in part, via a c-di-GMP-dependent pathway.

Many virulent bacteria, including Aeromonas caviae (1), Campylobacter jejuni (2), Clostridium difficile (3), Helicobacter pylori (4), Legionella pneumophila (5), Salmonella enterica serovar Typhimurium (6), and Vibrio cholerae (7), use flagellar motility to avoid unfavorable environments and to establish replication niches at different stages of infection. In Enterobacteriaceae, flagellar type III secretion and assembly are strictly dependent on the organization of a hierarchy that controls the sequential expression of structural and regulatory genes. In Escherichia coli and Salmonella typhimurium, the heterotetrameric transcription factor FlhDC2 (8) is positioned at the top of the flagellar expression hierarchy, where the decision to produce flagella is made (9, 10). The flhDC operon encoding FlhDC2 is transcribed from a class 1 flagellar promoter. FlhDC2, in turn, activates σ28-dependent transcription from the class 2 flagellar promoters that drive expression of the structural subunits required for the hook-basal body structure and expression of regulatory subunits (10, 11). One of these regulatory subunits, σ28, or FliA, is encoded by the fliAZ operon. FliA can associate with the core RNA polymerase to drive transcription of the class 3 flagellar genes (12). The activity of FliA depends on its interaction with the cytoplasmic anti-sigma factor FlgM, which inhibits the FliA-RNA polymerase association until completion of the hook-basal body assembly, at which point the anti-sigma factor is secreted (13). It has been suggested that additional negative feedback loops exist to ensure that every stage of flagellar assembly is signaled prior to synthesis of the components for the next stage. This feedback control allows cells to avoid costly production of unnecessary flagellar subunits (14).

Global transcriptional profiling in E. coli (15), S. typhimurium (16), and Yersinia enterocolitica (17, 18) demonstrated that flagellar regulators FlhD2C2 and FliA control numerous genes other than those involved in flagellar biogenesis. These flagellar regulators have been shown to affect the synthesis of virulence factors, directly and indirectly, such as secreted hemolysin in Proteus mirabilis (19), the type III secretion system-1 in Salmonella (20), the Lap phospholipase in Y. enterocolitica (21–23), an exoenzyme in Xenorhabdus nematophila (24, 25), an invasion factor in C. jejuni (26), and factors involved in the intracellular growth of L. pneumophila in amoebas and determinants for the cytotoxicity against macrophages (27, 28). Together, these findings indicate that coordinated regulation of motility and virulence factor synthesis is not limited to Enterobacteriaceae.
Our study concerns a new pathogenic group of *E. coli* associated with ileal lesions of Crohn disease (29, 30). The strains belonging to this pathovar, designated adherent-invasive *E. coli* (AIEC), are able to adhere to and invade intestinal epithelial cells and replicate within macrophages (29). AIEC adhesion to and invasion depend on the type 1 pili that are involved in triggering membrane extensions in epithelial cells (31). However, the type 1 pili of AIEC reference strain LF82 are not able to confer invasiveness to a nonpathogenic *E. coli* strain K-12, which proves that the genetic background of AIEC is essential.

Flagella play important roles in the adhesion to and invasion of strain LF82 (32). The nonmotile flagellar LF82-*ΔflIC* mutant shows a drastic down-regulation of type 1 pili synthesis, a decrease in adhesion and invasion abilities, and a feedback-induced decrease in the flagellar regulator *flhDC* mRNA levels. This demonstrates that, in strain LF82, as in other bacteria mentioned above, flagellar motility and other factors are coregulated. In this report, we gained insights into the coregulated transcriptional regulation of genes encoding type 1 pili. We further show that Flia affects phase variation, which results in increased type 1 synthesis via a regulatory pathway involving a novel second messenger, cyclic dimeric GMP (c-di-GMP).²

### EXPERIMENTAL PROCEDURES

**Bacterial Strains, Plasmids, and Cell Lines**—Strain AIEC LF82 was isolated from a chronic ileal lesion of a patient with Crohn disease and belongs to *E. coli* serotype O83:H1. It adheres to and invades HEp-2, Intestine-407, and Caco-2 cells (29). *E. coli* strain JM109 was used as host strain for cloning experiments. Bacterial strains and plasmids used in this study are listed in Table 1.

