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Involvement of Lipoprotein NlpI in the Virulence of Adherent Invasive *Escherichia coli* Strain LF82 Isolated from a Patient with Crohn's Disease

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Escherichia coli **strain LF82 recovered from a chronic lesion of a patient with Crohn's disease (CD) is able to adhere to and invade cultured intestinal epithelial cells and to replicate within macrophages. One mutant selected for its impaired ability to invade epithelial cells had an insertion of a Tn *phoA* transposon within the *nlpI* gene encoding the lipoprotein NlpI. A NlpI-negative isogenic mutant showed a 35-fold decrease in its ability to adhere to and a 45-fold decrease in its ability to invade Intestine-407 cells, but its ability to survive and to replicate within macrophages was similar to that of wild-type strain LF82. In addition, this mutant did not express flagella and synthesized very small amounts of type 1 pili. Downregulation of type 1 pili in the NlpI-negative mutant resulted from a preferential switch toward the OFF position of the invertible DNA element located upstream of the *fim* operon. The FimB and FimE recombinases act in concert to control the switch, and a large decrease in *fimB* and *fimE* mRNA levels was observed. The absence of flagellar structures correlated with a drastic 19-fold decrease in the *fliC* mRNA level, regardless of the $FlhD_2C_2$ transcriptional regulator and of the σ^{28} transcription factor. The key role of NlpI in virulence is independent of type 1 pili and motility, since induced type 1 pilus expression and/or forced contact between bacteria and intestinal epithelial cells did not restore the ability of the NlpI mutant to adhere to and to invade intestinal epithelial cells.**

Crohn's disease (CD) is an inflammatory bowel disease characterized by chronic transmural, segmental, and granulomatous inflammation of the intestine in humans (10). CD has features that might be the result of a microbial process in the gut. Various studies have addressed the hypothesis that pathogenic bacteria contribute to the pathogenesis of inflammatory bowel disease (4, 22, 23, 33, 36). Bacterial adhesion to intestinal epithelial cells represents the first step in the pathogenicity of many organisms involved in infectious bowel diseases since it allows the bacteria to colonize the gut mucosa and resist mechanical removal from the intestine. The ileal mucosa of patients with CD is abnormally colonized with adherent *Escherichia coli* strains, which suggests that these bacteria are involved in the initiation of the inflammatory process (7).

Colonization of host tissues is usually mediated by adhesins on the surface of the bacteria, responsible for binding to specific receptor moieties of the host cell. Enterobacteria elaborate either adhesive filamentous appendages from their surface, termed fimbriae or pili (such as type 1 pili, pap pili, or type IV pili), or nonfimbrial adhesins (for a review, see references 34 and 39). We previously reported that type 1 pili play an important role in the interaction between *E. coli* isolated from patients with CD and intestinal epithelial cells (2). However, other structures expressed on the bacterial surface could play a direct or indirect role in the adhesion process. For example, flagella allow some microorganisms to adhere to epithelial cells via active motility, in particular enteropathogenic *E. coli* (12) and *Salmonella enterica* (35). In addition, outer membrane proteins, such as OmpA, promote *E. coli* K1 adhesion to and invasion of brain microvascular endothelial cells (29). Finally, bacterial lipoproteins are also involved in adhesion to and invasion of epithelial cells (42). For example, the surface-exposed lipoprotein JlpA specific to *Campylobacter jejuni* mediates adherence to HEp-2 epithelial cells and Lsp, which belongs to the LraI lipoprotein family, is involved in *Streptococcus pyogenes* adhesion to and invasion of A549 cells (11, 16).

E. coli strain LF82, isolated from a chronic ileal lesion of a patient with CD, belongs to a new pathogenic group of invasive *E. coli* strains mainly associated with CD and designated AIEC (for "adherent invasive *E. coli*") (3). It is a true invasive pathogen since its uptake by HEp-2 epithelial cells and Intestine-407 intestinal epithelial cells is dependent on actin microfilaments and microtubules, it survives intracellularly, and it replicates in the host cell cytoplasm after lysis of endocytic vacuoles (3). However, strain LF82 has none of the invasive determinants of *Salmonella*, *Shigella*, and invasive *E. coli* strains, which are known to be involved in gastrointestinal infections. In addition, *E. coli* strain LF82 is able to survive and/or replicate intracellularly within J774-A1 macrophage cells, murine peritoneal macrophages, and human monocyte-derived macrophages (13). Type 1 pilus-mediated adherence plays an essential part in the invasive ability of strain LF82 by inducing membrane extensions, which surround the bacteria at the sites of contact between the entering bacteria and the epithelial cells (2). However, type 1 pili have to be expressed in the genetic background of strain LF82 to promote bacterial uptake since their expression in *E. coli* K-12 is not sufficient to confer invasiveness. We

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TABLE 1. Bacterial strains, plasmids, and cosmids used in this study

Strain or plasmid	Relevant characteristic(s)	Source or reference
Strains		
LF82	*E. coli* isolated from an ileal biopsy specimen from a CD patient	7
LF82-Δ*nlpI*	LF82 isogenic mutant with *nlpI* deleted	This study
LF82-Δ*fliC*	LF82 isogenic mutant with *fliC* deleted	1
Plasmids and cosmids		
pUC18	*E. coli* cloning vector, *ori*ColE1, ampicillin resistant	Biolabs
pHSG575	*E. coli* cloning vector, chloramphenicol resistant	Biolabs
pKOBEG	pBAD cloning vector harboring λ phage *red*γβα operon	5
pHC79	*E. coli* cloning cosmid, ampicillin resistant	6
pPBI01	pHSG575 harboring the 11.2-kb SalI fragment with the entire *fim* operon of *E. coli* K-12 strain J96	2
pPBI02	pUC18 containing the 1.3-kb PstI fragment with the complete *nlpI* gene of LF82	This study
pPBI03	pHSG575 containing the 1.3-kb PstI fragment with the complete *nlpI* gene of LF82	This study
Cosmid 14C11	Cosmid pHC79 harboring the entire *nlpI* gene of LF82	This study

recently showed that flagella also play a direct role in the adhesion process via active motility and an indirect role in the interaction between bacteria and epithelial cells by downregulating the expression of type 1 pili (1). Finally, a study of the ability of the LF82-Δ*fliC* isogenic mutant to adhere to and invade intestinal epithelial cells showed that another factor, in addition to flagella and type 1 pili, is involved in adherence and invasion of strain LF82 (1).

To identify new genetic determinants involved in the ability of strain LF82 to adhere and/or invade, we screened 5,329 Tn5 *phoA* mutants for a decreased ability to interact with HEp-2 epithelial cells. Sixteen mutants were identified that showed a decreased invasion and/or adhesion to HEp-2 cells, and analysis of transposon insertion sites revealed mutated genes associated with type 1 pilus biogenesis, genes encoding a putative outer membrane protein and a putative serine-threonine protein kinase, and the *nlpI* gene encoding the NlpI lipoprotein (2). The present study focuses on the NlpI lipoprotein. We showed that NlpI, for which no biological role has been described, is a key component of AIEC strain LF82 virulence.

MATERIALS AND METHODS

Bacterial strains, plasmids, and cell lines. Strain LF82 was isolated from a chronic ileal lesion of a patient with CD and belongs to *E. coli* serotype O83:H1. It is sensitive to most antibiotics but not to amoxicillin. It adhered to and invaded HEp-2, Intestine-407, and Caco-2 cells (3). *E. coli* strain SM10 (λpir) was used for Tn *phoA* mutagenesis as a source of the mini-Tn5 *phoA* donor plasmid pUTKml (9). *E. coli* strains JM109 and C600 were used as host strains for cloning experiments. The bacterial strains, plasmids, and cosmids used in this study are listed in Table 1.

