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

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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 $FlhD_2C_2$, 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 LF82-Δ*flhD* and LF82-Δ*fliA* mutants. We related the loss of type 1 pili to the decreased expression of the FliA-dependent *yhjH* gene in the LF82-Δ*fliA* 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 LF82-Δ*fliA* mutant. Deletion of the GGDEF domain diguanylate cyclase gene, *yaiC*, involved in c-di-GMP synthesis in the LF82-Δ*fliA* 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 $FlhD_2C_2$ (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 $FlhD_2C_2$ is transcribed from a class 1 flagellar promoter. $FlhD_2C_2$, in turn, activates σ^{70}-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, σ^{28} 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 $FlhD_2C_2$ 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*.

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Table 1.

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TABLE 1
Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Source or reference
Strains		
LF82	*E. coli* isolated from an ileal biopsy of a patient with CD	Ref. 54
LF82-Δ*fliC*	LF82 mutant deleted of *fliC* gene	Ref. 32
U306	*E. coli* K-12 mutant deleted of *flhD* gene, chloramphenicol^r	Ref. 55
U309	*E. coli* K-12 mutant deleted of *fliA* gene, chloramphenicol^r	Ref. 55
LF82-Δ*flhD*	LF82 mutant deleted of *flhD* gene from U306	This study
LF82-Δ*fliA*	LF82 mutant deleted of *fliA* gene from U309	This study
LF82-*fimA*:: Tn5 *phoA*	Tn5-*phoA* insertion into *fimA* of strain LF82	Ref. 31
LF82-Δ*fliZ*	LF82 mutant deleted of *fliZ* gene	This study
LF82-Δ*yaiC*	LF82 mutant deleted of *yaiC* gene	This study
LF82-Δ*fliAD yaiC*	LF82 mutant deleted of *fliA and yaiC* genes	This study
LF82-Δ*ycgR*	LF82 mutant deleted of *ycgR* gene	This study
LF82-Δ*fliAD ycgR*	LF82 mutant deleted of *fliA and ycgR* genes	This study
LF82-Δ*BcsC*	LF82 mutant deleted of *BcsC* gene	This study
Plasmids		
pKOBEG	pBAD cloning vector harboring l phage *red*γβα operon, chloramphenicol^r	Ref. 36
pKD46	pBAD cloning vector harbouring l phage *red*γβα operon, ampicillin^r	Ref. 35
pKD4	Plasmid carrying a kanamycin resistance cassette	Ref. 35
pBAD18	*E. coli* cloning vector, ampicillin^r	Ref. 56
pBAD*flhDC*	pBAD18 harboring the entire *flhDC* operon of LF82 *E. coli*, amplified with *flhDC*F*Hind* and *flhDC*R*Xba*	This study
pBAD*fliA*	pBAD18 harboring the entire *fliA* gene of LF82 *E. coli*, amplified with *fliA*H*ind* and *fliA*R*Xba*	This study
pBAD*yhjH*	pBAD18 harboring the entire *yhjH* gene of LF82 *E. coli*, amplified with *yhjHEco*F and *yhjHHind*R	This study
pBAD*yaiC*	pBAD18 harboring the entire *yaiC* gene of LF82 *E. coli*, amplified with *yaiCXba*F and *yaiCHind*R	This study
pLITMUS28	Ampicillin^r vector	NEB
p*LyahA*Ω	pLITMUS28 harboring the *yahA* gene from MG1655 *E. coli*	Gomelsky
pUC19	*E. coli* cloning vector, *ori*ColE1, ampicillin^r	Biolabs
p*ycgR*	pMS258 harboring the entire *ycgR* gene of *E. coli* K12 cloned in pUC19	Ref. 39
pORN104	Plasmid harboring the entire *fim* operon of K12 *E. coli* strain J96 cloned in pRN2010	Ref. 57
pRN2010	Cloning plasmid	Ref. 57

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 to 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 aflagellar LF82-Δ*fliC* 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 coregulation of flagella, type 1 pili synthesis, adhesion, and invasion in strain LF82. We disrupted the *flhD* and *fliA* genes to test whether $FlhD_2C_2$ and FliA are involved in the direct or indirect 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.

Plasmid vector pBAD18 was used in cloning experiments. Bacteria were grown routinely in LB broth or on LB agar plates overnight at 37 °C. Antibiotics were added at the following concentrations: ampicillin (50 μg/ml), kanamycin (50 μg/ml), and chloramphenicol (25 μg/ml).