**Plasmids**

| Strains | Relevant characteristics | Source or reference |
|---------|--------------------------|---------------------|
| LF82    | *E. coli* isolated from an ileal biopsy of a patient with CD | Ref. 54 |
| LF82-*ΔflIC* | *E. coli* K-12 mutant deleted of *flhD* gene, chloramphenicol² | Ref. 32 |
| U306    | *E. coli* K-12 mutant deleted of *flhD* gene, chloramphenicol² | Ref. 55 |
| U309    | *E. coli* K-12 mutant deleted of *flhD* gene, chloramphenicol² | Ref. 55 |
| LF82-*ΔflhD* | LF82 mutant deleted of *flhD* gene from U306 | This study |
| LF82-*ΔflA* | LF82 mutant deleted of *fla* gene from U306 | This study |
| LF82-*ΔfimA*; Tn5 *phoA* | Tn5-*phoA* insertion into *fimA* of strain LF82 | Ref. 31 |
| LF82-*Δflz* | LF82 mutant deleted of *flz* gene | This study |
| LF82-*ΔyaiC* | LF82 mutant deleted of *yaiC* gene | This study |
| LF82-*ΔflADyaiC* | LF82 mutant deleted of *fla* and *yaiC* genes | This study |
| LF82-*ΔyejR* | LF82 mutant deleted of *yejR* gene | This study |
| LF82-*ΔflADyejR* | LF82 mutant deleted of *fla* and *yejR* genes | This study |
| LF82-*ΔBcsC* | LF82 mutant deleted of *BcsC* gene | This study |

² The abbreviation used is: c-di-GMP, cyclic dimeric GMP.
determine the number of intracellular bacteria, fresh cell culture medium containing 100 μg/ml gentamicin (Sigma) was added for 1 h to eliminate extracellular bacteria. Monolayers were then lysed with 1% Triton X-100. The bacteria were quantified as described above.

**Immunoblotting**—Bacteria were grown overnight at 37 °C in LB broth without agitation. 700 μl of culture were centrifuged, and the pellet of bacteria was suspended in 100 μl of SDS sample buffer. Western immunoblotting was performed according to the procedure of Towbin et al. (33) with minor modifications. The total protein extracts were heated for 5 min with 0.23% HCl, and proteins were resolved by SDS-PAGE using 12% polyacrylamide gels and electroblotted onto nitrocellulose membranes (Amersham Biosciences). The membranes were blocked with 2% (w/v) bovine serum albumin in TBST at room temperature for 2 h. The membranes were reacted with the rabbit antisera raised against purified type 1 pili preparations, a generous gift from Karen Krogfelt, diluted in 1% (w/v) bovine serum albumin in TBST at room temperature for 2 h. Immunoreactants were detected using horseradish peroxidase-conjugated anti-rabbit immunoglobulin G antibody (1:10,000), enhanced chemiluminescence reagents (Amersham Biosciences) and autoradiography.

**Yeast Cell Aggregation Assay**—Commercial baker’s yeast (Saccharomyces cerevisiae) was suspended in phosphate-buffered saline (10 mg, dry weight/ml). E. coli strains were resuspended to an optical density of 0.6 at 620 nm in phosphate-buffered saline. Equal volumes of fixed yeast cell suspension and decreasing concentrations of E. coli suspension were mixed in a 96-well plate. Aggregation was monitored visually, and the titer was recorded as the last dilution of bacteria giving a positive aggregation reaction.

**Transmission Electron Microscopy**—Bacteria were grown overnight in Luria-Bertani broth without shaking and were fixed and negatively stained with 1% ammonium molybdate on carbon-Formvar copper grids. Gold immunolabeling was performed by the method of Levine et al. (34). A washed bacterial suspension was placed on carbon-Formvar copper grids. Excess liquid was removed, and the grids were placed face down on antiserum (1:1000) raised against purified type 1 pili for 15 min. After 10 washings, the grids were placed on a drop of gold-labeled goat anti-rabbit serum (Janssen Life Sciences Products, Olen, Belgium) for 15 min. After a further thorough washing, the grids were negatively stained with 1% ammonium molybdate for 1 min.