Plasmid vectors pUC18 and pHSG575 and cosmid vector pHC79 (6) were used in cloning procedures. Bacteria were grown routinely in Luria-Bertani (LB) broth or on LB agar plates (Institut Pasteur Production) overnight at 37°C. Antibiotics were added to the media 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 medium (Seromed; Biochrom KG, Berlin, Germany) supplemented with 10% (vol/vol) fetal calf serum (FCS) (Seromed), 1% nonessential amino acids (Life Technologies, Cergy-Pontoise, France), 1% L-glutamine (Life Technologies), 200 U of penicillin, 50 mg of streptomycin, 0.25 mg of amphoterocin B per liter, and 1% minimal essential medium (MEM) vitamin mix X-100 (Life Technologies).

The murine macrophage-like cell line J774-A1 (American Type Culture Collection no. TIB67) was maintained in an atmosphere containing 5% CO_2 at 37°C in RPMI 1640 medium (Seromed) supplemented with 10% (vol/vol) FCS, 1% nonessential amino acids, 1% L-glutamine, and 1% MEM vitamin mix X-100.

Assays of adhesion to and invasion of Intestine-407 cells and bacterial survival and replication within macrophages. The assays of bacterial invasion of Intestine-407 epithelial cells and bacterial survival and replication within macrophages were performed using the gentamicin protection assay as described previously (3, 13). Briefly, monolayers were seeded in 24-well tissue culture plates (Polylabo, Strasbourg, France) with 4×10^5 cells per well for Intestine-407 cells and 2×10^5 cells per well for J774-A1 macrophages and incubated for 20 h. Monolayers were then infected in 1 ml of the cell culture medium without antibiotics and with heat-inactivated FCS at a multiplicity of infection of 10 bacteria per cell for adhesion and invasion assays and 100 bacteria per cell for bacterial uptake, survival, and replication within macrophages.

For assay of adhesion to Intestine-407 cells, after a 3-h incubation period at 37°C, monolayers were washed five times in phosphate-buffered saline (PBS; pH 7.2). The epithelial cells were then lysed with 1% Triton X-100 (Sigma Chemical Co., St. Louis, Mo.) in deionized water. Samples were diluted and plated onto Muller-Hinton agar plates to determine the number of CFU corresponding to the total number of cell-associated bacteria (adherent and intracellular bacteria). To determine the number of intracellular bacteria, fresh cell culture medium containing 100 μg of gentamicin per ml (Sigma) was added for 1 h to kill extracellular bacteria. The monolayers were then lysed with 1% Triton X-100, and the bacteria were quantified as described above. When needed, adhesion and invasion assays were performed after centrifugation for 8 min at 1,000 × g.

For bacterial survival and replication within macrophages, infected monolayers were centrifuged at 1,000 × g for 10 min at 20°C and then incubated for 10 min at 37°C. The monolayers were washed twice in PBS, and the number of intracellular bacteria were determined after a 1-h and a 24-h incubation period at 37°C in fresh cell culture medium containing 20 μg of gentamicin per ml.

Colony and Western immunoblotting. The rabbit antiserum raised against purified type 1 pilus preparations was a generous gift from Karen Krogfelt (18). Bacteria were grown overnight at 37°C in LB broth without agitation. A 1-ml volume of culture was centrifuged, and the pellet of bacteria was suspended in 100 μl of PBS. A 5-μl sample was spotted onto nitrocellulose membranes (Amersham International). The membranes were blocked with 2% (wt/vol) bovine serum albumin (Sigma) in Tris-buffered saline–0.05% Tween (TBST) at room temperature for 2 h. The membranes were incubated with the type 1 pilus antiserum diluted in 1% (wt/vol.) bovine serum albumin in TBST at room temperature for 2 h. Immunoreactants were then detected using a secondary anti-rabbit antibody conjugated with alkaline phosphatase. A substrate composed of 5-bromo-4-chloro-3-indolyl phosphate and 4-nitroblue tetraolium chloride (Sigma) in a detection buffer (100 mM Tris, 100 mM NaCl, 5 mM $MgCl_2$ [pH 9.5]) was used to visualize reaction products.

TABLE 2. Oligonucleotides used and PCR product sizes

Primer	Oligonucleotide sequence (5′-3′)	PCR product size (bp)	Use
MI *nlpI*-1	AGCAACCGGGAACAGGACGTTCATTCAACCGTGGTCTTCGGGAGTGGGAAAAAGCCACGTTGTGTCTCAA	1,057	LF82-Δ*nlpI* isogenic mutant construction
MI *nlpI*-2	CGTTAAGGTGATGGCAATCAAAAAAGATTACGGGCTGATGTGTACGTCAGTTAGAAAAACTCATCGAGCA		
nlpI-1	AACTCGACAATGTTCCATCCCC	244	nlpI probe
nlpI-2	CTTGATCCAACTTACAACTACC		
nlpI-3	AACCGCCAGTTTGAACAGTGCC	735	Allelic replacement of *nIpI* in LF82-Δ*nIpI*
nlpI-4	AGGATGCAGTAATACTTCCTGGC		
nlpI-5	AACGCGTTGAGAAAGTGACCG	1,348	Allelic replacement of *nIpI* in LF82-Δ*nIpI*
nlpI-6	CAGCCATGTAGTACGTGTGCC		
A2-GBL-3	AAAGCCACGTTGTGTCTCAA	957	Kanamycin resistance cassette amplification
B2-GBLnp-5	TTAGAAAAACTCATCGAGCA		
FIME	GCAGGCGGTTTGTTACGGGG	750	OFF-oriented invertible element
INV	GAGGTGATGTGAAATTAATTTAC		
FIMA	GATGCGGTACGAACCTGTCC	450	ON-oriented invertible element
INV	GAGGTGATGTGAAATTAATTTAC		
RNA 16S 1	ATGACCAGCCACACTGGAAC	157	RT-PCR
RNA 16S 2	CTTCCTCCCCGCTGAAAGTA		
flhDC1	CACCAATGTCCAGGCACGGG	211	RT-PCR
flhDC2	GATGCTGCCATTCTCAACCG		
fliA-F	CGCCGTGCTCTTCGCGCC	202	RT-PCR
fliA-R	CGTGCGACGCAACGCGCG		
motB-F	TGAAAGCCCAATTCCCGGCG	509	RT-PCR
motB-R	CTTAAGCGCATCGTTGCCGC		
fliC-5	TGGTGCTGCAACTGCTAACGC	212	RT-PCR
fliC-6	TTATCGGCATATTTTGCGCTAGC		
fimB-F	CCTGACCCATAGTGAAATCG	159	RT-PCR
fimB-R	CTTTGCCTTAAGATCAATATC		
fimE-F	GGCATATCGGCATGGGATGC	170	RT-PCR
fimE-R	CGTTCCTGGGTCCAGCGTTC		

Western immunoblotting using *E. coli* antiserum H1 (a generous gift from the World Health Organization International *Escherichia coli* Centre) was performed by the procedure of Towbin et al. (40) with minor modifications. Bacterial in stationary growth were harvested, suspended in sodium dodecyl sulfate sample buffer, and heated for 5 min. Proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis using 12% polyacrylamide gels (21) and electroblotted onto nitrocellulose membranes. The membranes were then dried and processed as described above.

Motility assays. Motility assays were used to analyze the motility function of AIEC strain LF82 and different mutants. Bacterial strains were grown overnight at 37°C in LB broth. A 5-μl volume of this culture was inoculated onto 0.3% agar plates. These plates were incubated at 37°C for 16 h, and motility was assessed qualitatively by examining the circular swim formed by the growing motile bacterial cells.

Construction of the LF82-Δ*nlpI* isogenic mutant. The LF82-Δ*nlpI* isogenic mutant with the *nlpI* gene deleted was generated with a PCR product, using the method described by Datsenko and Wanner (8) and modified by Chaveroche et al. (5). Briefly, the basic strategy was to replace a chromosomal sequence with a selectable antibiotic resistance gene (kanamycin) generated by PCR. This PCR product was generated by using primers with 50-nucleotide extensions that are homologous to regions adjacent to the *nlpI* and template *E. coli* strain harboring the kanamycin resistance gene (Table 2). In addition, strain LF82 was transformed with plasmid pKOBEG, a plasmid that encoded Red proteins from phage λ, which protect linear DNA in bacteria, expressed under the control of an inductible promoter in the presence of 1 mM L-arabinose. This plasmid was maintained in bacteria at 30°C with 25 μg of chloramphenicol per ml and was killed at 37°C.