Intestine-407 cells (derived from human intestinal embryonic jejunum and ileum) were purchased from Flow Laboratories, Inc. (McLean, VA). Cultured cells were maintained in an atmosphere containing 5% CO_2 at 37 °C in modified Eagle's medium (Seromed, Biochrom KG, Berlin, Germany) supplemented with 10% (v/v) fetal calf serum (Seromed), 1% nonessential amino acids (Invitrogen), 1% L-glutamine (Invitrogen); 200 units of penicillin, 50 mg of streptomycin, and 0.25 mg of amphoterocin B per liter; and 1% minimal essential medium vitamin mix X-100 (Invitrogen).

Adhesion and Invasion Assays—The bacterial invasion was performed using the gentamicin protection assay. Briefly, monolayers were seeded in 24-well tissue culture plates (Polylabo, Strasbourg, France) with 4×10^5 cells/well and incubated for 20 h. Monolayers were then infected in 1 ml of the cell culture medium without antibiotics and with heat-inactivated fetal calf serum at a multiplicity of infection of 10 bacteria per epithelial cell. The infected monolayers were centrifuged for 10 min at 1000 × *g* before the 3-h infection period at 37 °C and washed three times in phosphate-buffered saline (pH 7.2). The epithelial cells were then lysed with 1% Triton X-100 (Sigma) in deionized water. Samples were diluted and plated onto Mueller-Hinton agar plates to determine the number of colony-forming units corresponding to the total number of cell-associated bacteria (adherent and intracellular bacteria). To

[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 (Sigma) in Tris-buffered saline, 0.05% Tween (TBST) at room temperature for 2 h. The membranes were reacted with the rabbit antiserum 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 (Jansen 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) gener-

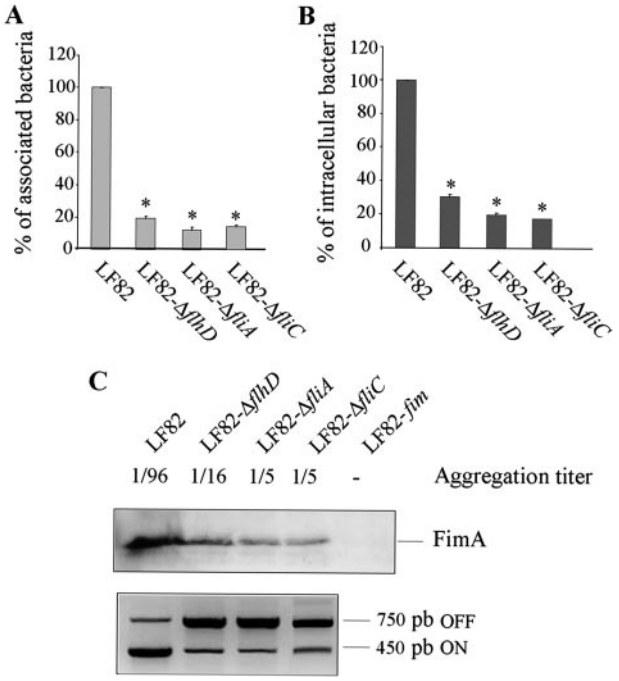

FIGURE 1. **Effects of *fliA*, *flhD*, or *fliC* disruptions on adhesion to (*A*) and invasion of (*B*) Intestine-407 cells and type 1 pili regulation (*C*).** Adhesion and invasion levels of AIEC strain LF82, LF82-Δ*flhD*, LF82-Δ*fliA*, and LF82-Δ*fliC* mutants were measured. Cell-associated bacteria were quantified after a 3-h infection period. Invasion was determined after gentamicin treatment for an additional 1 h. The mean number of cell-associated LF82 bacteria was $4.2 \times 10^5 \pm 2.5 \times 10^5$ colony-forming units/well. The mean number of intracellular LF82 bacteria was $3.2 \times 10^3 \pm 0.9 \times 10^3$ colony-forming units/well. Results are expressed as cell-associated (adherent + intracellular) or intracellular bacteria relative to those obtained for strain LF82, taken as 100%. Each value is the mean ± S.E. of at least four separate experiments. *C*, determination of the type 1 pili yeast aggregation titer, evaluation 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-Δ*flhD*, LF82-Δ*fliA*, and LF82-Δ*fliC* mutants. Orientation was determined by PCR analysis, as described under "Experimental Procedures." A 450-bp product revealed ON orientation and 750-bp product OFF-orientation of the invertible element. *, $p < 0.05$.

ated 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 *flhD* 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 $A_{620\,nm}$ 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 electroporated. 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.

