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Role of Decreased Levels of Fis Histone-Like Protein in Crohn's Disease-Associated Adherent Invasive *Escherichia coli* LF82 Bacteria Interacting with Intestinal Epithelial Cells▽

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The interaction of Crohn's disease (CD)-associated adherent-invasive *Escherichia coli* (AIEC) strain LF82 with intestinal epithelial cells depends on surface appendages, such as type 1 pili and flagella. Histone-like proteins operate as global regulators to control the expression of these virulence factors. We evaluated the role of histone-like proteins in AIEC reference strain LF82 during infection of intestinal epithelial cells, Intestine-407, and observed that the *fis* mRNA level was decreased. The role of Fis in AIEC LF82 was determined by studying the phenotype of an LF82 *fis*::Km mutant. This was the first mutant of strain LF82 that has been described thus far that is unable to express flagellin but still able to produce type 1 pili. The cyclic-di-GMP pathway linking flagella and type 1 pilus expression is not involved in Fis-mediated regulation, and we identified in the present study Fis-binding sites located upstream of the *fimE* gene and in the intergenic region between *fimB* and *nanC* of the *fim* operon encoding type 1 pili. The major consequence of decreased Fis expression in AIEC bacteria in contact with host cells is a direct downregulation of *fimE* expression, leading to the preferential ON phase of the *fimS* element. Thus, by maintaining type 1 pilus expression, AIEC bacteria, which interact with the gut mucosa, have greater ability to colonize and to induce inflammation in CD patients.

Crohn's disease (CD) is an inflammatory bowel disease occurring in individuals with a genetic predisposition in whom an environmental or infectious trigger causes an abnormal immune response (53). Several lines of evidence suggest that bacteria play a role in the onset and perpetuation of inflammatory bowel disease (IBD) (45). *Escherichia coli* has been assigned a putative role in CD. The bacteria are abnormally predominant in early and chronic ileal lesions of CD, and most *E. coli* strains isolated from the ileal mucosa of CD patients adhere to intestinal epithelial cells (17, 32). In addition to their ability to adhere, *E. coli* cells are able to invade intestinal epithelial cells and belong to the pathogenic group of *E. coli*, adherent-invasive *E. coli* (AIEC) (10). Many independent studies have reported the abnormal presence of AIEC bacteria associated with ileal mucosa of CD patients (16, 36, 37) owing to increased ileal expression of CEACAM6 (carcinoembryonic antigen-related cell adhesion molecule 6), which acts as a receptor for AIEC binding to the intestinal mucosa (6).

Various bacterial factors act in concert for full virulence of AIEC, including flagella, OmpC, and type 1 pili (5, 9, 13, 43). Experiments performed *in vitro* with cultured intestinal epithelial cells, *ex vivo* with human isolated enterocytes from CD patients, or *in vivo* using transgenic mice expressing human CEACAM receptors showed that type 1 pili play a key role in the ability of AIEC bacteria to adhere to and to invade intestinal epithelial cells and to induce intestinal inflammation (6, 9, 11). Nucleotide sequences of *fim* genes revealed that strain LF82 produces variants of type 1 pili that differ from those of *E. coli* K-12. In the absence of type 1 pili, AIEC bacteria are no longer able to adhere to and to invade intestinal epithelial cells. Similar loss of adhesion-invasion was observed in flagellum-negative mutants, following coregulation between flagella and type 1 pilus expression, which depends on the level of intracytoplasmic cyclic di-GMP (c-di-GMP) (5, 13). We previously reported that these type 1 pilus variants have to be expressed in the genetic background of strain LF82 in order to promote bacterial uptake since their expression in *E. coli* strain K-12 is not sufficient to confer invasiveness (9). The search for factors involved in the regulation of AIEC virulence led us to focus on the global regulators, histone-like proteins.

In addition to RNA polymerase and transcription factors, the chromosome of *E. coli* cells is bound by a battery of proteins, called histone-like proteins, involved in DNA repair, replication, protection, and folding. Many of these proteins can also act as global transcription factors that bind to the DNA with little or no sequence specificity and operate at a high level in the hierarchy; a relatively small number of DNA binding proteins influences the transcription of large numbers of genes (for a review, see reference 20). Variations in the concentration of these proteins play a part, along with other regulators, in fine-tuning the expression of many genes, including those involved in pathogenesis. Histone-like proteins are generally involved in various delicately balanced processes, and some antagonism between these effects exists. The histone-like proteins known to regulate bacterial virulence are the integration

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)	Source or reference
Strains		
AIEC LF82	*E. coli* isolated from an ileal biopsy of a patient with CD	17
K-12 *fis*::Km	*E. coli* K-12 with a kanamycin resistance cassette in the *fis* gene	23
LF82 *fis*::Km	LF82 isogenic mutant deleted of *fis* gene	This study
LF82 Δ*fliA*	LF82 isogenic mutant deleted of *fliA* gene	13
Plasmids		
pKOBEG	pBAD cloning vector harboring λ phage *red*γβα operon; chloramphenicol resistant	12
pKD4	Plasmid with a kanamycin resistance cassette	18
pBAD18	*E. coli* cloning vector; ampicillin resistant	24
pBAD30	*E. coli* cloning vector; ampicillin resistant	24
pBAD*yhjH*	pBAD18 harboring the entire *yhjH* gene of LF82 *E. coli*	13
pBAD*fis*	pBAD30 harboring the entire *fis* gene of LF82 *E. coli*, amplified with *fis*EcoF and *fis*HindR primers	This study
pUC18	*E. coli* cloning vector; ampicillin resistant	NEB[a]

[a] New England Biolabs, Beverly, MA.

host factor (IHF), the histone-like dimeric HU protein, the histone-like nucleoid structuring protein (H-NS), the leucine-responsive regulatory protein (Lrp), and the factor for inversion stimulation (Fis) (19, 30, 35, 39). Fis binds to and bends DNA and associates as a homodimer on the DNA containing a consensus sequence (for a review, see reference 41). It is a regulator of transcription that can act positively or negatively, depending on the location of its binding site with respect to the target promoter. In many cases, the Fis protein can influence transcription by modulating promoter activity through its ability to affect the level of DNA supercoiling. The Fis protein acts as a modulator in the expression of virulence genes during intracellular growth in *Salmonella* (39) and acts as a negative regulator of curli expression in attaching and effacing *E. coli* (44). We show here that decreased levels of Fis in AIEC LF82 bacteria associated with intestinal epithelial cells result in loss of flagellum expression but continuance of type 1 pilus expression through *fimE*-dependent phase variation and independently of the c-di-GMP pathway.

MATERIALS AND METHODS

Bacterial strains, plasmids, and cell lines. Strain LF82, which belongs to *E. coli* serotype O83:H1, was isolated from a chronic ileal lesion of a patient with CD. Strain LF82 is sensitive to most antibiotics but not to amoxicillin. It adhered to and invaded HEp-2, Intestine-407, and Caco-2 cells (10). *E. coli* strain JM109 was used as the host strain for cloning experiments. Bacterial strains and plasmids used in this study are listed in Table 1.

Plasmid vectors pBAD18 and pBAD30 Amp^r were used in cloning experiments. Bacteria were grown routinely in Luria-Bertani (LB) broth or on LB agar plates (Institut Pasteur Production, Marnes-la-Coquette, France) overnight at 37°C. Antibiotics were added to the medium at the following concentrations: ampicillin, 50 μg/ml; kanamycin, 50 μg/ml; and chloramphenicol, 25 μg/ml. Sialic acid was used at 0.1% (wt/vol) (Sigma).

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 medium (Seromed; Biochrom KG, Berlin, Germany) supplemented with 10% (vol/vol) fetal bovine serum (PAA), 1% nonessential amino acids (Life Technologies, Cergy-Pontoise, France), 1% L-glutamine (Life Technologies), 200 U of penicillin, 50 mg of streptomycin, and 0.25 mg of amphotericin B per liter and with 1% of minimal essential medium (MEM) vitamin mix X-100 (Life Technologies).

Separation of plasmid topoisomers by gel electrophoresis. AIEC LF82 was transformed by electroporation with the pUC18 multicopy plasmid and used to infect the intestinal epithelial cell line Intestine-407. Plasmid DNA was recovered either from bacteria directly or from cells containing intracellular bacteria using a plasmid purification kit (Macherey-Nagel), and plasmids were separated by electrophoresis on a 1% agarose gel containing 2.5 μg/ml of chloroquine. Under these conditions topoisomers that are more supercoiled run faster in the gel than more relaxed topoisomers (26). Samples were transferred to a nylon membrane. A probe targeting the ampicillin resistance cassette on pUC18 was generated using a PCR digoxigenin (DIG) labeling kit (Roche) and the puc18f1 and puc18r1 primers (Table 2), hybridized on the blot, and detected using an anti-DIG antibody, which was visualized using CDP-star (Roche) to reveal the different topoisomers.