Strain LF82/pKOBEG was cultivated at 30°C with 1 mM L-arabinose to induce Red expression. When the optical density at 620 nm reached 0.5, the bacterial culture was incubated for 10 min at 42°C to kill the plasmid. The bacteria were washed three times with 10% glycerol, and PCR products were electroporated. The LF82-Δ*nlpI* isogenic mutant was selected onto LB agar containing 50 μg of kanamycin per ml. Replacement of the *nlpI* gene by the kanamycin resistance cassette in the LF82-Δ*nlpI* isogenic mutant was confirmed by PCR (Fig. 1).

Cosmid cloning and transcomplementation assays. A cosmid library of 2,300 clones of total genomic DNA from strain LF82 was generated in cosmid vector pHC79 (2). Cosmids harboring the *nlpI* gene were detected by hybridization with a 244-base *nlpI* probe generated by PCR (Table 2). Cosmid 14C11 harboring the *nlpI* gene was extracted and digested with PstI (Boehringer, Mannheim, Germany). A 1.3-kb fragment containing the entire *nlpI* gene was cloned into the pUC18 and pHSG575 vectors, and the products were named pPBI02 and pPBI03, respectively. The pPBI03 construct was used to transform the LF82-Δ*nlpI* isogenic mutant.

DNA sequencing and sequence analysis. Plasmid DNA templates were prepared using the Qiagen plasmid minikit. Single-stranded DNA was sequenced by Euro Sequence Gene Service (Cybergene) by the dideoxynucleotide chain termination method. DNA sequences were translated and analyzed using GENE JOCKEY II software. DNA and amino acid sequence comparisons were performed with BLAST programs available from the National Center for Biotechnology Information (http://www.ncbi.nlm.nhi.gov).

DNA manipulations, hybridization, and PCR experiments. Standard DNA procedures and hybridization were performed as described elsewhere (32). PCR conditions and all PCR primer sequences are listed in Table 2.

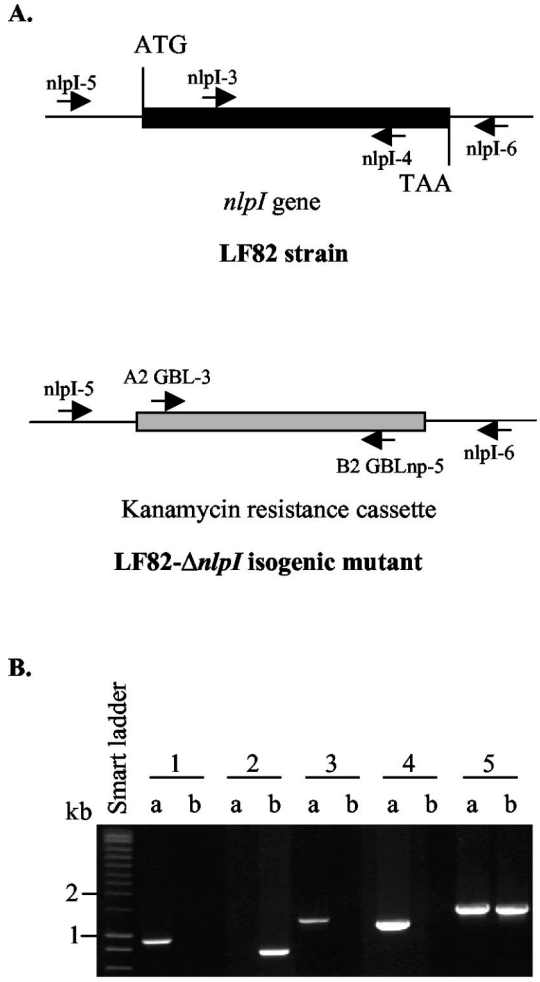

FIG. 1. Allelic replacement of the *nlpI* gene by the kanamycin resistance cassette in the LF82-Δ*nlpI* isogenic mutant. (A) Schematic representation of the locations of different primers on the DNA of strain LF82 and the LF82-Δ*nlpI* isogenic mutant. (B) PCR amplification product analysis. Amplification products were generated by using specific primers for kanamycin resistance cassette sequence (A2-GBL-3 and B2-GBLnp-5) (lanes 1), for the intragenic region of the *nlpI* gene (nlpI-3 and nlpI-4) (lanes 2), for location of the kanamycin resistance cassette (nlpI-5 and B2-GBLnp-5) (lanes 3) and (nlpI-6 and A2-GBL-3) (lanes 4), and to amplify the extragenic region of *nlpI* gene (nlpI-5 and nlpI-6) (lanes 5). DNA to be amplified was released by boiling from LF82-Δ*nlpI* bacteria (lanes a) and LF82 bacteria (lanes b).

RNA manipulations, RT, and real-time RT-PCR. Total RNA was extracted from bacteria and treated with DNase (Roche Diagnostics, Mannheim, Germany) to remove any contaminating genomic DNA. Total RNA was reverse transcribed and amplified using primers specific to *fimB*, *fimE*, *flhDC*, *fliA*, *motB*, and *fliC* mRNAs or 16S rRNA (Table 2). Amplification of a single expected PCR product was confirmed by electrophoresis on 2% agarose gels and ethidium bromide staining. Real-time reverse transcription-PCR (RT-PCR) was performed using a light cycler (Roche Diagnostics). Quantification of mRNA levels or 16S rRNA (as controls) was done using RNA master SYBR Green I (Roche Diagnostics) with 0.5 μg of total RNA.

Statistical analysis. For analysis of the significance of differences in adhesion and invasion levels, and uptake and survival within J774-A1 macrophages, Student's t-test was used for comparison of two groups of data. All experiments were repeated at least three times. A *P*-value less than or equal to 0.05 was considered statistically significant.

RESULTS

Identification of the Tn *phoA* insertional site in mutant 2D2. Nucleotide sequence analysis of the Tn *phoA* insertion region of mutant 2D2 revealed that this transposon was inserted in the putative *yhbM* open reading frame (2), which is annotated as coding for a putative regulator in *E. coli* K-12 strain MG1655 (GenBank accession no. AE000397). This open reading frame was later reported to encode a lipoprotein and has been renamed gene *nlpI*, for "new lipoprotein I" (27). The nucleotide sequence of the LF82 *nlpI* gene demonstrated 99% identity to that of *E. coli* K-12. Alignment of deduced amino acid sequences revealed 100% identity between NlpI of strain LF82 and that of *E. coli* K-12. To study the role of the *nlpI* gene in AIEC strain LF82, an isogenic mutant with the *nlpI* gene deleted was constructed. PCR controls confirmed deletion of the *nlpI* gene (Fig. 1).

Growth of the LF82-Δ*nlpI* isogenic mutant in vitro. A previously published study of the *nlpI* mutant WU62 of *E. coli* K-12 bearing an insertion of a chloramphenicol cassette within the *nlpI* gene showed that the NlpI lipoprotein may be involved in an undefined step in the overall process of cell division (27). In contrast to results reported by Ohara et al. for the *nlpI* insertion mutant WU62 (27), microscopic examination of the LF82-Δ*nlpI* isogenic mutant showed that the bacteria were not elongated (data not shown). Therefore, we checked whether deletion of the *nlpI* gene in strain LF82 could interfere with the bacterial growth rate. Growth of wild-type strain LF82 and the LF82-Δ*nlpI* isogenic mutant took place at 37°C without shaking in LB broth (data not shown) and in the bacterium-cell incubation medium used for adhesion and invasion experiments (Eagle minimal essential medium cell culture medium supplemented with 10% heat-inactivated FCS). The growth curves for wild-type strain LF82 and the LF82-Δ*nlpI* isogenic mutant were similar all time points (from 1 to 6 h) in both media. With an initial inoculum of 3×10^6 CFU/ml and a 6-h incubation in Eagle minimal essential medium, the number of bacteria reached $1.06 \times 10^8 \pm 0.06 \times 10^8$ and $1.21 \times 10^8 \pm 0.10 \times 10^8$ for the wild-type strain LF82 and the LF82-Δ*nlpI* isogenic mutant, respectively. Thus, *nlpI* gene deletion had no effect on the ability of the LF82-Δ*nlpI* isogenic mutant to grow in vitro. Differences in the number of cell-associated bacteria (adherent and invasive bacteria) between wild-type strain LF82 and isogenic mutant LF82-Δ*nlpI* observed in subsequent experiments were not due to growth deficiency of the isogenic mutant.