**DNA Manipulations, Hybridization, and PCR Experiments**—PCR conditions and all PCR primer sequences are listed in Table S1. DNA to be amplified was released from whole organisms by boiling. Bacteria were harvested from 1.5 ml of an overnight broth culture, suspended in 150 μl of sterile water, and incubated at 100 °C for 20 min. After centrifugation of the lysate, 5 μl of the supernatant were used in the PCR assays.

**Construction of Isogenic Mutants**—Isogenic mutants were generated using PCR products, as described by Datsenko et al. (35) and modified by Chaveroche et al. (36). The basic strategy was to replace a chromosomal sequence with a selectable antibiotic resistance gene (kanamycin or chloramphenicol) generated by PCR. This PCR product was generated by using primers with 50-nucleotide extensions that are homologous to regions adjacent to the target gene and template E. coli strain harboring the kanamycin resistance gene on the pKD4 plasmid. For the construction of fliD and fliA mutants in AIEC strain LF82, the chloramphenicol resistance cassette was amplified from E. coli K12 mutants carrying deletions. In addition, strain AIEC LF82 was transformed with pKOBEG or pKD46 plasmid, a plasmid that encoded Red proteins from phage λ, synthesized under the control of an L-arabinose-inducible promoter. This plasmid was maintained in bacteria at 30 °C with 25 μg/ml chloramphenicol and was eliminated at 37 °C.

Strain LF82/pKOBEG or pKD46 was grown at 30 °C with 1 mM L-arabinose to induce Red expression. When A620 nm reached 0.6, the bacterial culture was incubated for 20 min at 42 °C to eliminate the plasmid. Bacteria were washed three times with 10% glycerol, and PCR products were electrophoresed. Isogenic mutants were selected on LB agar containing 50 μg/ml kanamycin or 25 μg/ml chloramphenicol. Replacement of the gene by the kanamycin or chloramphenicol resistance cassette in isogenic mutant was confirmed by PCR.
Transcomplementation Assays—The genes were amplified by PCR (PerkinElmer Life Sciences thermal cycler) from AIEC LF82 genomic DNA (1–10 ng) using 2.5 units of Pfu DNA polymerase (Promega) and appropriate primers in Pfu DNA polymerase buffer containing a 200 μM concentration of each deoxynucleoside triphosphate. The amplified DNAs were purified using a NucleoSpin extract kit (Macherey-Nagel, Düren, Germany), digested with HindIII and XbaI or EcoRI, and ligated in cloning vector pBAD18.

RNA Manipulations and Real Time Reverse Transcription-PCR—Total RNA was extracted from bacteria and treated with DNase I (Roche Applied Science). The mRNA was reverse transcribed and amplified using gene-specific primers (Table S1 of supplemental materials). Real time reverse transcription-PCR was performed using a Light Cycler (Roche Applied Science), digested with intestinal epithelial cells Intestine-407. See the legend to Fig. 1. *, p < 0.05.

Calcofluor Binding Assays—5 μl of an overnight culture suspended in water (A600 of 5) were spotted onto LB agar plates without NaCl supplemented with calcofluor (fluorescence brightener 28; 50 μg/ml). Plates were incubated at 37 °C for 48 h. The dye binding was analyzed over time.

Statistical Analysis—For analysis of the significance of differences in adhesion and invasion levels, Student’s t test was used for comparison of two groups of data. All experiments were performed at least three times. A p value less than or equal to 0.05 was considered as statistically significant.