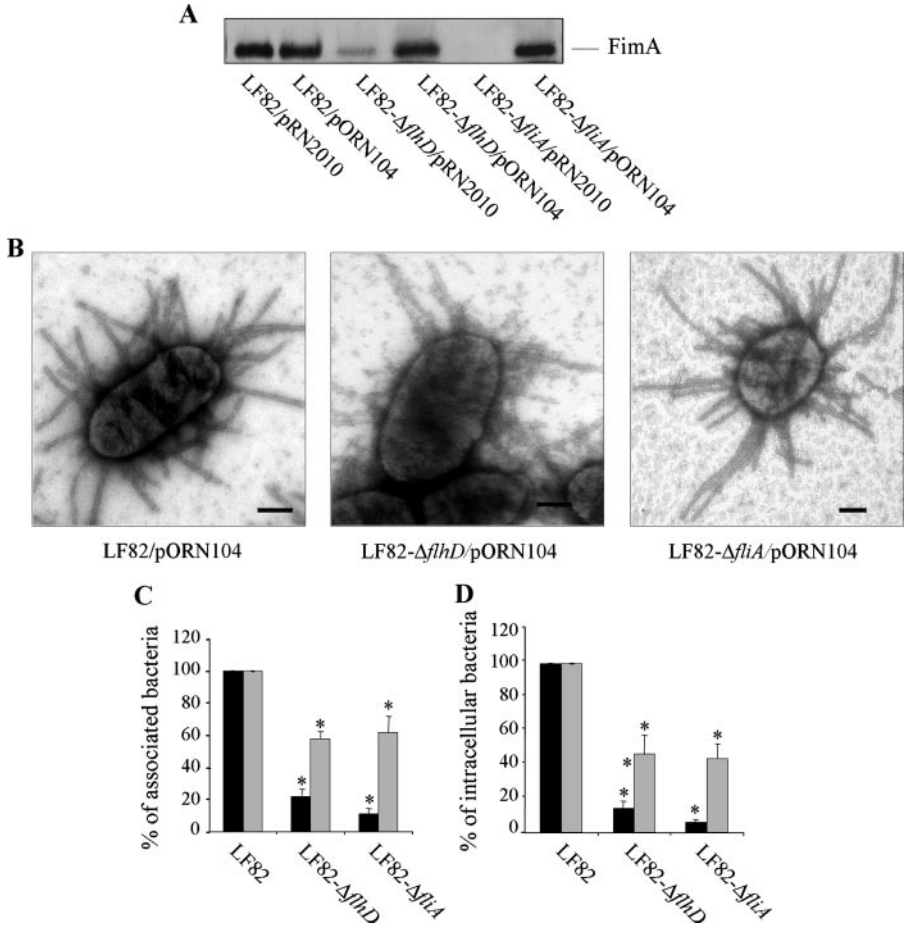

FIGURE 2. **Type 1 pili synthesis (*A*), transmission electron microscope (*TEM*) examination of bacteria (*B*), adhesion (*C*), and invasion (*D*) abilities of AIEC strain LF82, LF82-Δ*flhD*, and LF82-Δ*fliA* mutants, transformed by pRN2010 empty vector (*black bars*) or pORN104 harboring the entire *fim* operon cloned in pRN2010 (*gray bars*).** Determination of the amount of FimA subunit using Western blot and type 1 pili antiserum. Selected images of individual bacteria stained using immunogold labeling with polyclonal antibodies raised against purified type 1 pili. The *black scale bar* indicates 500 nm. Adhesion and invasion were measured with intestinal epithelial cells Intestine-407. See the legend to Fig. 1. *, $p < 0.05$.

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 (Machery-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), and quantification of the mRNA level or 16 S rRNA (as a control) was performed using RNA master SYBER Green 1 (Roche Applied Science) with 0.5 μg of total RNA. Amplification of a single expected reverse transcription-PCR product was confirmed by electrophoresis on a 2% agarose gel.