Phenotype of the LF82-Δ*nlpI* isogenic mutant. Quantitative adhesion assays showed that the LF82-Δ*nlpI* isogenic mutant was consistently reduced in its ability to adhere to Intestine-407 epithelial cells, having a 2.8% ± 0.8% residual adhesion level compared to wild-type strain LF82, taken as 100% (Fig. 2A). In addition, the LF82-Δ*nlpI* isogenic mutant was unable to invade Intestine-407 epithelial cells, with 2.2% ± 0.3% residual invasion level compared to wild-type strain LF82 (Fig. 2B). Transcomplementation experiments were performed with the *nlpI* gene cloned into plasmid vector pHSG575, forming pPBI03. The adhesion level of the LF82-Δ*nlpI* isogenic mutant transcomplemented with the cloned *nlpI* gene (pPBI03) was 111.7% of that of strain LF82, while the adhesion level of the LF82-Δ*nlpI* isogenic mutant transcomplemented with the vec-

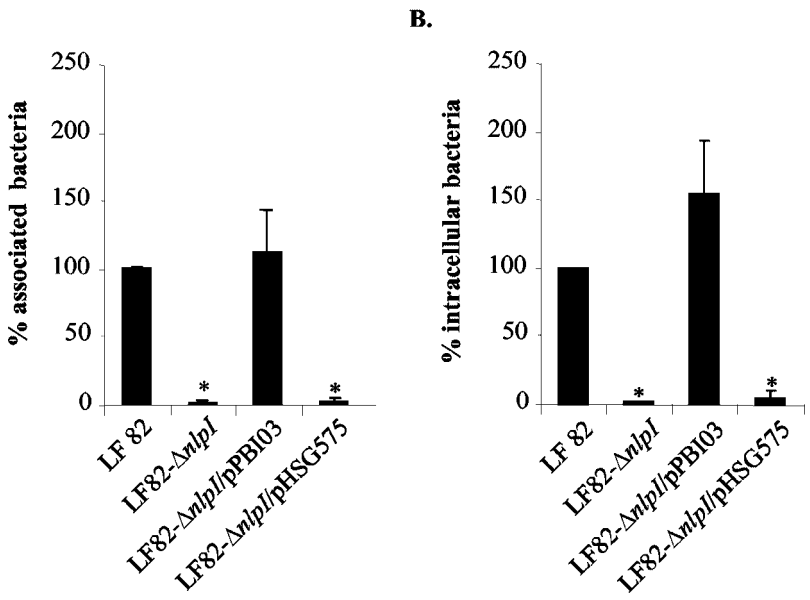

FIG. 2. Transcomplementation of the adhesion (A) and invasion (B) defects of the LF82-Δ*nlpI* isogenic mutant with plasmid pPBI03 harboring the entire LF82 *nlpI* gene. Cell-associated bacteria were quantified after a 3-h infection. Invasion was determined after gentamicin treatment for an additional 1 h. The results are expressed as cell-associated (adherent plus intracellular) or intracellular bacteria relative to those obtained for wild-type strain LF82, taken as 100%. Each value is the mean of at least five separate experiments. Error bars indicate standard errors of the means for five separate experiments. *, $P < 0.001$.

tor alone was 3.8%. The invasion level of the LF82-Δ*nlpI* isogenic mutant transcomplemented with the cloned *nlpI* gene was 153.7% of that of strain LF82, while the invasion level of the LF82-Δ*nlpI* isogenic mutant transcomplemented with the vector alone was 4.4% (Fig. 2). Hence, the *nlpI* gene plays a crucial role in the ability of AIEC strain LF82 to adhere to and invade intestinal epithelial cells. Based on similar residual adhesion and invasion levels of the LF82-Δ*nlpI* isogenic mutant, the role of the *nlpI* gene in strain LF82 invasion could be an indirect consequence of the loss of adhesion.

AIEC strains, in addition to being able to adhere to and invade epithelial cells, are able to survive and replicate intracellularly within J774-A1 macrophages. We therefore investigated the role of *nlpI* gene in such abilities. Bacterial uptake was determined after 1 h of gentamicin treatment, and the number of intracellular bacteria surviving the macrophage killing was determined after 24 h of gentamicin treatment. The level of intracellular bacteria at 24 h postinfection was expressed as a mean percentage of the number of bacteria recovered after 1 h of gentamicin treatment, defined as 100%. As shown in Fig. 3A, the uptake of the LF82-Δ*nlpI* isogenic mutant by J774-A1 macrophages was fourfold less efficient than that of strain LF82. The number of internalized bacteria was $3.35 \times 10^5 \pm 0.79 \times 10^5$ CFU per well for the LF82-Δ*nlpI* isogenic mutant and $14.9 \times 10^5 \pm 1.70 \times 10^5$ CFU per well for wild-type strain LF82. Transcomplementation with the cloned *nlpI* gene restored the uptake of the isogenic mutant to a level not significantly different of that of strain LF82. The LF82-Δ*nlpI* isogenic mutant survived within macrophages at the same levels as wild-type strain LF82, suggesting that the *nlpI* gene was not involved in resistance to macrophage killing (Fig. 3B). Similar rates of replication of intracellular bacteria were observed for wild-type strain LF82 and the LF82-Δ*nlpI* isogenic mutant, indicating that *nlpI* gene deletion had no effect on the ability of the bacteria to grow intracellularly.

Loss of type 1 pilus expression in LF82-Δ*nlpI* isogenic mutant. A decreased ability to adhere to and invade epithelial cells has already been observed and is characteristic of type 1 pilus negative mutants of strain LF82 (2). Type 1 pilus expression of the LF82-Δ*nlpI* isogenic mutant was assessed by colony immunobloting using polyclonal antibodies raised against type 1 pili and by determining the ability of the bacteria to aggregate yeast cells via binding to D-mannose residues. As shown in Fig. 4A, this mutant was unable to synthesize type 1 pili. It was also unable to aggregate yeast cells (Table 3). Type 1 pilus expression is mediated by a process called phase variation, in which the bacteria can switch between piliated and nonpiliated states under the control of a switch invertible element, located upstream of the type 1 pilus-encoding operon (*fim* operon). PCR experiments using two sets of primers specific to the phase-ON and phase-OFF orientations of the invertible element (37) clearly demonstrated that the decrease in type 1 pilus expression in the LF82-Δ*nlpI* isogenic mutant correlated with a switch of the invertible element to the phase-OFF orientation (Fig. 4B). Transcomplementation of the LF82-Δ*nlpI* isogenic mutant with the cloned *nlpI* gene (pPBI03) restored type 1 pili expression, while transformation with the vector alone (pHSG575) did not restore the wild-type phenotype (Fig. 4A and B; Table 3). Since the orientation of the invertible element is controlled by site-specific DNA inversion catalyzed by the two *fim* recombinases FimB and FimE, we performed RT-PCR experiments to quantify *fimB* and *fimE* mRNA levels. The levels of both *fimB* and *fimE* mRNAs in the LF82-Δ*nlpI* isogenic mutant were decreased (12- and 9-fold, respectively) compared to those in wild-type strain LF82 (Table 4). However, only the decrease in the *fimE* mRNA level was statisti-