RESULTS

FlhD2C2 and FliA Play Key Roles in Interactions of AIEC Strain LF82 with Intestinal Epithelial Cells—To analyze the role of major flagella regulators FlhD2C2 and FliA in the adhesion and invasion abilities of the AIEC strain LF82, we constructed mutants with deletions in the flhD or fliA genes. As expected, the LF82-ΔflhD and LF82-ΔfliA mutants were nonmotile (data not shown). We measured the adhesion and invasion levels of these flagellar mutants using an in vitro assay with Intestine-407 epithelial cells. Since the LF82-ΔflhD and LF82-ΔfliA mutants were nonmotile, we included a centrifugation step to bring bacterial and epithelial cells into close contact and thereby to enable bacteria to initiate infection. Both mutants were strongly impaired in adhesion and invasion compared with the wild-type strain LF82. The adhesion and invasion levels of the LF82-ΔflhD mutant were significantly lower (19 and 30%, respectively) than of those of strain LF82. Similarly, the adhesion and invasion levels of LF82-ΔfliA were significantly lower (13 and 20%, respectively) than those of strain LF82 (Fig. 1, A and B). These results are consistent with our earlier observation that the LF82-ΔfliC mutant, which lacks flagellin, is impaired in adhesion and invasion abilities. They further indicate that, in strain LF82, the FlhD2C2 and FliA regulators are instrumental in enabling bacteria to enter intestinal epithelial cells, irrespective of flagellar motility.

Decreased Adhesion and Invasion of the ΔflhD and ΔfliA Mutants Is a Consequence of Lowered Levels of Type 1 Pili—The decrease in the ability to adhere to and to invade epithelial cells has been observed in nonflagellated mutants of strain LF82 and was attributed to lower type 1 pili levels (32, 37). We therefore analyzed expression of type 1 pili in the LF82-ΔflhD and LF82-ΔfliA mutants by monitoring bacterial aggregation of yeast cells, which occurs as a result of pili binding to D-mannose residues located at the yeast surface. Both mutants were strongly impaired
in their ability to aggregate yeast cells compared with the wild type strain. We verified that this was a consequence of a decreased level of the FimA major subunit of type 1 pili by Western blot using a type 1 pili antiserum (Fig. 1C).

The regulation of type 1 pili expression is controlled by phase variation, which allows bacteria to switch between piliated and nonpiliated states by inverting a fimS DNA element located upstream of the fim operon. We used a PCR-based approach (38) to confirm that the deficiency in type 1 pili resulted from the shift of the DNA invertible element orientation toward the phase-OFF orientation (Fig. 1C).

To confirm the critical role of type 1 pili in the impaired adhesion and invasion of the LF82-ΔfliD and LF82-ΔfliA mutants, we transformed both mutants with plasmid pORN104, which contains the fim operon in vector pRN2010. The transformants expressed type 1 pili to levels close to those of strain LF82/pORN104, as shown by comparable reaction with the type 1 pili antiserum (Fig. 2A). Transmission electron microscope examination of gold immunolabeling of the LF82-ΔfliA/pORN104 and the LF82-ΔfliD/pORN104 transformants using anti-type 1 pili antibodies revealed that type 1 pili were properly assembled at the surface of bacteria even in the absence of flagella (Fig. 2B). The induced synthesis of type 1 pili partially restored adhesion and invasion levels to 57 and 44%, respectively, in LF82-ΔfliD and to 61 and 41%, respectively, in LF82-ΔfliA, compared with levels in LF82/pORN104 (Fig. 2, C and D). The incomplete restoration of adherence and invasiveness suggests that type 1 pili may not be the only adhesion/invasion determinant regulated by FlhD₂C₂ and FliA.

FliA Restores Adhesion/Invasion Defects in both ΔfliA and ΔfliD Mutants—In the flagellar gene hierarchy, expression levels of FlhD₂C₂ intimately depend on the presence of FliA and vice versa (9, 10). To further clarify the involvement of FlhD₂C₂ and FliA in the control of adhesion and invasion in strain LF82, the LF82-ΔfliD and LF82-ΔfliA mutants were transformed with plasmids pBADfliDC and pBADfliA expressing FlhD₂C₂ and FliA, respectively. We ensured that adhesion, invasion, and motility of LF82-ΔfliD and LF82-ΔfliA were fully complemented with pBADfliDC and pBADfliA, respectively (Table 2; data not shown). The overexpressed flhDC operon had no effect on adhesion and invasion in the LF82-ΔfliA mutant. However, the overexpressed fliA gene fully restored the defects in the LF82-ΔfliD mutant (Table 2). These results indicate that, in addition to type 1 pili synthesis, the full invasiveness of AIEC strain LF82 is likely to be controlled through the FliA-dependent gene expression.