DNA manipulations and PCR experiments. PCR conditions and all PCR primer sequences are listed in Table 2. 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 10 min. After centrifugation of the lysate, 5 μl of the supernatant was used in the PCR assays. Usually, 30 PCR cycles were performed. Amplification products were separated by electrophoresis on a 1% agarose gel.

Western blot analysis. Samples of bacteria were grown overnight at 37°C in LB broth without agitation. A total of 700 μl of culture was centrifuged; the pellet of bacteria was suspended in 100 μl of SDS sample buffer and heated for 5 min at 95°C, and equivalent amounts of protein extract were separated by SDS-PAGE (12 or 17% polyacrylamide). Proteins were electroblotted onto nitrocellulose membranes (Amersham International), and the membranes were immunoblotted for type 1 pili (rabbit antiserum raised against purified type 1 pilus preparations, diluted 1:7,500), and RNA polymerase alpha subunit (rabbit anti-alpha, 1:10,000). Immunoreactants were detected by autoradiography using horseradish peroxidase-conjugated anti-rabbit immunoglobulin G antibody (1:10,000) and enhancing chemiluminescence (Amersham Biosciences).

RNA manipulations and real-time RT-PCR. Total RNAs from bacteria associated with epithelial Intestine-407 cells and from bacteria grown in MEM were extracted using a protocol adapted from Lucchini et al. (34). After treatment with DNase (Roche Diagnostics, Mannheim, Germany) to eliminate contaminating DNA, total RNAs were reverse transcribed and amplified using specific primers (Table 2). Real-time reverse transcription-PCR (RT-PCR) was performed using an Eppendorf Replex, and quantification of the mRNA level or 16S rRNA (as a control) was performed using RNA Master SYBR Green 1 (Roche Diagnostics) with 0.25 μg of total RNA. Amplification of a single expected RT-PCR product was confirmed by electrophoresis on a 2% agarose gel.

Construction of *fis* insertion mutant. To construct *fis* insertion mutants in AIEC strain LF82, the resistance cassette was amplified from *E. coli* K-12 mutants carrying an insertion in the target gene. In addition, strain AIEC LF82 was transformed with pKOBEG plasmids, which encode Red proteins from phage λ under the control of an L-arabinose inducible promoter. Plasmids were maintained in bacteria at 30°C with 25 μg/ml of chloramphenicol and killed at 42°C. Strain LF82/pKOBEG was grown at 30°C in the presence of 1 mM L-arabinose to induce Red expression. When the optical density at 620 nm (OD_{620}) 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. Replacement of the gene by the kanamycin resistance cassette in mutants was confirmed by PCR.

TABLE 2. Oligonucleotides used and PCR product sizes

Primer	Oligonucleotide sequence (5′–3′)	PCR product size (bp)	Use	Annealing temp (°C)
K1	CAGTCATAGCCGAATAGCCT	473	Kanamycin resistance cassette amplification	54
K2	GGTGCCCTGAATGAACTGCA			
KT	CAGTCATAGCCGAATTACCCT		Localization verification of kanamycin resistance cassette	
*hupB*Fint	TTGATCGACAAGATTGCTGC	199	RT-PCR amplification	52
*hupB*Rint	CGCGTCTTTCAGTGCTTTAC			
*hns*Fint	GAACAACATCCGTACTCTTC	334	RT-PCR amplification and	52
*hns*Rint	GATTACAGCTGGAGTACGGC			
*ihfA*Fint	TTGATAAGCTTGGGCTTAGC	248	RT-PCR amplification and	52
*ihfA*Rint	CGAAGCGTTTTCGACCCGGC			
*fis*F	GAACAGCTGGAGGCGTTGGAG	438	LF82 *fis*::Km insertion mutant construction	55
*fis*R	AATCAAGCATTTAGCTAACCTG			
*fis*Fint	CGAACAACGCGTAAATTCTG	260	RT-PCR amplification and	52
*fis*Rint	GCAGCGTACCACGGTTGATG		LF82 *fis*::Km insertion mutant verification	
*lrp*Fint	CAAGAAGCGCCCTGGCAAAG	467	RT-PCR amplification and	52
*lrp*Rint	CCAGACGATTACTCTGCTTG			
puc18f1	CCTATCTCAGCGATCTGTCT		Synthesis of pUC18 probe	55
puc18r1	GATGGCATGACAGTAAGAGA			
*yhjH*Fint	GCGCCAGATTGAGGATCTTC	241	RT-PCR amplification	55
*yhjH*Rint	GAAAGAAACCAGCCTTGTGC			
*yaiC*Fint	CTGGTGTTGATGGTGGTTTC	291	RT-PCR amplification	55
*yaiC*Rint	GTTGCATCGCGATTATGCCG			
*ycgR*Fint	CCATGAGCAGTTCCTGAAAC	262	RT-PCR amplification	55
*ycgR*Rint	CTGCTGTAGTTGTTCAACAG			
*fliC*Fint	TGGTGCTGCAACTGCTAACGC	212	RT-PCR amplification	55
*fliC*Rint	TTATCGGCATATTTTGCGCTAGC			
*csgD*Fint	ACGCCTTGCAACCCATTGAC	160	RT-PCR amplification	55
*csgD*Rint	GGCAGGATACTTTGAGCAGG			
*MICsgE*F	AGAACTACTGCATGTTTGACACGCATTGCA GCCTCCGAACAATTTTTTATGTAG	1573	LF82-D*csgE* isogenic mutant construction	55
*MICsgE*R	CCGGACACAAGCGGTTTCCTGGGCAAACG ATAACCTCAGGCGATAAAGCCCATATGA ATATCCTCCTTAGTTC			
*csgE*F	AATCTTTTCAAGAAGATAGCCG	552	LF82-D*csgE* isogenic mutant verification	55
*csgE*R	CCAGCCCAACTTAATGGCG			
*csgE*Fint	CTTAACGATTAATGAAAGGCCC	208	LF82-D*csgE* isogenic mutant verification	55
*csgE*Rint	ATCATGCGCCAAATCGCCCG			
*fisEco*F	CCGGAATTCCCATTGAGGATGCCAGCGAAC	391	Cloning of the *fis gene*	55
*fisXba*R	GCTCTAGAGCACCTGAATTAGTTCATGCCG			
pro*fimE*F	ACTTCGGGTGTGGTTATCTT	75	Synthesis of *fimE* promoter containing the Fis binding site	50
pro*fimE*R	TTGAATGAATACCTATAGG			
pro*nanC*F	TCGCGGTGCACCTTTATACC	75	Synthesis of *nanC* promoter containing the Fis binding site	55
pro*nanC*R	AATTGCAAATCTCGTTTTGT			
Fis-*nanC*-F-FAM	GGTCTCCTGTGAAAATTCTC	567	Footprinting assay containing the Fis binding site	55
Fis-*nanC* R	TGATGAGAATTATGATCAGC			
Fis-*fimE*-F-FAM	TAAGTTAACCATATCCATAC	582	Footprinting assay containing the Fis binding site	55
Fis-*fimE*-R	ACGTAACGGGTGAACGGTAG			
*fimE*Fint	GGCATATCGGCATGGGATGC	168	RT-PCR amplification	50
*fimE*Rint	GAACGCTGGACCCAGGAACG			
*nanC*Fint	GTAAGTGACATATCCATCCC	180	RT-PCR amplification	56
*nanC*Rint	CATTTTAGCAGCAACGGCAC			
*fimB*Fint	CCTGACCCATAGTGAAATCG	156	RT-PCR amplification	55
*fimB*Rint	CTTTGCCTTAAGATCAATATC			
FIME	GCAGGCGGTTTGTTACGGGG	750	Off-oriented invertible element	55
INV	GAGGTGATGTGAAATTAATTTAC			
FIMA	GATGCGGTACGAACCTGTCC	450	On-oriented invertible element	55
INV	GAGGTGATGTGAAATTAATTTAC			
16S 1	ATGACCAGCCACACTGGAAC	157	RT-PCR amplification	55
16S 2	CTTCCTCCCCGCTGAAAGTA			

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 as follows. A washed bacterial suspension was placed on carbon-Formvar copper grids. Excess liquid was removed, and the grids were placed face down on antiserum (1:1,000) 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.

Bacterial growth and motility assay. Each strain was tested for the ability to grow under static conditions in MEM at 37°C for 8 h. The experiment was monitored by measuring the OD_{620}. For motility assays, bacteria were grown overnight at 37°C on LB broth, and 2 μl of this culture was inoculated onto 0.3% agar plates. The plates were incubated at 37°C for 10 h, and motility was assessed qualitatively by examining the circular swim formed by the growing motile bacterial cells.