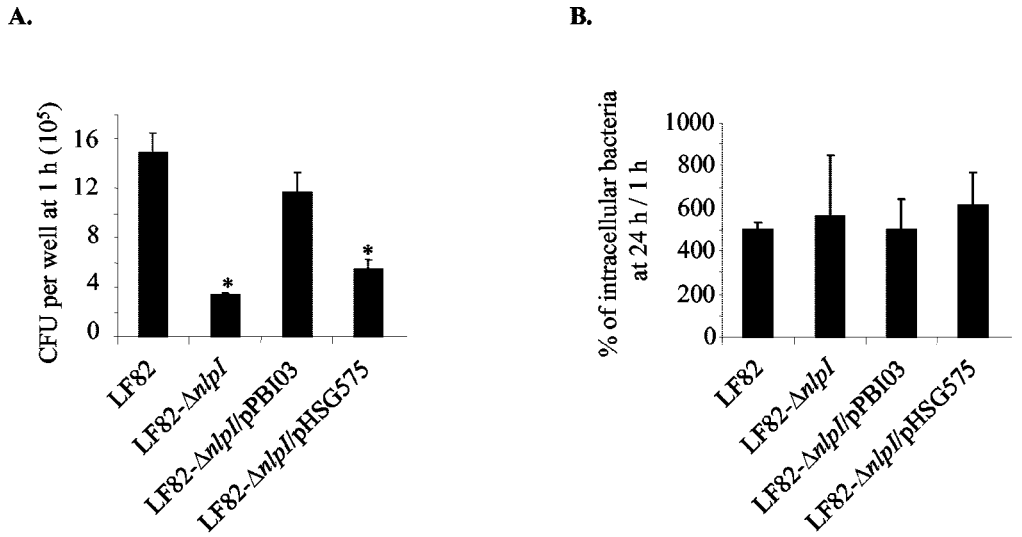

FIG. 3. Phenotype of the LF82-Δ*nlpI* isogenic mutant within J774-A1 macrophages cells. (A) Uptake of LF82, the LF82-Δ*nlpI* isogenic mutant, the LF82-Δ*nlpI* isogenic mutant transformed with the cloned *nlpI* gene (pPBI03), and the LF82-Δ*nlpI* isogenic mutant harboring the vector alone (pHSG575). Results were expressed in CFU per well after 1 h of gentamicin treatment. (B) Bacterial survival and replication after 24 h of gentamicin treatment. Results are expressed as the number of intracellular bacteria at 24 h relative to that obtained at 1 h after gentamicin exposure, taken as 100%. Data are means ± standard errors of the means for five separate experiments. *, $P < 0.05$.

cally significant ($P < 0.05$). The *fimB* and *fimE* mRNA levels in the LF82-Δ*nlpI* isogenic mutant transcomplemented with the cloned *nlpI* gene (pPBI03) were similar to those observed in the wild-type strain LF82.

Biogenesis of type 1 pili in the LF82-Δ*nlpI* isogenic mutant. Since some lipoproteins play a role in the biogenesis of fimbriae (30, 31, 41), we investigated whether the LF82-Δ*nlpI* isogenic mutant could express functional type 1 pili on the bacterial surface. The LF82-Δ*nlpI* isogenic mutant was transformed with plasmid pPBI01 harboring the entire *fim* operon to force the bacteria to express these pili. As a consequence of induced type 1 pilus expression, the LF82-Δ*nlpI*/pPBI01 bacteria aggregated yeast cells (Table 3) and showed a similar amount of type 1 pili to that of strain LF82 by colony immunoblotting assays with antibodies raised against type 1 pili (data not shown). Thus, the absence of biogenesis of type 1 pili in the LF82-Δ*nlpI* isogenic mutant was not directly related to the absence of NlpI in the outer membrane.

Loss of flagellum expression in LF82-ΔnlpI isogenic mutant. We recently observed that the lack of expression of flagella in the LF82-Δ*fliC* isogenic mutant induced the switch of the type 1 pilus invertible element to the phase-OFF orientation (1); we therefore decided to analyze whether the decreased type 1 pilus expression was due to the absence of flagella in a NlpI-negative mutant. The LF82-Δ*nlpI* isogenic mutant was nonmotile onto 0.3% agar plates (Fig. 4C). In addition, electron microscope examination of negatively stained bacteria indicated that no bacteria synthesized flagella in the LF82-Δ*nlpI* isogenic mutant whereas 78% of the bacteria were flagellated in the wild-type strain LF82. The lack of flagella was confirmed by Western immunobloting using polyclonal antibodies raised against *E. coli* flagellin H1 (Fig. 4D). Transcomplementation of the LF82-Δ*nlpI* isogenic mutant with the cloned *nlpI* gene (pPBI03) restored motility and syn-

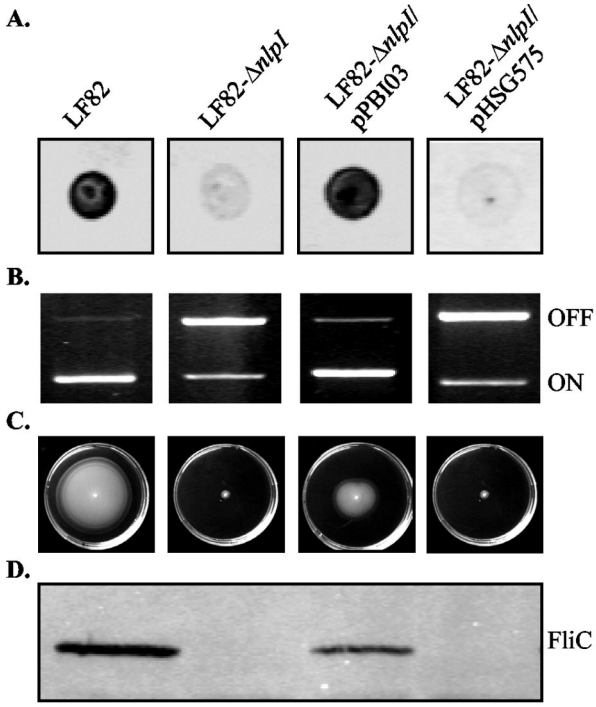

FIG. 4. Type 1 pilus and flagellum expression and/or regulation. Experiments were performed with strain LF82, the LF82-Δ*nlpI* isogenic mutant, the LF82-Δ*nlpI* isogenic mutant transcomplemented with the cloned *nlpI* gene (pPBI03), and the LF82-Δ*nlpI* isogenic mutant transformed with the vector alone (pHSG575). (A) Colony immunoblotting using polyclonal antibodies raised against purified type I pili. (B) Determination of the invertible element orientation of the *fim* operon. The orientation was determined by PCR analysis, as described in Materials and Methods. A 450-bp product revealed the ON-orientation and a 750-bp product revealed the OFF-orientation of the invertible element. (C) Motility on a 0.3% agar plate after 16 h at 37°C. Motility was visualized as a halo of radial diffusion of bacteria around the primary inoculum. (D) Western immunoblot analysis of FliC synthesis, using polyclonal antibodies raised against purified flagellin H1.

TABLE 3. Expression of pili in wild-type strain LF82 compared to that in *nlpI* mutants

Strain	Yeast aggregation[a]	% Positive for pili[b]
LF82	1/8	96
LF82-Δ*nlpI*	1	9
LF82-Δ*nlpI*/pPBI03	1/8	88
LF82-Δ*nlpI*/pPBI01	1/8	ND[c]

[a] Aggregation was monitored visually, and the titer was recorded as the last dilution giving a positive aggregation reaction.
[b] The percentage of bacteria expressing pili was monitored by electron microscopy examination of negatively stained bacteria.
[c] ND, not done.

thesis of the FliC flagellin, while transformation with the vector alone (pHSG575) did not (Fig. 4C and D).

Molecular analysis of flagellar downregulation. Since three levels of transcriptional regulation of flagellar biogenesis exist, we measured the mRNA levels of the *flhDC* operon encoding the class I master transcriptional regulator $FlhD_2C_2$, the *fliA* gene encoding the class II σ^{28} sigma factor, and the class III *fliC* and *motB* genes encoding the FliC flagellin and the MotB protein (necessary for motor rotation), respectively. The levels of the *flhDC* and *fliA* mRNAs in the LF82-Δ*nlpI* isogenic mutant were similar to those measured in wild-type strain LF82 (Table 4). In contrast, the mRNA level of *fliC* was drastically decreased (19-fold) in the LF82-Δ*nlpI* isogenic mutant. Surprisingly, the mRNA level of *motB*, which, like *fliC*, belongs to the class III genes of the flagellar regulon and whose transcription is also controlled by the σ^{28} sigma factor, was not modified. These results showed that the absence of the NlpI lipoprotein induces a decrease in *fliC* transcription or *fliC* mRNA stability.