Overexpression of the yhjH Gene Encoding an EAL Domain c-di-GMP Phosphodiesterase Alleviates

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### TABLE 2

| Strain                  | Yeast aggregation | Adhesion | Invasion |
|-------------------------|-------------------|----------|----------|
| LF82                    | 1/96              | 100      | 100      |
| LF82-ΔfliD              | 1/16              | 21 ± 3   | 34 ± 6   |
| LF82-ΔfliD/pBADfliDC    | 1/96              | 95 ± 4   | 95 ± 2   |
| LF82-ΔfliD/pBADfliA     | 1/96              | 77 ± 15  | 80 ± 1   |
| LF82-ΔfliA/pBADfliB     | 1/12              | 18 ± 1   | 25 ± 7   |
| LF82-ΔfliA              | 1/5               | 12 ± 5   | 17 ± 4   |
| LF82-ΔfliA/pBADfliA     | 1/96              | 99 ± 3   | 102 ± 7  |
| LF82-ΔfliA/pBADfliB     | 1/6               | 12 ± 3   | 17 ± 5   |
| LF82-ΔfliA/pBADfliDC    | 1/5               | 10 ± 5   | 19 ± 4   |

*Aggregation was monitored visually, and the titer was recorded as the last dilution giving a positive aggregation reaction.

*a* See Fig. 1 legend.

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**FIGURE 3.** Regulatory role of EAL domain proteins in the LF82-ΔfliA mutant. Adhesion (A) and invasion (B) abilities of AIEC strain LF82, LF82-ΔfliZ, and LF82-ΔfliA mutants. The latter mutant was transformed by pBADyhjH, pLYaA1, pLITMUS28, or pBAD18 with intestinal epithelial cells Intestine-407. C, determination of the amount of FimA subunit using Western blot and type 1 pili antiserum and orientation of the fim operon invertible element in strain LF82, LF82-ΔfimA mutant, and ΔfliA transformed with pYHJH, pLYaA1, or control empty vectors. D, adhesion (light gray bars) and invasion (dark gray bars) abilities of AIEC strain LF82/pORN104, LF82-ΔfliA/pORN104/pBAD18, and LF82-ΔfliA/pORN104/pBADyhjH mutants with intestinal epithelial cells Intestine-407. See the legend to Fig. 1. *p < 0.05.
**FliA, c-di-GMP and Adherent-Invasive E. coli**

**The Adhesion/Invasion Defect in the ΔfliA Mutant—**To identify putative mediators of the effect of FliA on the synthesis of type 1 pili in strain LF82, we investigated the involvement of two genes, *fliZ*, which belongs to the *fliAZ* operon, and *yjhH*, whose expression depends on FliA in *E. coli* K-12 and *S. typhimurium* (25, 39). FliZ exerts a control checkpoint between the synthesis of flagella and invasion factors in *S. typhimurium* (40). YjhH stimulates flagellar motility when it is overexpressed (39). YjhH belongs to a group of proteins carrying an EAL domain and functions as a phosphodiesterase of the second messenger c-di-GMP (41, 42).

A deletion mutant, LF82-Δ*fliZ*, was thus created, and the LF82-Δ*fliA* mutant was transformed with the pBAD*yjhH* plasmid carrying the *yjhH* gene under an arabinose-inducible promoter. The adhesion and invasion levels of these strains were measured after a centrifugation step. The LF82-Δ*fliZ* mutant was not impaired in adhesion and invasion compared with the wild type (Fig. 3, A and B). Conversely, the transformation of LF82-Δ*fliA* mutant with pBAD*yjhH* restored adhesion and invasion levels to 40 and 48%, respectively, of those of strain LF82. This restoration was due to increased type 1 pili synthesis, as verified by the increased accumulation of the FimA subunit and the shift in orientation of the DNA invertible element for type 1 pili toward the ON position (Fig. 3C). Restoration of piliation was confirmed by electron microscopic examination of strain LF82-Δ*fliA*/pBAD*yjhH* (Fig. 4).

To further analyze whether higher expression of type 1 pili observed in LF82-Δ*fliA*/pBAD*yjhH* was responsible for the partial restoration of adhesion and invasion, we investigated these parameters in LF82-Δ*fliA*/pBAD*yjhH* transformed with pORN104. In this strain, we observed full restoration of adhesion and invasion (Fig. 3D). These results suggest that in strain LF82, FliA regulates type 1 pili expression and adhesion and invasion abilities by acting via the YjhH expression.