Yeast cell aggregation assay. Commercial baker's yeast (*Saccharomyces cerevisiae*) was suspended in phosphate-buffered-saline (PBS; 10 mg dry weight/ml). *E. coli* strains were resuspended to an optical density of 0.2 at 620 nm in PBS. Equal volumes of fixed yeast cell suspension and decreasing concentrations of *E. coli* suspension were used, and aggregation was monitored visually.

Adhesion assays. 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 bovine serum at a multiplicity of infection (MOI) of 10 bacteria per epithelial cell. After a 3-h incubation period at 37°C, monolayers were washed three times in PBS (pH 7.2). The epithelial cells were then lysed with 1% Triton X-100 (Sigma Chemical Company, St. Louis, MO) in deionized water. Samples were diluted and plated onto Mueller-Hinton agar plates to determine the number of CFU corresponding to the total number of cell-associated bacteria (adherent and intracellular bacteria). The infected monolayers were centrifuged for 10 min at $1,000 \times g$ before the 3-h infection period.

Consensus elaboration. The MEME (http://meme.sdsc.edu/meme/meme.html) software program (version 4.1.0) was used for the identification of motifs within 65 sequences (Table 3) previously reported to bind Fis protein (1, 25, 48, 51). MEME uses the method of Bailey et al. to identify likely motifs within the input set of sequences (2, 4). It produces a consensus sequence and a position-specific probability matrix, which has probabilities associated with each base at each position. A range of motif widths (≥15 nucleotides in length) and zero or one motif per sequence were specified in our queries.

We then applied the MAST program (version 4.0), using the motif weight matrix from the MEME program, to search for the motif in the *fim* operon as well as for a random sequence model based on the letter composition of the target sequence. The algorithm in MAST calculates position scores for the motif at each possible position within a sequence (3). These scores are translated into position *P* values, which represent the probability of a randomly selected position in a randomly generated sequence having a match score at least as large as that of the given position. The best (i.e., lowest) position *P* values are then adjusted to take into account the length of the sequence. Only the motif hits with a position-specific goodness-of-fit *P* value of less than 10^{-4} were considered to identify putative Fis-binding sites (FBSs).

Electrophoretic mobility shift assay (EMSA). DNA binding by the purified Fis protein was assayed by incubating the 75-bp DNA fragment that harbors a Fis-binding site in its center with increasing concentrations of Fis for 15 min in the presence of excess poly(dI-dC) double-stranded DNA to prevent nonspecific interactions. After separation of the products on a 12% native polyacrylamide gel, complexed and uncomplexed DNA fragments labeled with DNA DIG ddUTP by a terminal transferase enzyme fill-in reaction were visualized using an alkaline-phosphatase-coupled anti-DIG antibody (DIG Gel Shift Kit, 2nd Generation; Roche).

DNase I footprinting by an automated DNA sequencer. Probes were generated by PCR with the primers Fis-*nanC*-F-FAM/Fis-*nanC*-R and Fis-*fimE*-F-FAM/Fis-*fimE*-R (where FAM is 6-carboxyfluorescein) (Table 2). The PCRs were performed for 30 cycles under the following conditions: 95°C for 30 s, 55°C for 30 s, and 72°C for 60 s. PCR products, FAM labeled at the 5′ end, were purified using a NucleoSpin Extract II (Macherey-Nagel) kit and quantified using an Eppendorf BioPhotometer. Each 10-μl reaction mixture contained 10 mM Tris-HCl, pH 7.5, 5 mM KCl, 1 mM EDTA, 2 mM dithiothreitol (DTT), 0.1 mg/ml bovine serum albumin (BSA), 8% glycerol, 20 ng of FAM-labeled DNA fragment, and 0.45 nM purified Fis protein. After incubation at 25°C for 15 min, DNase I (Roche Diagnostics, Mannheim, Germany), freshly prepared by a 1/10 dilution, was added. After 5 min at 25°C, digestion was stopped by chilling the sample on ice and the addition of stop solution (0.3 M sodium acetate [NaAc] and 5 mM EDTA). DNA fragments were further purified using a NucleoSpin Extract II (Macherey-Nagel) kit and analyzed using an ABI Prism 3100 Genetic

TABLE 3. The 65 sequences selected to define the motif corresponding to the FBSs

Sequence no.	Gene name or DNA region[a]	Position *P* value	FBS[b]
1	*fis5*	3.26e−06	ATT **GGTCAAAGTTTGGCC** TTT
2	7	4.04e−06	TA **GGTCAAATTTTGACC** TATA
3	*thru3*	1.36e−05	GTC **GCATAAAATGTGACC** AAT
4	*mu1* left end	1.62e−05	AAC **GACTAAAATTTGCAC** TAC
5	*nhd1*	1.94e−05	ATT **GTTTATTATTTGAGC** GAA
6	*hin3* distal	2.28e−05	TGC **GGTCACAATTTGCAC** TAG
7	*ndr*	3.18e−05	TATGCC **GTTCAAGAAATCGCC**
8	*rrnb1*	3.72e−05	AAC **GGGCAATAATTGTTC** AGC
9	6	3.72e−05	TA **GATCAAATTTTGATC** TATA
10	*ndr4*	4.36e−05	CCC **GTTCAAGAAATCGGC** CGA
11	*thru1*	4.36e−05	GAT **GTTGAAAAAGTGTGC** TAA
12	*hin2* proximal	5.07e−05	GGG **TGTCAACAATTGACC** AAA
13	*tn51*	5.07e−05	GGG **GATCAAGATCTGATC** AAG
14	13	5.07e−05	TA **GCTTAAATTTTAAGC** TATA
15	*aldb4*	5.90e−05	ACT **GGCGAAGATTTCGCC** AGT
16	*fis2*	7.87e−05	AGT **GACTAAAATTTACAC** TCA
17	*cin2* distal	9.04e−05	AAA **GCGCAGGATGTGAGC** TAA
18	*prop1*	1.03e−04	AAA **GGTCATTAACTGCCC** AAT
19	*aldb3*	1.03e−04	AGC **GGCTAACAATTTGCC** AGC
20	*rrnb3*	1.34e−04	ACC **GCGCAACATTCAACC** AAA
21	*tyrt3*	1.96e−04	TAC **GGATGAAAATTACGC** AAC
22	*fis6*	2.21e−04	ATG **AGTTAAGAAATGACC** ATA
23	*fis*	2.21e−04	CTA **GTGCAAATTGTGACC** GCA
24	*nhd2*	2.49e−04	GTT **GCTGAAAAGATAGGC** GAC
25	*gin1* proximal	2.79e−04	GGG **TATCAACAAATGACC** AGA
26	*tgt-sec1*	3.13e−04	TGA **GCTAAAAAATTCATC** GAT
27	*hin1*	3.50e−04	AGC **GACTAAAATTCTTCC** TTA
28	*prop2*	3.50e−04	TCC **GGTTAAGGAATGTAC** AAT
29	*mu2* right end	3.91e−04	ATA **GTTTGGTATTTAGCC** GCT
30	*tyrt1*	3.91e−04	GGC **GATTAAAGAATAATC** GTT
31	*gin3*	4.36e−04	TTT **GAAGATAAATTAAGC** GGA
32	*gin2* distal	4.36e−04	TTT **GTGCAGGATGTGAAC** AAA
33	*aldb1*	4.36e−04	GCT **GCGCGATAAATCGCC** ACA
34	*tgt-sec2*	5.39e−04	TTT **GGATAGAATATAATC** GAT
35	*fis1*	5.39e−04	TTT **GCCGATTATTTACGC** AAA
36	*hin4*	5.98e−04	GCG **GACTGGGATTTGTTC** AGG
37	*ndr3*	5.98e−04	AAA **GATTAATAAGCCATC** TAT
38	*thru2*	5.98e−04	CAC **GATGAAGAAACAGCC** GAA
39	*oric1*	5.98e−04	ACA **ACTCAAAAACTGAAC** AAC
40	*rrnb2*	7.32e−04	AAC **GCTCGAAAAACTGGC** AGT
41	*puc19 lacP*	8.08e−04	GGT **GCCTATTGAGTGAGC** TAA
42	*cin1* proximal	8.08e−04	GCG **TATCAACAAATGAAC** AAA
43	*hin5*	8.90e−04	AGT **GTTGATTACTTGTGC** CAT
44	*fis3*	8.90e−04	GTG **GTGCGATAATTACTC** ATA
45	*lambda1 att*	9.80e−04	TTT **GCATAAAAAACAGAC** TAC
46	*hns2*	1.08e−03	TTG **GGGTAGCAATAGAGC** CTT
47	*ndr1*	1.18e−03	ACC **GAATAGAAAACAACC** ATT
48	*hns3*	1.30e−03	AGG **AATTACAAATTGTTC** TTT
49	*hns7*	1.56e−03	AAT **GATGAAAAGTAGAAC** AAG
50	*hns6*	1.86e−03	GAA **GACTGAAAGGTCGTC** AGG
51	*fis4*	1.86e−03	ATT **GCATTTAAAATGAGC** GTG
52	*hns4*	2.61e−03	TCT **TTTCATAAAATTAGC** CAG
53	*oric2*	3.07e−03	TAA **GTATACAGATCGTGC** GAT
54	*cin3*	3.33e−03	CTG **GGTTAAAAAAGTTAC** TCC
55	*aldb2*	3.61e−03	TGT **AATCATCATTTCCAC** AAC
56	*hns1*	4.22e−03	TAA **GTTTGAGATTACTAC** AAT
57	*cin4*	4.55e−03	GTC **GTATGGAAGTTAGAC** CGT
58	*hns5*	5.70e−03	TGA **GGTTAAAACTTCCTG** ATT
59	*lambda3 OLII*	5.70e−03	TGC **GGTGATAAATTATCT** CTG
60	*hns5*	6.13e−03	AAC **GAATTAAAGGTAACC** AGT
61	*nhd3*	8.13e−03	AAT **GGTTATTAACATAGC** TCT
62	3	9.32e−03	TA **TCTCAAATTTTGAGA** TATA
63	*lambda2 OLI*	1.56e−02	TTC **ATATAAAAAACATAC** AGA
64	*ndr2*	1.56e−02	ATT **GACCACAACTGATAC** ATC
65	*ndr5*	1.87e−02	GAA **GAGAAAAATTTGTTA** AAA