Calcofluor Binding Assays—5 μl of an overnight culture suspended in water (A_{600} 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

$FlhD_2C_2$ and FliA Play Key Roles in Interactions of AIEC Strain LF82 with Intestinal Epithelial Cells—To analyze the role of major flagella regulators $FlhD_2C_2$ 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-Δ*flhD* and LF82-Δ*fliA* 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-Δ*flhD* and LF82-Δ*fliA* 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-Δ*flhD* mutant were significantly lower (19 and 30%, respectively) than of those of strain LF82. Similarly, the adhesion and invasion levels of LF82-Δ*fliA* 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-Δ*fliC* mutant, which lacks flagellin, is impaired in adhesion and invasion abilities. They further indicate that, in strain LF82, the $FlhD_2C_2$ 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-Δ*flhD* and LF82-Δ*fliA* 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. 1*C*).

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. 1*C*).

To confirm the critical role of type 1 pili in the impaired adhesion and invasion of the LF82-Δ*flhD* and LF82-Δ*fliA* 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. 2*A*). Transmission electron microscope examination of gold immunolabeling of the LF82-Δ*fliA*/pORN104 and the LF82-Δ*flhD*/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. 2*B*). The induced synthesis of type 1 pili partially restored adhesion and invasion levels to 57 and 44%, respectively, in LF82-Δ*flhD* and to 61 and 41%, respectively, in LF82-Δ*fliA*, 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 $FlhD_2C_2$ and FliA.

TABLE 2
Regulation of type 1 pili and adhesion and invasion abilities of AIEC strain LF82 compared with LF82-Δ*flhD* and LF82-Δ*fliA* mutants

Strain	Yeast aggregation[a]	Adhesion[b]	Invasion[b]
		%	%
LF82	1/96	100	100
LF82-Δ*flhD*	1/16	21 ± 3	34 ± 6
LF82-Δ*flhD*/pBAD*flhDC*	1/96	95 ± 4	95 ± 2
LF82-Δ*flhD*/pBAD*fliA*	1/96	77 ± 15	80 ± 1
LF82-Δ*flhD*/pBAD18	1/12	18 ± 1	25 ± 7
LF82-Δ*fliA*	1/5	12 ± 5	17 ± 4
LF82-Δ*fliA*/pBAD*fliA*	1/96	99 ± 3	102 ± 7
LF82-Δ*fliA*/pBAD18	1/6	12 ± 3	17 ± 5
LF82-Δ*fliA*/pBAD*flhDC*	1/5	10 ± 5	19 ± 4

[a] Aggregation was monitored visually, and the titer was recorded as the last dilution giving a positive aggregation reaction.
[b] See Fig. 1 legend.

FliA Restores Adhesion/Invasion Defects in both ΔfliA and ΔflhD Mutants—In the flagellar gene hierarchy, expression levels of $FlhD_2C_2$ intimately depend on the presence of FliA and vice versa (9, 10). To further clarify the involvement of $FlhD_2C_2$ and FliA in the control of adhesion and invasion in strain LF82, the LF82-Δ*flhD* and LF82-Δ*fliA* mutants were transformed with plasmids pBA*DflhDC* and pBA*DfliA* expressing $FlhD_2C_2$ and FliA, respectively. We ensured that adhesion, invasion, and motility of LF82-Δ*flhD* and LF82-Δ*fliA* were fully complemented with pBAD*flhDC* and pBAD*fliA*, respectively (Table 2; data not shown). The overexpressed *flhDC* operon had no effect on adhesion and invasion in the LF82-Δ*fliA* mutant. However, the overexpressed *fliA* gene fully restored the defects in the LF82-Δ*flhD* 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