The c-di-GMP Turner Over Is Involved in the Adhesion and Invasion of AIEC Strain LF82—Genetic analysis has provided firm evidence of the c-di-GMP phosphodiesterase activity of YjhH, yet its enzymatic activity has not been tested *in vitro*. To ascertain that c-di-GMP phosphodiesterase activity of YjhH affects adhesion and invasion, we transformed the LF82-Δ*fliA* mutant with a plasmid carrying the *E. coli* K-12 yahA gene encoding a well characterized c-di-GMP phosphodiesterase (43). We observed that, with overexpression of *yjhH*, overexpression of *yahA* partially restored the adhesion/invasion defects and type 1 pili synthesis, as evidenced by the increased FimA subunit accumulation and the shift in orientation of the DNA invertible element for type 1 pili toward the ON position (Fig. 3, A–C).

To further explore the role of c-di-GMP in adhesion and invasion, we constructed two mutants, LF82-Δ*yaiC* and LF82-Δ*fliA*Δ*yaiC*, each containing a deletion in the *yaiC* gene, which encodes a diguanylate cyclase involved in c-di-GMP synthesis (Fig. 5, A and B). The homolog of YaiC in *S. typhimurium* (41), AdrA, which shares 75% identity with YaiC, is one of the major diguanylate cyclases in this bacterium. We anticipated that the absence of YaiC would decrease c-di-GMP synthesis in the *Δ*yaiC* mutants, which may mimic the effect of increased c-di-GMP hydrolysis by overexpressed YjhH or YahA. In the genetic background of strain LF82, deletion of the *yaiC* gene did not affect adhesion and invasion (data not shown), and neither did *yaiC* overexpression (Fig. 5, A and B). Similarly, in the LF82-Δ*fliA* harboring pORN104, overexpression of *yaiC* did not decrease adhesion and invasion levels (Fig. 5D). However, deletion of *yaiC* in the genetic background of LF82-Δ*fliA* (where c-di-GMP levels may be elevated due to the lower expression of c-di-GMP phosphodiesterase YjhH) resulted in increased adhesion and invasion levels, reaching 37 and 83%, respectively, of those in strain LF82. This partial increase correlated with a shift in the orientation of the DNA invertible element toward the phase-ON position (Fig. 5C). It seems that a decrease in c-di-GMP levels caused either by an overexpression of a c-di-GMP phosphodiesterase or by a loss of a major diguanylate cyclase affects type 1 pili expression but only in the absence of FliA.

Cellulose biosynthesis in certain strains of *E. coli* and *S. typhimurium* strains is strongly up-regulated by c-di-GMP (41). Using calcofluor-binding assays, we observed that overexpression of YaiC in strain LF82 increased cellulose synthesis (data not shown), as reported elsewhere in *S. typhimurium* upon overexpression of the YaiC homolog AdrA (41). However, as shown above, this increased cellulose synthesis did not affect adhesion and invasion (Fig. 5, A and B). Further, the LF82-Δ*bcgC* mutant impaired in cellulose synthesis had adhesion and
and observed that the absence of YcgR did not modify adhesion and invasion. We then explored the effect of overexpressed YcgR and observed that in strain LF82 carrying plasmid pycgR, the levels of type 1 pili were decreased (Figs. 4 and 5C). The adhesion and invasion levels of LF82/pycgR were significantly decreased, to 22 and 37%, respectively, of those of strain LF82 (Fig. 5C). Interestingly, analysis of the double mutant LF82-ΔfliAΔycgR indicated that, in the absence of YcgR, the FliA null mutant recovered its abilities to adhere to and to invade, reaching 49 and 67%, respectively, of those of the wild-type strain LF82 (Fig. 5D). However, we did not observe any further decrease in adhesion and invasion levels in the LF82-ΔfliA mutant that overexpressed YcgR (data not shown). This indicates that the effect of FliA on type 1 pili is mediated to a large extent via the negatively regulating YcgR/c-di-GMP pathway.