[a] As described by Shao et al. (48).
[b] The letters in bold indicate the bases included in the 15-residue FBS motif.

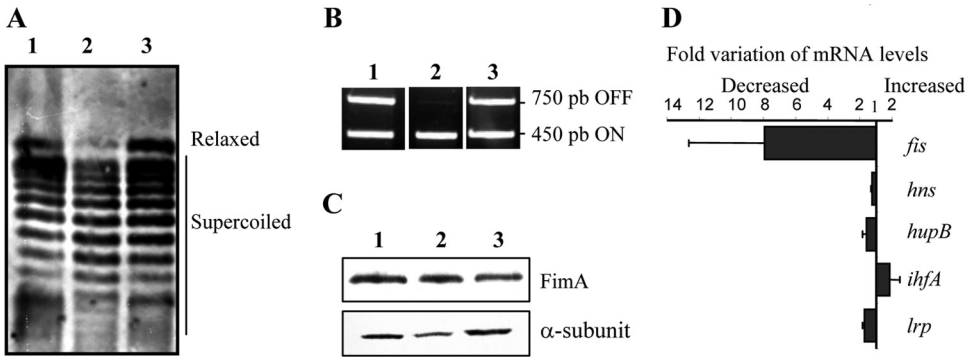

FIG. 1. DNA supercoiling, orientation of the *fimS* element, and quantification of *fis*, *hns*, *hupB*, *ihfA*, and *lrp* mRNA levels in AIEC LF82 associated with intestinal epithelial cells. (A) The wild-type strain harboring pUC18 reporter was used to infect Intestine-407 cells for 3 h or was grown in MEM or conditioned MEM. Plasmid DNA was separated by electrophoresis on 1% agarose gel containing 2.5 μg/ml chloroquine, and topoisomers were detected by hybridization with DIG-labeled pUC18 probe. (B) Determination of the orientation of the *fim* operon invertible element in wild-type AIEC LF82 strain grown in MEM or in conditioned MEM for 3 h or after a 3-h infection period of Intestine-407 cells. A 450-bp product revealed ON orientation, and the 750-bp product revealed the OFF orientation of the invertible element. (C) Determination of the amount of the FimA subunit by Western blotting using antibodies raised against type 1 pili was performed. As a control, Western blot analysis was performed using antibodies raised against the α-subunit of RNA polymerase. (D) mRNA levels of *fis*, *hns*, *hupB*, *ihfA*, and *lrp* genes encoding histone-like proteins in AIEC LF82 bacteria after a 3-h infection period of Intestine-407 cells relative to levels in AIEC LF82 bacteria grown for 3 h in MEM cell culture medium using real-time RT-PCR. As controls, 16S rRNA levels were measured. Only experiments showing the same levels of 16S rRNA for each sample were taken into account. Data represent means of at least three separate experiments; bars indicate and standard errors of the means (SEM). Lanes 1, wild-type AIEC LF82 strain grown in MEM; lanes 2, AIEC LF82 after a 3-h infection period of Intestine-407 cells; lanes 3, AIEC LF82 grown in conditioned MEM for 3 h.

Analyzer (Applied Biosystems) capillary sequencer. A GeneScan 400HD ROX size standard (Applied Biosystems) ladder was used as an internal control.

Statistical analysis. To analyze the significance of differences between adhesion and invasion levels, a Student's *t* test was used for comparison of two groups of data. All experiments were made at least three times. A *P* value of less than or equal to 0.05 was considered statistically significant.

RESULTS

Expression of histone-like protein-encoding genes in cell-associated bacteria. To explore the involvement of global regulators in the ability of AIEC bacteria to adhere to intestinal epithelial cells, we first compared the variations in DNA supercoiling in cell-associated bacteria and in bacteria cultured in cell culture medium. We compared changes in supercoiling levels of reporter plasmid pUC18 control DNA in Intestine-407 cell-associated LF82 bacteria and in bacteria grown in MEM or conditioned MEM. In cell-associated AIEC LF82 bacteria, the supercoiling levels of plasmid topoisomers were higher than those from AIEC LF82 bacteria grown in cell culture medium or conditioned medium (Fig. 1A). As type 1 pili are the major bacterial factors responsible for the ability of AIEC LF82 bacteria to adhere, we analyzed AIEC type 1 pilus expression in bacteria associated with intestinal epithelial cells. The regulation of type 1 pilus expression is controlled by a process called phase variation that allows bacteria to switch between piliated (ON) and nonpiliated (OFF) states by inverting an *fimS* DNA element located upstream of the *fim* operon. Thus, we analyzed the orientation of *fimS* using a PCR-based approach (47). In cell-associated AIEC bacteria, we observed a preferential ON-phase orientation, whereas in AIEC bacteria grown in cell culture medium alone or in conditioned cell culture medium approximately half of the bacteria in the population were in OFF phase (Fig. 1B). However, Western blot analysis did not reveal an increased level of FimA, the major subunit of type 1 pili, in cell-associated AIEC bacteria (Fig. 1C), which is certainly due to the fact that LF82 bacteria grown in MEM were highly piliated and any increase in piliation was difficult to measure. Type 1 pilus expression depends on DNA relaxation in *E. coli* (15), and histone-like proteins participate in the organization of the nucleoid by affecting DNA supercoiling (50). We therefore measured the levels of *hupB*, *ihfA*, *fis*, *hns*, and *lrp* mRNAs, encoding HUβ, IHFα, Fis, H-NS, and Lrp proteins, respectively, by RT-PCR in LF82 bacteria after a 3-h infection period of Intestine-407 epithelial cells or after 3 h of bacterial growth in MEM (Fig. 1D). The levels of mRNA encoding H-NS, HUβ, IHFα, and Lrp histone-like proteins were not significantly different between cell-associated AIEC LF82 bacteria and bacteria grown in MEM, indicating that these histone-like proteins were unchanged during the infection process. In contrast, the *fis* mRNA level was 7.9- ± 4.7-fold decreased in bacteria interacting with Intestine-407 cells, but due to a large dispersion of the values (ranging from 1.3- to 31.1-fold), only a trend toward significance was observed ($P = 0.09$). Thus, these findings suggest that decreased levels of Fis in AIEC LF82 bacteria in contact with intestinal epithelial cells could be responsible for variations in DNA supercoiling and thus for the preferential ON-phase orientation of type 1 pilus expression.