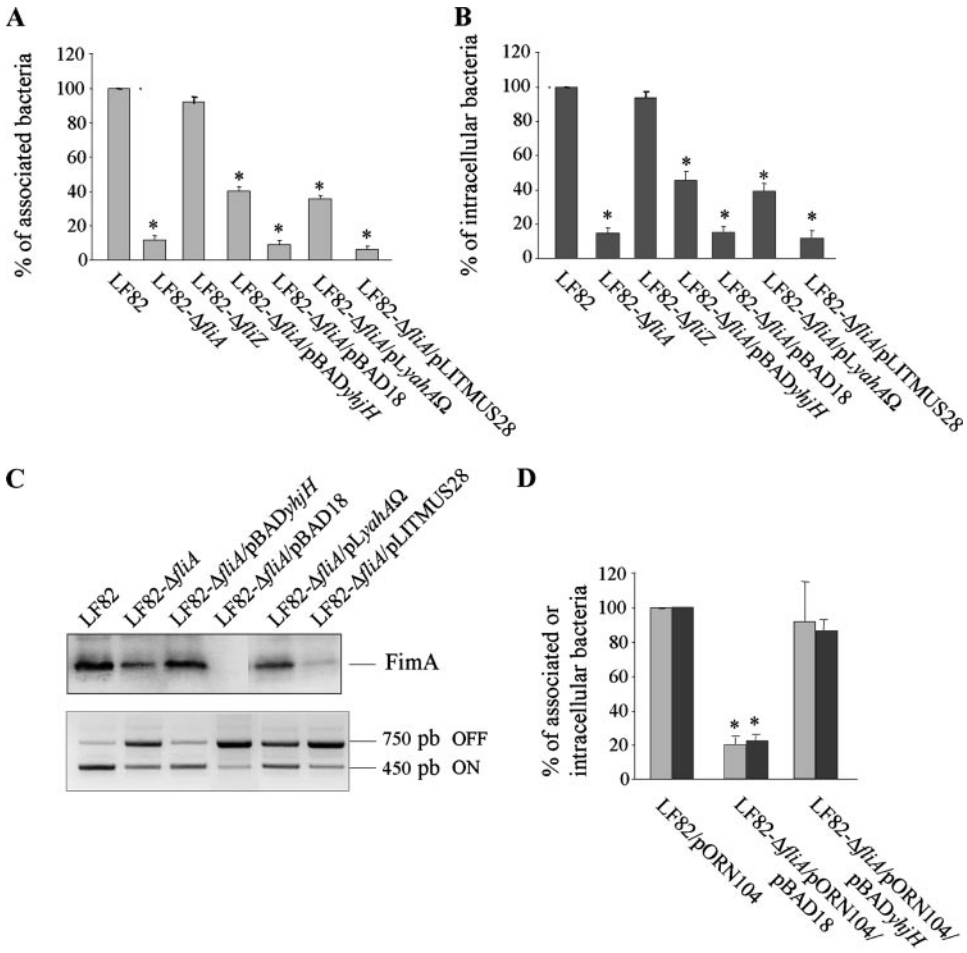

FIGURE 3. **Regulatory role of EAL domain proteins in the LF82-Δ*fliA* mutant.** Adhesion (*A*) and invasion (*B*) abilities of AIEC strain LF82, LF82-Δ*fliZ*, and LF82-Δ*fliA* mutants. The latter mutant was transformed by pBAD*yhjH*, pL*yahA*Ω, pLITMUS28, or pBAD18 with intestinal epithelial cells Intestine-407. *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-Δ*fliA* mutant, and Δ*fliA* transformed with p*yhjH*, pL*yahA*Ω, or control empty vectors. *D*, adhesion (*light gray bars*) and invasion (*dark gray bars*) abilities of AIEC strain LF82/pORN104, LF82-Δ*fliA*/pORN104/pBAD18, and LF82-Δ*fliA*/pORN104/pBAD*yhjH* mutants with intestinal epithelial cells Intestine-407. See the legend to Fig. 1. *, $p < 0.05$.

*the Adhesion/Invasion Defect in the Δ*fliA *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 *yhjH*, 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). YhjH stimulates flagellar motility when it is overexpressed (39). YhjH 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*yhjH* plasmid carrying the *yhjH* 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*yhjH* 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. 3*C*). Restoration of piliation was confirmed by electron microscopic examination of strain LF82-Δ*fliA*/pBAD*yhjH* (Fig. 4).

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

The c-di-GMP Turnover 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 YhjH, yet its enzymatic activity has not been tested *in vitro*. To ascertain that c-di-GMP phosphodiesterase activity of YhjH 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, as with overexpression of *yhjH*, 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 YhjH or YahA. In the genetic