**FliA-dependent Control of Genes Involved in c-di-GMP Regulation—** Although it has been shown that FliA affects yhjH expression in *E. coli* K12 and *S. typhimurium* strains (16, 39), it was unclear whether this holds true for strain LF82. According to the reverse transcription-PCR assays, the levels of yhjH mRNA were up to 111-fold higher in LF82 than in LF82-ΔfliA (Table 3). However, FliA did not affect the levels of yahA and yaiC transcripts. Interestingly, the ycgR transcript levels were much higher (14-fold) in LF82 than in LF82-ΔfliA. These results suggest that the role of FliA in c-di-GMP-dependent control over type 1 pili is complex; nevertheless, we have established a link between the flagellar hierarchy and type 1 pili synthesis.

**DISCUSSION**

In this study, we uncovered a regulatory pathway linking flagellar motility and type 1 pili synthesis, which is required for efficient adhesion to and invasion of intestinal epithelial cells by the AIEC strain LF82 associated with Crohn disease. As a first step in deciphering this pathway, we used our earlier observation that, in strain LF82, a mutation in the flagellin gene flhC results in decreased expression of the flhDC operon, which encodes FlhD2C2, master regulator of flagellar biogenesis, and concomitantly a loss of type 1 pili (32). In the present study, we found that the mutants deleted for the regulatory genes encoding the FlhD2C2 transcriptional activator or the downstream sigma factor FliA were severely impaired in type 1 pili synthesis. This suggests that regulators of flagellar synthesis control type 1

![Image](https://example.com/image.png)

**TABLE 3**

Quantification of *yaiC*, *yaiH*, *yahA*, and *ycgR* expression in AIEC strain LF82 and *fliA* mutant strain

| mRNA levels relative to those of wild-type strain LF82* | Domain         |
|-------------------------------------------------------|----------------|
| *yaiC*                                                 | 0.8 ± 0.2      |
| *yaiH*                                                 | 0.0 ± 0.0*     |
| *yahA*                                                 | 1.2 ± 0.2      |
| *ycgR*                                                 | 14.3 ± 0.0*    |

*Fold decrease in mRNA levels relative to that of wild-type strain LF82 using real-time reverse transcription-PCR. 16S rRNA levels were measured as controls. Only experiments showing the same levels of 16S rRNA for each sample were taken into account. Data are mean ± S.E. of at least three separate experiments.

* $p < 0.05$. 

invasion abilities similar to those of strain LF82 (data not shown). Together, these results show that cellulose is not involved in adhesion and invasion of the LF82 strain.

The *c-di-GMP* Receptor YcgR Acts as an Inhibitor of Adhesion and Invasion in Strain LF82—Recently, a PilZ domain protein was predicted (44) and verified (42) to function as a c-di-GMP receptor. The PilZ domain protein YcgR from *E. coli* was shown to bind c-di-GMP in *vitro* and affect flagellar motility in *vivo* (39, 42). The *ycgR* gene was also demonstrated to have the opposite effect on motility to that of the *yhjH* gene (39). However, the mechanism by which c-di-GMP affects flagellar motility is still uncertain. We constructed the LF82-ΔycgR mutant
pili synthesis in strain LF82. Hence, decreased FlhD$_2$C$_2$ levels are likely to be responsible for the lack of type 1 pili in the ΔfliC mutant. Forced contact between bacteria and host cells (through centrifugation), which bypasses the need for flagellar motility, and overexpression of type 1 pili did not fully restore the adhesion/invasion defects of the LF82-ΔflhD or LF82-ΔfliA mutants. This suggests that the range of virulence targets controlled by flagellar regulators in strain LF82 is not limited to type 1 pili.