Phenotype of AIEC LF82 Fis-negative mutant. In order to evaluate the role of Fis histone-like protein in the ability of AIEC strain LF82 to adhere to Intestine-407 cells, an insertion mutant interrupted in the *fis* gene was constructed. Growth curves during an 8-h period in MEM of the Fis histone-like protein-negative mutant were similar to those of wild-type strain LF82 (data not shown). Analysis of the LF82 *fis*::Km mutant phenotype indicated that it was nonmotile (data not shown) and did not express FliC, and no flagella were observed in the LF82 *fis*::Km mutant by electron microscopy examination (Fig. 2A). Immunogold labeling assays using polyclonal

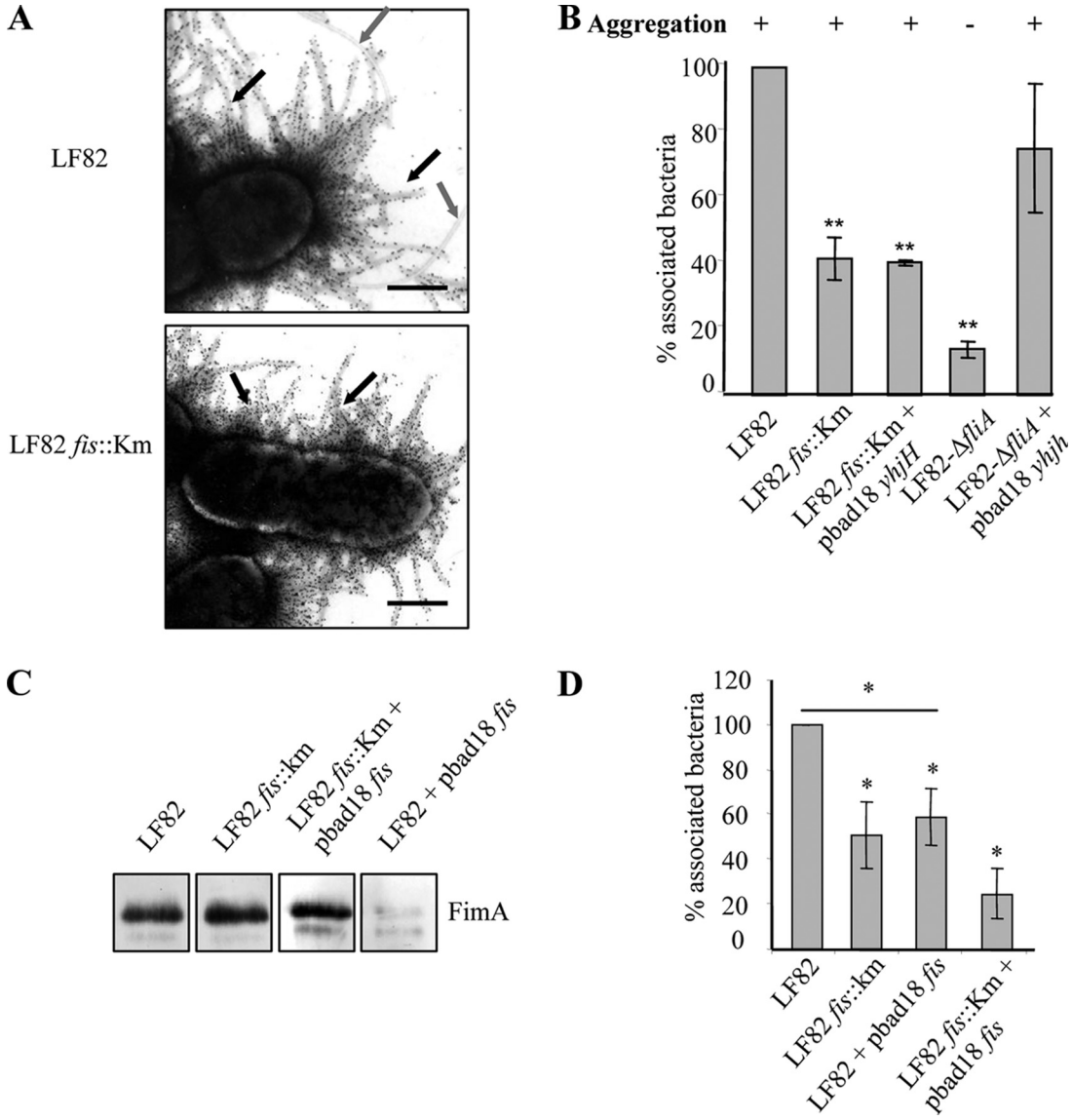

FIG. 2. Type 1 pilus expression and adhesion ability of LF82 wild-type strain and LF82 *fis*::Km mutant with induced YhjH and Fis expression. (A) Transmission electron microscopy (TEM) examination of bacteria stained using immunogold labeling with antibodies raised against purified type 1 pili (black arrow). Flagella (gray arrow) were observed only on the surface of wild-type bacteria. The black scale bar represents 500 nm. (B) In LF82 Δ*fliA* and LF82 *fis*::Km mutants harboring the pBAD*yhjH* construct, expression of YhjH protein was induced using 0.1% arabinose. Intestine-407 cell-associated bacteria were quantified after centrifugation and a 3-h infection period. The mean number of cell-associated LF82 bacteria was $7.2 \times 10^6 \pm 3.9 \times 10^5$ CFU. Results are expressed as cell-associated (adherent plus intracellular) bacteria relative to those obtained for strain LF82, taken as 100%. Each value is the mean ± SEM of at least four separate experiments. The expression of functional type 1 pili was evaluated by determination of the yeast aggregation. (C) Determination of the amount of FimA subunit by Western blotting using antibodies raised against type 1 pili. (D) Overexpression of Fis was induced in LF82 *fis*::Km and the wild-type strain harboring pBAD*fis* construct with 0.1% arabinose. Intestine-407 cell-associated bacteria were quantified after centrifugation and a 3-h infection period. The mean number of cell-associated LF82 bacteria was $7.8 \times 10^6 \pm 7.8 \times 10^5$ CFU. *, $P < 0.05$; **, $P < 0.01$.

antibody raised against purified type 1 pili revealed that the Fis-negative mutant produced amounts of type 1 pili similar to those of the wild-type strain (Fig. 2A). Unexpectedly, a significant decrease in adhesion was observed for the LF82 *fis*::Km mutant, with residual adhesion of 41.6% (Fig. 2B). The decrease in the ability of the LF82 *fis*::Km mutant to adhere was type 1 pilus independent since the LF82 *fis*::Km mutant still expressed functional type 1 pili on the bacterial surface, as assessed by the ability of the bacteria to aggregate yeast cells via binding to D-mannose residues located on the yeast surface (Fig. 2B). We previously reported that nonflagellated LF82 bacteria were unable to produce type 1 pili (5) and that the intracellular level of cyclic di-GMP is involved in the coregulation between flagella and type 1 pilus expression in AIEC strain LF82 (13). Data reported here indicate that this coregulation is impaired in the LF82 *fis*::Km mutant and suggest that the Fis protein could regulate an unidentified adhesion factor. This is not curli fimbriae, whose expression depends on the

TABLE 4. Quantification of *yaiC*, *yhjH*, *ycgR*, and *fliC* expression in AIEC strain LF82 and *fis* mutant strain

Gene	Decrease in mRNA[a]	Function
fliC	1776.72 ± 0.01*	Flagellin protein
yaiC	1.74 ± 0.19	Cyclase
yhjH	22.37 ± 0.04*	Phosphodiesterase
ycgR	8.03 ± 0.07*	c-di-GMP receptor
csgD	1.11 ± 0.23	Activator of curli

[a] Fold decrease in mRNA levels in the LF82 *fis*::Km mutant strain 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 ± standard error of at least three separate experiments. *, $P < 0.01$.

intracellular level of cyclic di-GMP since an AIEC LF82 Δ*csgE* negative mutant presented no defect in its adhesion ability (data not shown), and no difference in *csgD* mRNA level was observed between the *fis* mutant and wild-type strain LF82 (Table 4).

To evaluate whether the c-di-GMP pathway could be involved in the impaired ability of the LF82 *fis*::Km mutant to adhere, we measured mRNA levels in the LF82 *fis*::Km mutant of genes *yhjH* encoding the EAL domain phosphodiesterase YhjH, *yaiC* encoding the GGDEF domain diguanylate cyclase YaiC, and *ycgR* encoding the c-di-GMP specific receptor YcgR in the LF82 *fis*::Km mutant and in wild-type strain LF82. The *yaiC* mRNA level was not modified in the LF82 *fis*::Km mutant (Table 4). Conversely, those of *yhjH* and *ycgR* were 22.3-fold and 8.0-fold lower, respectively, in the LF82 *fis*::Km mutant than in wild-type strain LF82. These decreases were similar to those observed in the LF82 Δ*fliA* isogenic mutant (13). Thus, we induced expression of the phosphodiesterase YhjH in the LF82 *fis*::Km mutant to decrease the intracellular concentration of c-di-GMP. The pBAD*yhjH* plasmid carrying the *yhjH* gene under an arabinose-inducible promoter was therefore transformed into the LF82 *fis*::Km mutant (Fig. 2B). Similar experiments were performed with the LF82 Δ*fliA* mutant as a control to confirm that overexpression of YhjH restored its ability to interact with Intestine-407 cells (Fig. 2B). The induced expression of YhjH in the LF82 *fis*::Km mutant did not restore its ability to adhere, which suggests that there could exist an as yet unidentified adhesive factor whose expression is decreased in an AIEC LF82 *fis*-negative mutant independently of the intracellular concentration of c-di-GMP.