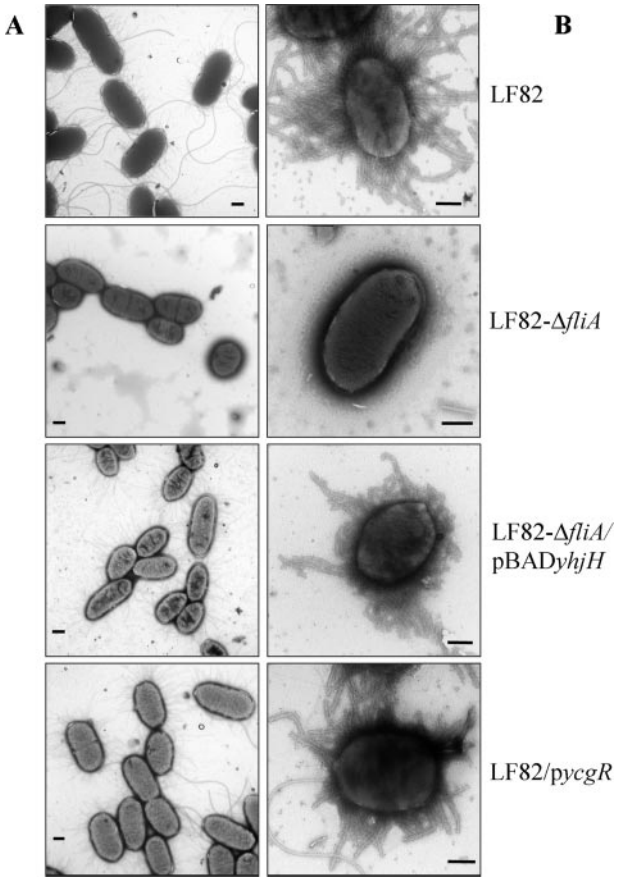

FIGURE 4. **Transmission electron microscope (*TEM*) examination of bacteria.** *A*, typical field of view of negatively stained bacteria; *B*, selected images of individual bacteria stained using immunogold labeling with polyclonal antibodies raised against purified type 1 pili. The *black scale bar* indicates 500 nm.

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. 5*D*). 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 YhjH) 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. 5*C*). 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*bcsC* mutant impaired in cellulose synthesis had adhesion and