Involvement of NlpI in adhesion and invasion of strain LF82. We analyzed the involvement of NlpI in adhesion to and invasion of Intestine-407 epithelial cells with the LF82-Δ*nlpI* isogenic mutant transformed with the cloned *fim* operon (pPBI01) in order to force the bacteria to express type 1 pili. The adhesion and invasion levels of the transformed mutant were compared to those of strain LF82 also transformed with the multicopy plasmid pPBI01 harboring the *fim* operon, since we previously reported that the adhesion and invasion levels of strain LF82/pPBI01 were 1.6- and 6.9-fold increased, respectively, compared to the levels of wild-type strain LF82 (1). The adhesion and invasion levels of the LF82-Δ*nlpI* isogenic mutant transformed with pPBI01 were 8.3 and 27.1%, respectively, of those of strain LF82/pPBI01 (Fig. 5). Thus, the large decreases in adhesion and invasion levels observed in the LF82-Δ*nlpI* isogenic mutant were not related only to the absence of type 1 pilus expression.

Since the decreases in adhesion and invasion of this NlpI-negative mutant may be related to bacterial motility, we added a centrifugation step to establish a close contact between bacteria and epithelial cells. As controls, we performed experiments with the LF82-Δ*fliC* isogenic mutant with induced type 1 pilus expression. As already reported (1), centrifugation fully restored the adhesion level of LF82-Δ*fliC*/pPBI01. In contrast, after centrifugation, the adhesion level of LF82-Δ*nlpI*/pPBI01 was not significantly increased in comparison with that in assays performed without centrifugation (Fig. 5A). In addition, there was no significant increase in the invasion level of LF82-Δ*nlpI*/pPBI01 (Fig. 5B). Thus, the lack of flagella and/or motility, and consequently the lack of type 1 pili, cannot explain the decreased adhesion and invasion abilities of LF82-Δ*nlpI* isogenic mutant.

DISCUSSION

A Tn *phoA* insertion within the gene encoding the NlpI lipoprotein induced a loss of the ability of mutant 2D2 to invade intestinal epithelial cells (2). For this study, an isogenic mutant with the *nlpI* gene deleted was constructed. The LF82-Δ*nlpI* isogenic mutant showed a 35-fold decrease in its ability to adhere to and a 45-fold decrease in its ability to invade Intestine-407 epithelial cells. Phagocytosis by J774-A1 macrophages of the LF82-Δ*nlpI* isogenic mutant showed a fourfold decrease, but the mutant was still able to survive and replicate intracellularly at the same level as that of wild-type strain LF82. In addition, the LF82-Δ*nlpI* isogenic mutant was nonmotile and did not express flagella. It synthesized very small amounts of type 1 pili, as confirmed by immunoblotting and electron microscope examination of negatively stained bacteria. The impaired functions were specifically linked to the lack of NlpI since transcomplementation of the LF82-Δ*nlpI* isogenic mutant with the cloned *nlpI* gene restored the wild-type phenotype.

Surface-exposed lipoproteins can play a role as adhesins. Such a role has been reported for the surface-exposed lipoprotein JlpA expressed by *Campylobacter jejuni* (16). NlpI is supposed to be targeted to the outer membrane in the *E. coli* cell envelope (M. Ohara, personal communication). This was confirmed by the protein sequence analysis indicating that the second amino acid residue of the NlpI protein is not an aspartate, a characteristic of inner membrane-anchored proteins (25, 43). In contrast to a *jlpA* isogenic mutant of *C. jejuni*, which lost the ability to adhere to HEp-2 cells but was still

TABLE 4. Quantification of *fimB*, *fimE*, *flhDC*, *fliA*, *motB*, and *fliC* expression by RT-PCR in wild-type strain LF82 and mutant strains

Mutant	Fold decrease in mRNA levels relative to those of wild-type strain LF82[a]					
	fimB	*fimE*	*flhDC*	*fliA*	*motB*	*fliC*
LF82-Δ*nlpI*	12.54 ± 9.16	9.45 ± 3.98[b]	0.95 ± 0.05	0.99 ± 0.05	0.98 ± 0.18	19.13 ± 3.60[b]
LF82-Δ*nlpI*/pPBI03	1.55 ± 0.77	3.63 ± 0.92	2.22 ± 0.89	1.07 ± 0.02	0.89 ± 0.13	3.63 ± 0.92

[a] Fold decrease in mRNA levels relative to that of wild-type strain LF82 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 in to account. Data are mean ± SEM of at least three separate experiments.
[b] $P < 0.05$.

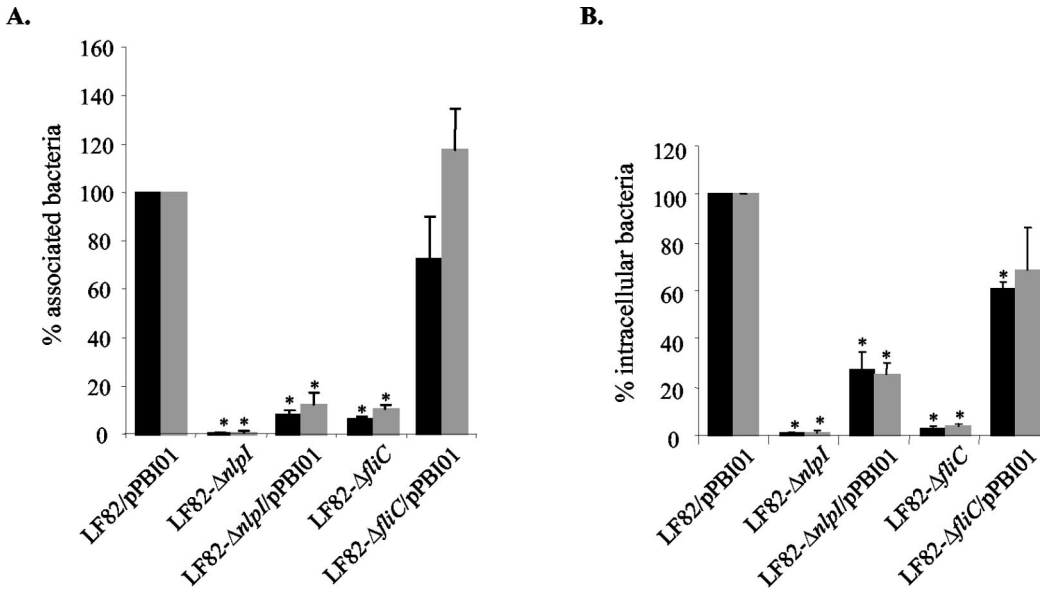

FIG. 5. Adhesion (A) and invasion (B) abilities of the isogenic mutant LF82-Δ*nlpI* with induced type 1 pilus expression and forced contact between bacteria and Intestine-407 epithelial cells. Induced type 1 pilus expression was determined by transformation with pPBI01 harboring the entire *fim* operon. Experiments were performed without (black) or with (grey) centifugation. See the legend to Fig. 2. Data are means ± standard errors of the means for five separate experiments. *, $P < 0.05$.

invasive, the LF82-Δ*nlpI* isogenic mutant was affected in various virulence phenotypes (adhesion, invasion, uptake by macrophages, and flagellar and type 1 pilus biogenesis). However, the effect of the absence of NlpI on the decreased invasion of the LF82-Δ*nlpI* isogenic mutant could be an indirect consequence of the loss of adhesion, since similar residual adhesion and invasion levels were observed. NlpI might function directly to promote strain LF82 adhesion to intestinal epithelial cells, but transformation of strain LF82 with a multicopy recombinant plasmid harboring the cloned *nlpI* gene did not lead to any significant increased adhesion levels (data not shown).