To further understand why the LF82 *fis*::Km mutant, which did not express flagella, did not behave like the LF82 Δ*fliA* mutant with regard to type 1 pilus expression, we investigated whether Fis could have a direct role in the expression of these fimbriae. We induced overexpression of Fis in the AIEC LF82 wild-type strain and observed impaired FimA expression (Fig. 2C) and, consequently, significantly decreased adhesion ability (Fig. 2D). Of note, overexpression of the Fis protein in the LF82 *fis*::Km mutant induced an even greater decrease in adhesion. Together, these results indicated that there could exist in AIEC strain LF82 two mechanisms that regulate the ability of LF82 bacteria to adhere to intestinal epithelial cells and that involve different Fis concentrations.

Regulation of AIEC strain LF82 type 1 pilus expression by the Fis histone-like protein. We therefore searched for motifs in Fis-binding sites (FBSs) using the MEME program. This analysis identified a 15-residue motif (5′-GGTCAAAAATTG ACC-3′) as the best possible FBS. Position *P* values of FBSs varied between 3.26×10^{-6} and 1.87×10^{-2} because of the low specificity of Fis-DNA interactions. The information content diagram provides information on which positions in the motif are the most highly conserved (Fig. 3A). We then used the MAST program to search for the presence of the motifs in the regulatory regions of the *fim* operon and in random sequences. One motif was observed 58 bp upstream of the *fimE* gene, encoding the FimE recombinase, which catalyzes the

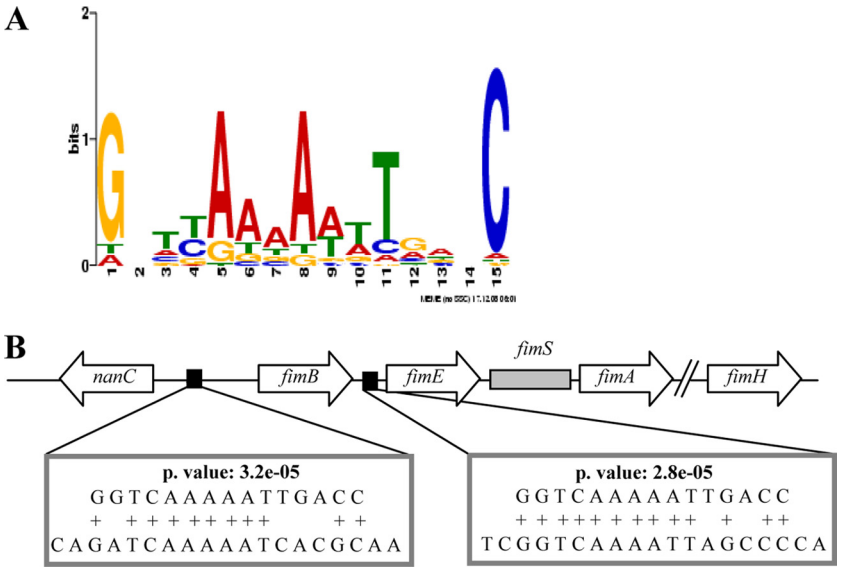

FIG. 3. Identification of specific FBSs in the *fim* operon. (A) Sequence logo generated from 65 Fis-defined FBSs and their complements displays the relative functional contribution of bases within the 15-bp core sequence of the Fis-binding site. Each column (position) in a motif can be characterized by the amount of information it contains (measured in bits). Highly conserved positions in the motif have high information content; positions where all letters are equally likely have low information content. (B) Location of putative identified FBS in regulatory regions of *fim* operon.

ON-to-OFF inversion of the *fimS* DNA element; the other motif was detected in the intergenic region between the *nanC* gene (440 bp upstream), encoding the *N*-acetylneuraminic acid (sialic acid) outer membrane channel protein, and the *fimB* gene (1,009 bp upstream), encoding the FimB recombinase which catalyzes both ON-to-OFF and OFF-to-ON inversions (Fig. 3B). Their position-specific goodness-of-fit *P* values were 2.8×10^{-5} and 3.2×10^{-5} for DNA sequence upstream of *fimE* and *nanC-fimB*, respectively. Among the 15 residues of the motif, only two mismatches were observed in comparison with the best possible FBS for the sequence upstream of *fimE*, and four mismatches were observed for the sequence in the intergenic region *nanC-fimB*. Of note, no putative FBS was found 1 kb upstream of the start codon of the other identified genes involved in adhesion and invasion of AIEC LF82, such as the *yfgI*, *ompC*, and *nlpI* genes, indicating that among the genes previously described in AIEC LF82 bacteria, only type 1 pilus expression could be regulated by Fis.

To confirm that the Fis protein is able to specifically bind DNA upstream of *fimE* and *nanC-fimB* and thus directly control their expression, EMSAs were performed using various Fis concentrations with a 75-bp DIG-labeled DNA fragment harboring putative FBSs. The purified Fis protein was able to bind specifically to the *fimE* promoter and in the intergenic *nanC-fimB* region, forming two complexes with low Fis protein concentrations (between 0.02 and 0.22 nM), and a 100-fold excess of non-labeled DNA competitor abrogated the shift (Fig. 4A and B). The two FBSs identified in the type 1 pilus regulatory region were bound with an affinity that was similar to that of Fis for its high-affinity binding sites (48) and required Fis concentrations of 0.033 nM to achieve more than 50% binding. As a control, Fis protein at concentrations similar to those used in the shift assays was unable to bind with similar affinity to an unrelated DNA sequence that did not harbor an FBS (Fig. 4C). DNase I footprinting with 0.45 nM purified Fis protein using 5′-FAM PCR products harboring the predicted FBS in a central position (567 bp for the intergenic region *nanC-fimB* and 582 bp in the region located upstream of *fimE*) was performed using a capillary DNA sequencer. The results revealed that Fis protein bound to FBS-predicted sequences, 5′-GATCAAAAATCAGCG-3′ in the intergenic *nanC-fimB* region and 5′-GGGCTAATTTTGACC-3′ in the region upstream of *fimE* (Fig. 5), confirming that the FBSs upstream of *fimE* and *nanC-fimB* were able to specifically bind Fis.

Fis positively controls *fimE* expression but negatively controls that of *nanC*. In order to verify that Fis could directly regulate the expression of genes encoding NanC and the two recombinases FimE and FimB, *fimE*, *fimB*, and *nanC* mRNA levels were measured in the LF82 *fis*::Km mutant and in wild-type strain LF82. Concerning *fimB*, only a 1.5-fold decrease in mRNA level was observed between the LF82 *fis*::Km mutant and wild-type strain LF82 (Fig. 6A). The *fimE* mRNA level was 2.7-fold lower in LF82 *fis*::Km mutant than in the LF82 wild-type strain. In contrast, the *nanC* mRNA level was increased 9.7-fold in the LF82 *fis*::Km mutant compared to wild-type strain LF82. We can speculate that in the wild-type strain LF82, Fis activates *fimE* expression and represses *nanC* expression. Western blot analysis using anti-type 1 pilus antibodies indicated similar levels of FimA proteins in wild-type LF82 bacteria and in the *fis*-negative mutant in the presence or absence of sialic acid (Fig. 6B). In addition, predominant ON-phase variation was observed in the *fis* mutant independently of sialic acid (Fig. 6C) and could be directly attributable to decreased FimE levels. In order to evaluate whether Fis interacts in the sialic acid-mediated activation of the *fim* operon, *nanC* mRNA levels were measured in the absence or presence of 0.1% of sialic acid in the LF82 *fis*::Km mutant and in wild-type strain LF82 during exponential growth phase. The expression of the *nanC* gene was increased 99.9- and 199.7-fold by sialic acid in the wild-type strain and the LF82 *fis*::Km mutant, respectively, indicating significantly increased expression of *nanC* in the *fis* mutant compared to that of the wild-type strain ($P = 0.01$) (Fig. 6D). In the LF82 *fis*::Km mutant, the absence of repression by Fis and the activation by sialic acid act in a synergic manner on the expression of *nanC*. As the membrane channel protein NanC, which allows sialic acid entry, regulates *fimB* expression through the regulators NanR and NagC (14), we measured *fimB* expression in the presence or absence of 0.1% sialic acid in the LF82 *fis*::Km mutant and in wild-type strain LF82. With regard to the absence of sialic acid, the *fimB*