The lack of FlhD$_2$C$_2$ in AIEC strain LF82 was compensated for by the increased expression of the downstream sigma factor FliA. It is likely that the genes involved in type 1 pili synthesis and other adhesion/invasion factors are controlled at the transcription level by FliA. Although the involvement of FliA in the regulation of virulence determinants other than flagella has been described in several pathogenic bacteria (18, 25, 26, 28), these studies did not identify intermediate steps linking FliA to the regulation of virulence determinants. In our study, we identified the FliA-dependent yhjH gene as such a mediator. YhjH is an EAL domain c-di-GMP phosphodiesterase required for the breakdown of the novel second messenger c-di-GMP (41). We show that it is the c-di-GMP phosphodiesterase activity of YhjH, and not any other property of YhjH, that was critical, because we were able to replace YhjH with an alternative EAL domain, c-di-GMP phosphodiesterase YahA (43), or achieve the same phenotype by deleting the diguanylate cyclase gene yaiC (37). We demonstrate that overexpressed YaiC did not change the adhesion and invasion abilities of the LF82 strain, which indicates that physiological amounts of the diguanylate cyclase are sufficient for its activity. Thus, in strain LF82, lower c-di-GMP levels seem to stimulate type 1 pili synthesis.

We observed that increased type 1 pili synthesis occurred via the phase variation mechanism (i.e., the shift in the orientation of the DNA invertible element fimS toward the phase-ON orientation). The pathway connecting lower c-di-GMP levels to phase variation remains unknown. In addition to controlling yhjH expression, FliA in strain LF82 controls expression of the c-di-GMP receptor YcgR, a finding consistent with observations made in E. coli K-12 and Chlamydia trachomatis (45, 46). YcgR is a c-di-GMP receptor involved in motility control. We suggest that YcgR is a good candidate for sensing the changes in cellular c-di-GMP levels and negatively controlling type 1 pili synthesis, adhesion, and invasion. The marked reduction in type 1 pili synthesis induced by overexpression of YcgR in LF82 strain is consistent with this suggestion. This was further confirmed by the increased adhesion and invasion abilities of the LF82-ΔfliA mutant when a second deletion was made in ycgR. The downstream partners of the YcgR protein are unknown. We speculate that either YcgR or its downstream partner affects the phase inversion of fimS DNA element by regulating the expression and/or activities of the FimB and FimE invertases (47). We cannot exclude the possibility that the regulation of type 1 pili also involves a factor that affects pili export or assembly. Further, it is possible that additional, as yet unidentified, c-di-GMP-binding proteins act as regulatory intermediates between FliA and adhesion and invasion factors in strain LF82. This hypothesis is included in the model depicted in Fig. 6.

Recent studies have linked c-di-GMP to virulence in several pathogenic bacteria. In S. typhimurium, the c-di-GMP phosphodiesterase CdgR is involved in resistance to phagocyte oxidative and in the cytotoxic effect in macrophages (48). In V. cholerae, the c-di-GMP phosphodiesterase VieA regulates expression of the cholera toxin genes ctxAB (49), whereas the CdgC protein carrying the EAL and GGDEF domains is involved in the control of extracellular protein secretion and flagellar biosynthesis (50). In Pseudomonas aeruginosa, the biofilm and cytotoxicity phenotypes are mediated by different GGDEF and EAL domain proteins involved in c-di-GMP metabolism (51). More specifically, the c-di-GMP phosphodiesterase FimX is involved in the assembly of Tfp type IV pili, which are required for twitching motility, biofilm formation, and adherence of Pseudomonas (52). In addition, increased c-di-GMP levels in S. typhimurium are associated with increased curli synthesis (53). Our work revealed yet another virulence factor, type 1 pili, whose expression is regulated via a c-di-GMP-dependent pathway. We also present the first evidence, to our knowledge, that such a pathway can influence invasiveness in a pathogenic bacteria. The novel role of c-di-GMP in the control of type 1 pili expression and, as a consequence, in the adhesion and invasion abilities of strain LF82 is in agreement with the general notion that lower c-di-GMP levels promote virulence.

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FIGURE 6. Model for a c-di-GMP-dependent coordinate regulation of the synthesis of flagella, type 1 pili in AIEC LF82. When the flagella cascade is activated, the accumulation of the FliA sigma factor leads to a concomitant strong induction of the yhjH and ycgR genes. The EAL domain YhjH degrades c-di-GMP to lower its local concentration and suppress the activity of a putative YcgR-c-di-GMP complex, finally leading to the synchronized activation of various adhesion and invasion factors of AIEC strain LF82. The FliA-induced YcgR protein could act in a feedback-like way to modulate the whole system by increasing the level of the YcgR-c-di-GMP complex when local c-di-GMP molecule concentration is high.
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