Some lipoproteins are described as playing a role in the biogenesis of fimbriae. For instance, the outer membrane lipoprotein BfpB is required for the biogenesis of type IV bundle-forming pili in enteropathogenic *E. coli* (30), lipoprotein HifD takes part in fimbria biogenesis in *Haemophilus influenzae* type b (41), and lipoprotein VirB7 is associated with the *Agrobacterium tumefaciens* T-pilus assembly (31). The very low level of type 1 pili observed in the LF82-Δ*nlpI* isogenic mutant could therefore be a direct consequence of the absence of lipoprotein NlpI in the outer membrane. We investigated whether the LF82-Δ*nlpI* isogenic mutant could express functional type 1 pili on the bacterial surface. The transformation of the LF82-Δ*nlpI* isogenic mutant with pPBI01 harboring the entire *fim* operon fully restored the expression of type 1 pili on the surface of bacteria, as shown by yeast cell aggregation assays. Thus, the NlpI lipoprotein is not essential for type 1 pilus biogenesis on the bacterial surface.

The decreased expression of type 1 pili in the LF82-Δ*nlpI* isogenic mutant correlated with a switch of the invertible element located upstream of the type 1 pilus-encoding *fim* operon to the preferential phase-OFF orientation. A similar result was recently shown for LF82 mutants deficient for flagellar biogenesis or motility, for which very small amounts of type 1 pili are expressed on the bacterial surface (1). Since a deletion of the *nlpI* gene in AIEC strain LF82 yielded bacteria unable to produce both type 1 pili and flagella, a genetic link probably exists between the expression of type 1 pili and that of flagella.

The orientation of the invertible element is determinated by the influence of two upstream, *trans*-acting gene products, FimB and FimE (17). FimB recombinase mediates both ON-to-OFF and OFF-to-ON inversion, whereas FimE recombinase mediates mainly ON-to-OFF inversion (26). A possible explanation for the specific ON-to-OFF phase transition in the LF82-Δ*nlpI* isogenic mutant could have been an increase of the FimE activity. However, the *fimE* mRNA levels were decreased 12-fold in the LF82-Δ*nlpI* isogenic mutant compared to the wild-type strain. An undetectable FimE recombinase activity and a marked decrease in levels of *fimE* mRNA when the *fim* switch is in the OFF orientation have already been reported (19, 38). Since it has been shown that switch OFF-specific *fimE* mRNAs are more susceptible to exoribonuclease digestion (38), the decrease in the levels of *fimE* and *fimB* mRNA observed in the LF82-Δ*nlpI* isogenic mutant compared to the wild-type strain could result from mRNA stability.

The absence of flagellar structures in the LF82-Δ*nlpI* isogenic mutant correlated with a lack of flagellin synthesis. The flagellar operons are organized as a hierarchy within the regulon, with the expression of operons at a given level affecting the expression of operons at lower levels (20, 24). The *flhDC* operon lies on the top of the hierarchy as the sole class I operon, with both of its gene products being absolutely required for the expression of all other genes of the flagellar regulon (15), i.e., those belonging to the class II and III operons. The σ^{28} sigma factor (a class II product) is required for the expression of the *fliC* gene and the *motABcheAW* operon, which belongs to the class III operons. We previously reported that the *flhDC* transcript level was downregulated in a *fliC*

LF82 isogenic mutant (1). We thus investigated whether the lack of flagella in the LF82-Δ*nlpI* isogenic mutant correlated with a decreased level of *flhDC* mRNAs. This level was similar in the nonflagellated LF82-Δ*nlpI* isogenic mutant and the wild-type strain, LF82. Moreover, the level of mRNAs encoding the σ^{28} sigma factor was identical to that for the wild-type strain, confirming that the absence of flagella was not dependent on transcriptional regulation *via* the $FlhD_2C_2$ complex. Since the transcription of both *fliC* gene and *motABcheAW* operon is known to be under the same control, similar levels of *fliC* and *motB* mRNA were expected. Surprisingly, the level of *fliC* mRNAs was drastically decreased (19-fold) in the LF82-Δ*nlpI* isogenic mutant in comparison with the wild-type strain LF82, while the *motB* mRNA level was not modified. That the levels of specific class III transcripts differ in the LF82-Δ*nlpI* isogenic mutant is intriguing, and this difference has not been reported previously. Since we have shown that $FlhD_2C_2$ and σ^{28} sigma factor are expressed and active in the absence of NlpI, the decrease in the *fliC* mRNA level is not dependent on the known flagellar transcriptional hierarchy. It can be speculated that the link between the lipoprotein NlpI and the *fliC* transcript level depends either on an unknown regulator involved in a transcriptional control or on mRNA stability.

The biological role of outer membrane lipoproteins in bacterial virulence has been previously reported (14, 28). Lipoproteins can be involved in two-component signal transduction pathways. As an example, the NlpE lipoprotein in *E. coli* K-12 is involved in the Cpx two-component signal transduction pathway, which plays a key role in the regulation of induced adhesion to hydrophobic surfaces (28). The loss in the NlpI negative mutant of a broad spectrum of virulence factors (lack of flagella and type 1 pili, reduced bacterial uptake by J774-A1 macrophages, and drastic decreases in the abilities to adhere to and to invade Intestine-407 epithelial cells) could indicate that a regulatory system is also involved in NlpI-mediated full virulence in strain LF82. Two-component signal transduction pathways in which NlpI could be involved are under investigation.