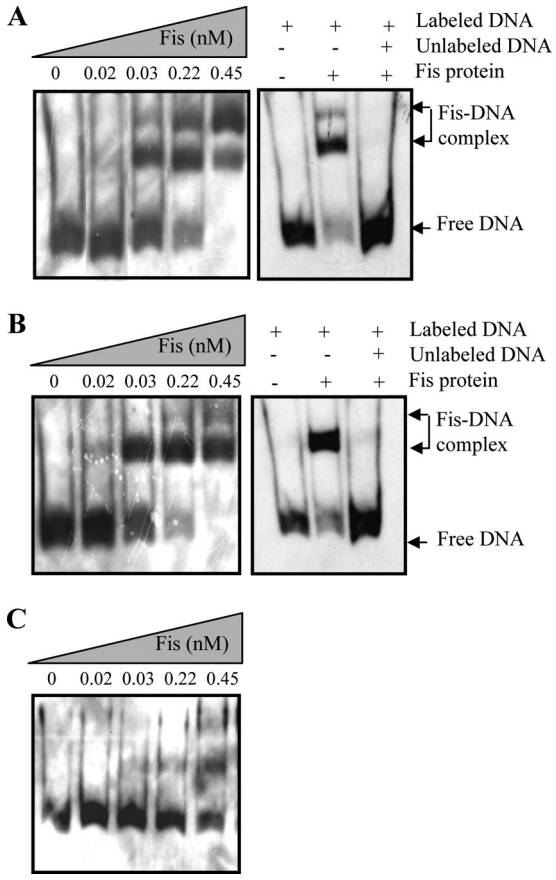

FIG. 4. Validation of putative FBS by EMSA. Binding of purified Fis to the 75-bp labeled DNA fragments and with an excess of unlabeled DNA bearing the *fimE* promoter (A), intergenic *nanC-fimB* region (B), or an unrelated DNA sequence (C). The DNA fragments were incubated with increasing concentrations of Fis or with 0.045 nM Fis in the presence of 1,000-fold excess of nonspecific competitor poly(dI-dC). The protein was incubated with the DNA for 15 min at room temperature, and samples were analyzed on a native 12% polyacrylamide gel.

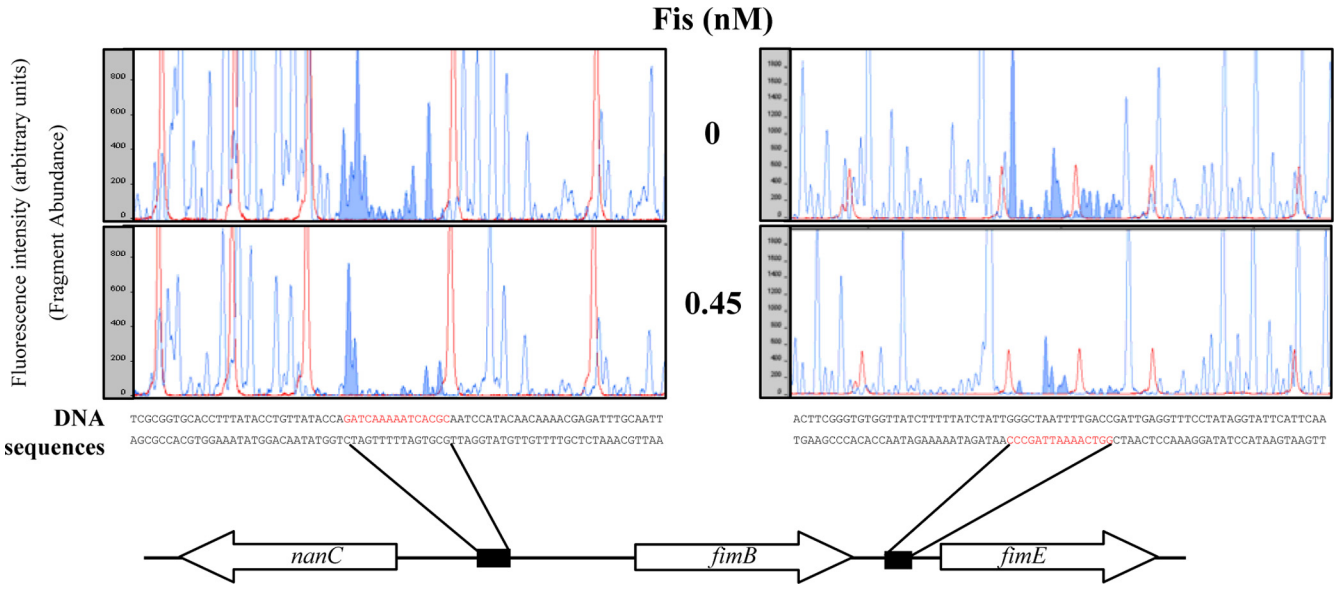

FIG. 5. DNase I footprinting using an automated capillary sequencer in the *fim* regulatory regions. Electropherograms showed a protection pattern of the *fimE* (left panel) and *nanC-fimB* (right panel) promoters after digestion with DNase I following incubation in the presence (bottom panel) or absence (top panel) of Fis (0.45 nM). Corresponding DNA sequences are given below the electropherograms and focus on the region corresponding to the *in silico* identified FBS (blue peaks). Red peaks represent the DNA ladder.

mRNA levels were 1.7- and 17.5-fold lower in the wild-type strain and the LF82 *fis*::Km mutant, respectively, in the presence of sialic acid (Fig. 6D). Thus, Fis via regulation of FimE expression could have a more dominant role than the effect of sialic acid on the regulation of type 1 pilus phase variation.

DISCUSSION

It is well established that the histone-like proteins operate as global regulators to control the expression of numerous genes, including virulence genes (21). The transcriptional adaptation of adherent-invasive *E. coli* (AIEC) was evaluated by comparing DNA supercoiling in bacteria associated with intestinal epithelial cells and in bacteria grown in cell culture medium. Direct contact between bacteria and cells led to an increase in bacteria DNA supercoiling. In addition, in cell-associated AIEC bacteria, a preferential ON-phase orientation of the *fimS* invertible element controlling type 1 pilus expression was observed. The investigation of histone-like protein expression in cell-associated AIEC LF82 bacteria showed that the *fis* mRNA level was much lower during interaction with intestinal epithelial cells than in bacteria grown in cell culture medium (MEM). This suggests that Fis plays a key role during infection that could be linked to DNA supercoiling observed in cell-associated bacteria. Fis has been reported to play a role in DNA relaxation (20, 50), and modification of *fis* expression has been observed during cell infection by *Enterobacteriaceae*. Downregulation of *fis* expression in AIEC during infection of epithelial cells was reminiscent of that observed in *Shigella* (34). In contrast, upregulation of Fis correlates with increased invasion of epithelial Caco-2 cells by *Salmonella enterica* serovar Typhimurium (40).

Type 1 pili are filamentous appendages produced by many species of enteric bacteria. They promote attachment to a variety of eukaryotic cells by a process inhibited by D-mannose. We previously demonstrated that type 1 pili play a key role in the ability of AIEC bacteria to adhere to and to invade intestinal epithelial cells (6, 9) and that in an AIEC genetic background, a *fliA* mutation that abolishes the biogenesis of flagella leads to a loss of type 1 pilus expression through the intracellular concentration of the c-di-GMP secondary messenger (13). The phenotype determination of the LF82 *fis*::Km negative mutant revealed that it is nonmotile. This could result from a negative feedback into the class III gene since we observed a downregulation of *fliC* gene expression. Interestingly, the lack of motility in the LF82 *fis*::Km negative mutant is not associated with a type 1 pilus defect. This is the first example of a flagellum-defective mutant in AIEC strain LF82 that does not show a loss of type 1 pilus expression (5, 43). We demonstrated in the present study that the Fis regulation bypassed the c-di-GMP pathway to control type 1 pilus expression since the overexpression of *yhjH* in the *fis* mutant did not restore the adhesion level.

The transcription of the *fim* operon encoding type 1 pili depends on the invertible 314-bp DNA element (the *fimS* switch), which is located upstream of the *fim* operon and which contains the *fim* promoter. In *E. coli*, the orientation of *fimS* is determined by the influence of two upstream, *trans*-acting gene products, FimB and FimE, that function as site-specific recombinases (31). FimB mediates inversion of the invertible element *fimS* in both directions, whereas FimE rearranges DNA in the OFF position (38). Efficient inversion of *fimS* by FimB and FimE recombinases requires the histone-like proteins Lrp, IHF, and H-NS, which induce sharp bends in DNA and DNA relaxation (8, 15, 29, 47, 49). In the present study, we observed a very high proportion of bacteria in the ON phase in the LF82 *fis*::Km mutant, and impaired type 1 pilus expression in wild-type AIEC overexpressing Fis was observed, indicating that Fis