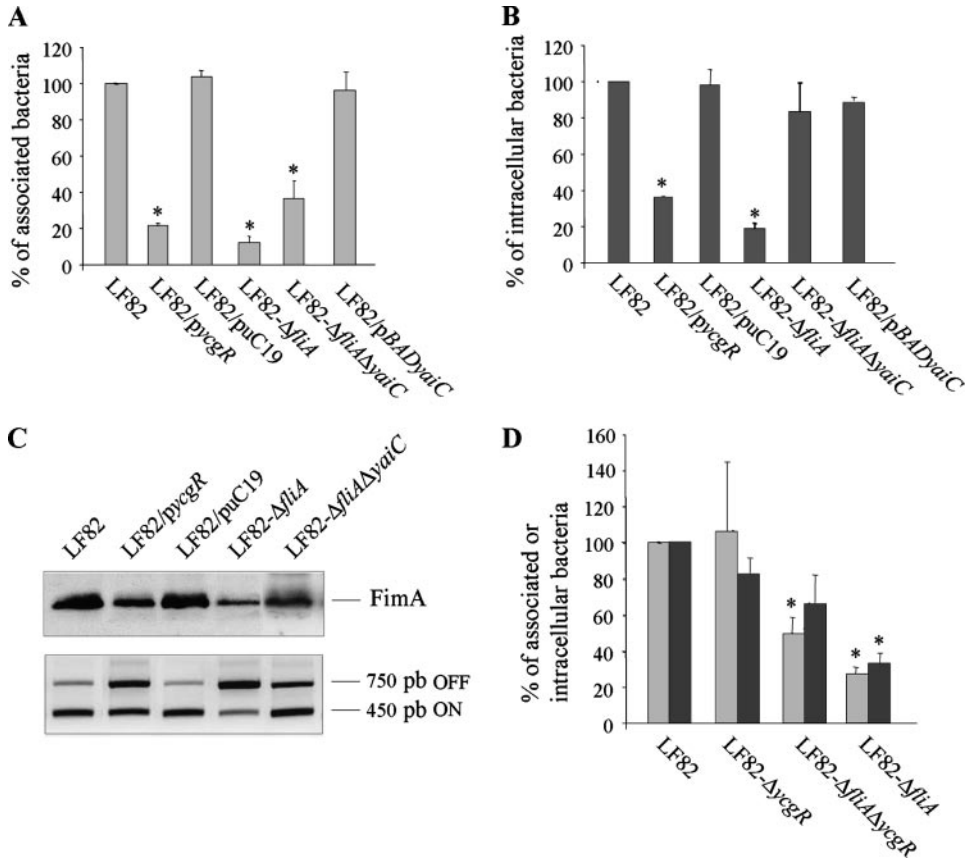

FIGURE 5. **Regulatory role of the *yaiC* gene encoding a GGDEF domain protein and the *ycgR* gene encoding a PilZ domain protein in AIEC LF82 adhesion and invasion abilities.** Adhesion (*A*) and invasion (*B*) abilities of AIEC strain LF82, LF82-Δ*fliA* and LF82-Δ*fliA*Δ*yaiC* mutants, and AIEC strain LF82 transformed by p*ycgR* or control empty vector 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 the same strains. *D*, adhesion (*light gray bars*) and invasion (*dark gray bars*) abilities of AIEC strain LF82, LF82-Δ*fliA*, LF82-Δ*ycgR*, and LF82-Δ*fliA*Δ*ycgR* mutants with intestinal epithelial cells Intestine-407. See the legend to Fig. 1. *, $p < 0.05$.

TABLE 3

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

	mRNA levels relative to those of wild-type strain LF82[a]	Domain
yaiC	0.8 ± 0.2	GGDEF
yhjH	111.0 ± 0.0[b]	EAL
yahA	1.2 ± 0.2	EAL
ycgR	14.3 ± 0.0[b]	PilZ

[a] -Fold decrease in mRNA levels relative to that of wild-type strain LF82 using real time reverse transcription-PCR. 16 S rRNA levels were measured as controls. Only experiments showing the same levels of 16 S rRNA for each sample were taken into account. Data are mean ± S.E. of at least three separate experiments.
[b] $p < 0.05$.

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 protein domain 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-Δ*ycgR* mutant 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 p*ycgR*, the levels of type 1 pili were decreased (Figs. 4 and 5*C*). The adhesion and invasion levels of LF82/p*ycgR* were significantly decreased, to 22 and 37%, respectively, of those of strain LF82 (Fig. 5*C*). Interestingly, analysis of the double mutant LF82-Δ*fliA*Δ*ycgR* 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. 5*D*). However, we did not observe any further decrease in adhesion and invasion levels in the LF82-Δ*fliA* 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-Δ*fliA* (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-Δ*fliA*. These results suggest that the role of FliA in c-di-GMP-dependent control over type 1 pili is complex; nevertheless, we have establish 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 *fliC* results in decreased expression of the *flhDC* operon, which encodes $FlhD_2C_2$, 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 $FlhD_2C_2$ 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

pili synthesis in strain LF82. Hence, decreased $FlhD_2C_2$ levels are likely to be responsible for the lack of type 1 pili in the Δ*fliC* 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-Δ*flhD* or LF82-Δ*fliA* 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 $FlhD_2C_2$ 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-Δ*fliA* 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 inverta-

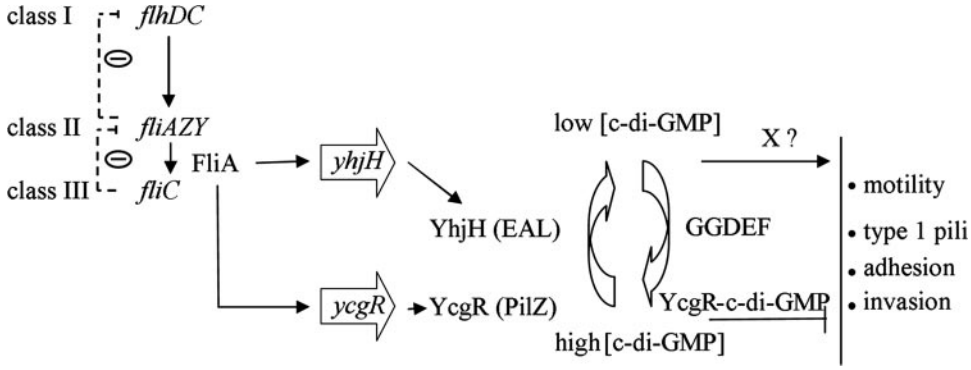

FIGURE 6. **Model for a c-di-GMP-dependent coordinate regulation of the synthesis of flagella, type 1 pili in AIEC LF82.** When the flagella cascade is activated, the accumulation of the FliA sigma factor leads to a concomitant strong induction of the *yhjH* and *ycgR* genes. The EAL domain YhjH degrades 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.

ses (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 oxidase 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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