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REFERENCES

1. **Barnich, N., J. Boudeau, L. Claret, and A. Darfeuille-Michaud.** 2003. Regulatory and functional co-operation of flagella and type 1 pili in adhesive and invasive abilities of AIEC strain LF82 isolated from a patient with Crohn's disease. Mol. Microbiol. **48:**781–794.
2. **Boudeau, J., N. Barnich, and A. Darfeuille-Michaud.** 2001. Type 1 pili-mediated adherence of *Escherichia coli* strain LF82 isolated from Crohn's disease is involved in bacterial invasion of intestinal epithelial cells. Mol. Microbiol. **39:**1272–1284.
3. **Boudeau, J., A. L. Glasser, E. Masseret, B. Joly, and A. Darfeuille-Michaud.** 1999. Invasive ability of an *Escherichia coli* strain isolated from the ileal mucosa of a patient with Crohn's disease. Infect. Immun. **67:**4499–4509.
4. **Burke, D. A., and A. T. Axon.** 1988. Adhesive *Escherichia coli* in inflammatory bowel disease and infective diarrhoea. Br. Med. J. **297:**102–104.
5. **Chaveroche, M. K., J. M. Ghigo, and C. d'Enfert.** 2000. A rapid method for efficient gene replacement in the filamentous fungus *Aspergillus nidulans*. Nucleic Acids Res. **28:**E97.
6. **Collins, J.** 1979. *Escherichia coli* plasmids packageable in vitro in lambda bacteriophage particles. Methods Enzymol. **68:**309–326.
7. **Darfeuille-Michaud, A., C. Neut, N. Barnich, E. Lederman, P. Di Martino, P. Desreumaux, L. Gambiez, B. Joly, A. Cortot, and J. F. Colombel.** 1998. Presence of adherent *Escherichia coli* strains in ileal mucosa of patients with Crohn's disease. Gastroenterology **115:**1405–1413.
8. **Datsenko, K. A., and B. L. Wanner.** 2000. One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. Proc. Natl. Acad. Sci. USA **97:**6640–6645.
9. **De Lorenzo, V., M. Herrero, U. Jakubzik, and K. N. Timmis.** 1990. Mini-Tn5 transposon derivatives for insertion mutagenesis, promoter probing, and chromosomal insertion of cloned DNA in gram-negative eubacteria. J. Bacteriol. **172:**6568–6572.
10. **Duchmann, R., and M. Zeitz.** 1999. Crohn's disease, p. 1055–1080. *In* P. L. Ogra et al. (ed.), Mucosal immunology, 2nd ed. Academic Press, San Diego, Calif.
11. **Elsner, A., B. Kreikemeyer, A. Braun-Kiewnick, B. Spellerberg, B. A. Buttaro, and A. Podbielski.** 2002. Involvement of Lsp, a member of the LraI-lipoprotein family in *Streptococcus pyogenes*, in eukaryotic cell adhesion and internalization. Infect. Immun. **70:**4859–4869.
12. **Giron, J. A., A. G. Torres, E. Freer, and J. B. Kaper.** 2002. The flagella of enteropathogenic *Escherichia coli* mediate adherence to epithelial cells. Mol. Microbiol. **44:**361–379.
13. **Glasser, A. L., J. Boudeau, N. Barnich, M. H. Perruchot, J. F. Colombel, and A. Darfeuille-Michaud.** 2001. Adherent invasive *Escherichia coli* strains from patients with Crohn's disease survive and replicate within macrophages without inducing host cell death. Infect. Immun. **69:**5529–5537.
14. **Hagiwara, D., M. Sugiura, T. Oshima, H. Mori, H. Aiba, T. Yamashino, and T. Mizuno.** 2003. Genome-wide analyses revealing a signaling network of the RcsC-YojN-RcsB phosphorelay system in *Escherichia coli*. J. Bacteriol. **185:**5735–5746.
15. **Helmann, J. D., and M. J. Chamberlin.** 1987. DNA sequence analysis suggests that expression of flagellar and chemotaxis genes in *Escherichia coli* and *Salmonella typhimurium* is controlled by an alternative sigma factor. Proc. Natl. Acad. Sci. USA **84:**6422–6424.
16. **Jin, S., A. Joe, J. Lynett, E. K. Hani, P. Sherman, and V. L. Chan.** 2001. JlpA, a novel surface-exposed lipoprotein specific to *Campylobacter jejuni*, mediates adherence to host epithelial cells. Mol. Microbiol. **39:**1225–1236.
17. **Klemm, P.** 1986. Two regulatory fim genes, *fimB* and *fimE*, control the phase variation of type 1 fimbriae in *Escherichia coli*. EMBO J. **5:**1389–1393.
18. **Krogfelt, K. A., and P. Klemm.** 1988. Investigation of minor components of *Escherichia coli* type 1 fimbriae: protein chemical and immunological aspects. Microb. Pathog. **4:**231–238.
19. **Kulasekara, H. D., and I. C. Blomfield.** 1999. The molecular basis for the specificity of *fimE* in the phase variation of type 1 fimbriae of *Escherichia coli* K-12. Mol. Microbiol. **31:**1171–1181.
20. **Kutsukake, K., and T. Iino.** 1994. Role of the FliA-FlgM regulatory system on the transcriptional control of the flagellar regulon and flagellar formation in *Salmonella typhimurium*. J. Bacteriol. **176:**3598–3605.
21. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227:**680–685.
22. **Lamps, L. W., K. T. Madhusudhan, J. M. Havens, J. K. Greenson, M. P. Bronner, M. C. Chiles, P. J. Dean, and M. A. Scott.** 2003. Pathogenic yersinia DNA is detected in bowel and mesenteric lymph nodes from patients with Crohn's disease. Am. J. Surg. Pathol. **27:**220–227.
23. **Liu, Y., H. J. van Kruiningen, A. B. West, R. W. Cartun, A. Cortot, and J. F. Colombel.** 1995. Immunocytochemical evidence of *Listeria*, *Escherichia coli*, and *Streptococcus* antigens in Crohn's disease. Gastroenterology **108:**1396–1404.
24. **Macnab, R. M.** 1992. Genetics and biogenesis of bacterial flagella. Annu. Rev. Genet. **26:**131–158.
25. **Masuda, K., S. Matsuyama, and H. Tokuda.** 2002. Elucidation of the function of lipoprotein-sorting signals that determine membrane localization. Proc. Natl. Acad. Sci. USA **99:**7390–7395.
26. **McClain, M. S., I. C. Blomfield, and B. I. Eisenstein.** 1991. Roles of *fimB* and *fimE* in site-specific DNA inversion associated with phase variation of type 1 fimbriae in *Escherichia coli*. J. Bacteriol. **173:**5308–5314.
27. **Ohara, M., H. C. Wu, K. Sankaran, and P. D. Rick.** 1999. Identification and characterization of a new lipoprotein, NlpI, in *Escherichia coli* K-12. J. Bacteriol. **181:**4318–4325.
28. **Otto, K., and T. J. Silhavy.** 2002. Surface sensing and adhesion of *Escherichia coli* controlled by the Cpx-signaling pathway. Proc. Natl. Acad. Sci. USA **99:**2287–2292.
29. **Prasadarao, N. V., C. A. Wass, M. F. Stins, H. Shimada, and K. S. Kim.** 1999. Outer membrane protein A-promoted actin condensation of brain microvascular endothelial cells is required for *Escherichia coli* invasion. Infect. Immun. **67:**5775–5783.
30. **Ramer, S. W., D. Bieber, and G. K. Schoolnik.** 1996. BfpB, an outer membrane lipoprotein required for the biogenesis of bundle-forming pili in enteropathogenic *Escherichia coli*. J. Bacteriol. **178:**6555–6563.

31. **Sagulenko, V., E. Sagulenko, S. Jakubowski, E. Spudich, and P. J. Christie.** 2001. VirB7 lipoprotein is exocellular and associates with the *Agrobacterium tumefaciens* T pilus. J. Bacteriol. **183:**3642–3651.
32. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
33. **Sartor, R. B., H. C. Rath, S. N. Lichtman, and E. A. van Tol.** 1996. Animal models of intestinal and joint inflammation. Baillieres Clin. Rheumatol. **10:**55–76.
34. **Sauer, F. G., M. A. Mulvey, J. D. Schilling, J. J. Martinez, and S. J. Hultgren.** 2000. Bacterial pili: molecular mechanisms of pathogenesis. Curr. Opin. Microbiol. **3:**65–72.
35. **Schmitt, C. K., J. S. Ikeda, S. C. Darnell, P. R. Watson, J. Bispham, T. S. Wallis, D. L. Weinstein, E. S. Metcalf, and A. D. O'Brien.** 2001. Absence of all components of the flagellar export and synthesis machinery differentially alters virulence of *Salmonella enterica* serovar Typhimurium in models of typhoid fever, survival in macrophages, tissue culture invasiveness, and calf enterocolitis. Infect. Immun. **69:**5619–5625.
36. **Schultsz, C., M. Moussa, R. van Ketel, G. N. Tytgat, and J. Dankert.** 1997. Frequency of pathogenic and enteroadherent *Escherichia coli* in patients with inflammatory bowel disease and controls. J. Clin. Pathol. **50:**573–579.
37. **Schwan, W. R., H. S. Seifert, and J. L. Duncan.** 1992. Growth conditions mediate differential transcription of *fim* genes involved in phase variation of type 1 pili. J. Bacteriol. **174:**2367–2375.
38. **Sohanpal, B. K., H. D. Kulasekara, A. Bonnen, and I. C. Blomfield.** 2001. Orientational control of *fimE* expression in *Escherichia coli*. Mol. Microbiol. **42:**483–494.
39. **Soto, G. E., and S. J. Hultgren.** 1999. Bacterial adhesins: common themes and variations in architecture and assembly. J. Bacteriol. **181:**1059–1071.
40. **Towbin, H., T. Staehelin, and J. Gordon.** 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA **76:**4350–4354.
41. **van Ham, S. M., L. van Alphen, F. R. Mooi, and J. P. van Putten.** 1994. The fimbrial gene cluster of *Haemophilus influenzae* type b. Mol. Microbiol. **13:**673–684.
42. **Wu, H. C.** 1996. Biosynthesis of lipoproteins, p. 1005–1014. *In* F. C. Neidhardt, R. Curtiss III, J. L. Ingraham, E. C. C. Lin, K. B. Low, B. Magasanik, W. S. Reznikoff, M. Riley, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella*: cellular and molecular biology, 2nd ed. ASM Press, Washington, D.C.
43. **Yamaguchi, K., F. Yu, and M. Inouye.** 1988. A single amino acid determinant of the membrane localization of lipoproteins in *E. coli*. Cell **53:**423–432.

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