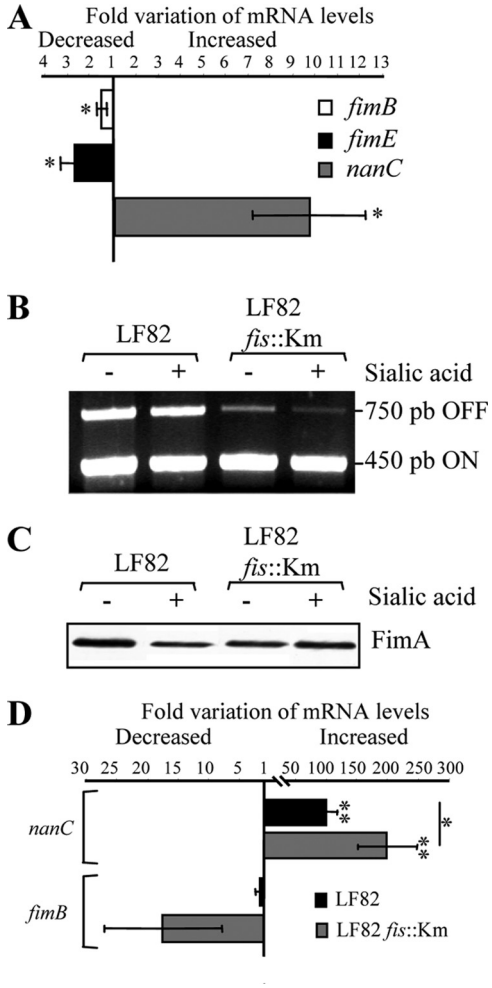

FIG. 6. Regulation of *fimE*, *fimB*, and *nanC* expression by Fis in the absence or presence of sialic acid. (A) Fold variation of *nanC*, *fimB*, and *fimE* mRNA levels in the LF82 *fis*::Km mutant relative to those in the wild-type LF82 strain using real-time RT-PCR. (B) Determination of the orientation of the *fimS* element in absence (+) or in presence (−) of 0.1% sialic acid in wild-type strain LF82 or in the LF82 *fis*::Km mutant (see the legend to Fig. 1B). Determination of the amount of FimA subunit by Western blotting using antibodies raised against type 1 pili. (D) Fold variation of *nanC* and *fimB* mRNA levels in LF82 *fis*::Km mutant and wild-type strain LF82 in the presence of 0.1% sialic acid relative to levels in the absence of sialic acid. As controls, 16S rRNA levels were measured. Only experiments showing the same levels of 16S rRNA for each sample were taken into account. Data represent means of at least three separate experiments; bars indicate SEM. *, $P < 0.05$; **, $P < 0.01$.

may negatively control type 1 pilus expression. The role of Fis was reported as participating in the regulation of fimbriae, such as curli or type IV pili (33, 44). However, to our knowledge, this is the first example showing that Fis is able to regulate type 1 pilus expression in *E. coli*.

Fis acts on DNA supercoiling and gene expression at three different levels. First, Fis can modify the activity of its own promoter in response to DNA supercoiling (46). Second, the Fis protein is a repressor of the transcription of gyrase genes *gyrA* and *gyrB* (28, 46) and is an activator of the topoisomerase *topA*, which modifies DNA topology (52). Third, Fis can directly bind to specific DNA sequences that may be located upstream of numerous genes and influence transcription by protein-protein contact with RNA polymerase (20). By investigating whether Fis could directly influence type 1 pilus expression, we identified a 15-residue motif, 5′-GGTCAAAAA TTGACC-3′, as the best possible Fis-binding site and found one motif upstream of the *fimE* gene, encoding the FimE recombinase. We enlarged the *in silico* survey of the *fim* regulatory region with the Fis consensus, and we found another Fis-binding site in the intergenic *nanC-fimB* region, which is exceptionally large for *E. coli* (1.4 kb). We verified by EMSAs that Fis is able to bind to the DNA sequence upstream of the *fimE* gene and in the intergenic *nanC-fimB* region identified *in silico* and showed by DNase I footprinting analysis that only one FBS was observed in the DNA region of the predicted FBS. We also observed partial protections near the two identified FBSs. They could correspond to tandem FBSs, but owing to lack of homologies with a degenerated FBS consensus, the likely explanations are that Fis can protect flanking FBS DNA regions (48) or create a ladder of complexes bound to nonspecific sequences around specific FBSs (7). Moreover, the latter hypothesis could account for the double shift observed in EMSAs.

In the present study, we report that Fis can regulate NanC and recombinase FimE expression in strain LF82. In addition, we observed that the expression of *nanC* is upregulated and that of *fimE* is downregulated in the Fis-negative mutant. The *nanC* gene encodes the *N*-acetylneuraminic acid (sialic acid) outer membrane channel protein NanC, thereby allowing the entry of this amino sugar into *E. coli*. In *E. coli* K-12 sialic acid downregulates FimB recombinase expression, and it has been suggested that *E. coli* recognizes the amino sugar as a harbinger of potential host defense activation, which leads to a decrease in type 1 pilus expression (22, 49). In contrast to the major effect of sialic acid on the *E. coli* K-12 strain documented in previous studies, we observed no difference in the expression of *fimB* in AIEC strain LF82 grown in the presence of sialic acid. We also observed no differences in the orientation of the *fimS* invertible DNA element in wild-type strain LF82 grown in the absence or presence of 0.1% sialic acid. The absence of regulation of type 1 pilus expression by sialic acid in CD-associated *E. coli* strains could be of advantage to the bacteria, allowing them to maintain type 1 pilus expression and, hence, to better adhere to the intestinal mucosa since it was reported that total sialic acid levels rise during inflammation in CD patients (42). In addition, we report here that Fis binds specifically to a DNA sequence located 58 bp upstream of the *fimE* translation start site and could positively control *fimE* expression since the *fimE* mRNA levels were decreased in the LF82 *fis*::Km negative mutant. The FimE recombinase catalyzing the ON-to-OFF changes of the *fimS* DNA element orientation (31) and downregulation of *fimE* expression in the LF82 Fis-negative mutant could explain the preferential ON phase of type 1 pili (Fig. 7). These results, together with the observation that FimE-mediated recombination of *fimS* was less stringent than that involving FimB (27), led us to speculate that in AIEC LF82 bacteria regulation by Fis of *fimE* is predominant compared to other mechanisms of regulation of type 1 pilus expression, such as the cyclic di-GMP or the sialic acid pathways.

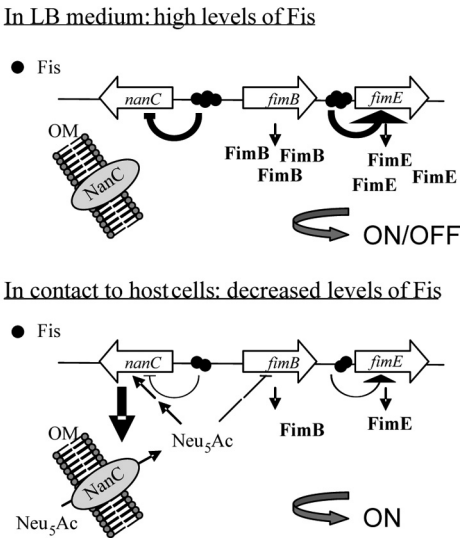

FIG. 7. Model of regulation of type 1 pilus expression in AIEC strain LF82 grown in cell culture medium (MEM) or in contact with intestinal epithelial cells. (A) In MEM, AIEC LF82 bacteria expressed high levels of Fis, leading to a repression of *nanC* and activation of *fimE* expression. As a consequence, there is equilibrium between the expression of the two recombinases FimE and FimB, and the population of AIEC LF82 bacteria in MEM is equally distributed among the ON and the OFF phases. (B) AIEC LF82 bacteria in contact with intestinal epithelial cells exhibited decreased Fis expression, leading to Fis-dependent decreased activation of *fimE*, and nullified inhibition of *nanC*, encoding NanC, the *N*-acetylneuraminic acid (Neu_5Ac) outer membrane channel, which allows entry of this amino sugar into bacteria. As a consequence, in addition to decreased levels of the recombinase FimE, FimB expression is decreased. Consequently, AIEC LF82 bacteria associated with intestinal epithelial cells are highly piliated due to the predominant ON phase of the *fim* promoter. OM, outer membrane.

Understanding the mechanism of regulation of type 1 pilus phase variation provides insights into how AIEC bacteria coordinate the expression of cell surface factors during infection. Under conditions encountered by bacteria in the gastrointestinal tract, it has been speculated that AIEC bacteria are hyperflagellated by activation of the σ^E regulatory pathway (43). This allows AIEC bacteria to move through the mucus layer and to reach the surface of the gut epithelium. In the present study, when the bacteria reached the intestinal epithelial cell surface, we observed a decrease in *fis* expression in AIEC LF82 and consequently a decrease in flagellum expression. As a consequence of Fis downregulation, the population of AIEC LF82 bacteria associated with intestinal epithelial cells was predominantly in the ON phase. Since AIEC bacteria strongly adhere via type 1 pili to the CEACAM6 molecule, which is overexpressed in the ileal mucosa of CD patients (6, 11), such changes in gene expression in cell-associated bacteria may enhance bacterial colonization and, hence, inflammation.

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