

The Flagella of an Atypical Enteropathogenic *Escherichia coli* Strain Are Required for Efficient Interaction with and Stimulation of Interleukin-8 Production by Enterocytes In Vitro[∇]

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The ability of some typical enteropathogenic *Escherichia coli* (EPEC) strains to adhere to, invade, and increase interleukin-8 (IL-8) production in intestinal epithelial cells in vitro has been demonstrated. However, few studies regarding these aspects have been performed with atypical EPEC (aEPEC) strains, which are emerging enteropathogens in Brazil. In this study, we evaluated a selected aEPEC strain (1711-4) of serotype O51:H40, the most prevalent aEPEC serotype in Brazil, in regard to its ability to adhere to and invade Caco-2 and T84 cells and to elicit IL-8 production in Caco-2 cells. The role of flagella in aEPEC 1711-4 adhesion, invasion, and IL-8 production was investigated by performing the same experiments with an isogenic aEPEC mutant unable to produce flagellin (FliC), the flagellum protein subunit. We demonstrated that this mutant (*fliC* mutant) had a marked decrease in the ability to adhere to T84 cells and invade both T84 and Caco-2 cells in gentamicin protection assays and by transmission electron microscopy. In addition, the aEPEC 1711-4 *fliC* mutant had a reduced ability to stimulate IL-8 production by Caco-2 cells in early (3-h) but not in late (24-h) infections. Our findings demonstrate that flagella of aEPEC 1711-4 are required for efficient adhesion, invasion, and early but not late IL-8 production in intestinal epithelial cells in vitro.

Typical enteropathogenic *Escherichia coli* (tEPEC) strains are a well-known cause of diarrhea in infants in developing countries (14, 27, 45) whereas atypical EPEC (aEPEC) strains are emerging enteropathogens (1, 3, 10, 14, 27, 28, 33, 36, 45). tEPEC and aEPEC produce a histopathological lesion on enterocytes that is known as the attaching and effacing (AE) lesion. This lesion is characterized by effacement of microvilli, intimate adherence between the bacterium and the cell membrane, and actin accumulation underneath adherent bacteria, forming a pedestal-like structure. Intimate adherence is mediated by intimin (an outer membrane bacterial adhesin) and its receptor Tir (translocated intimin receptor), which is translocated into the host cell by a type III secretion system (19). The proteins involved in the establishment of AE lesions are encoded on the chromosomal pathogenicity island called the locus of enterocyte effacement (LEE) (24). A functional LEE region is also found in enterohemorrhagic *E. coli* (EHEC) and probably plays a significant role in disease since patients with

hemolytic uremic syndrome develop a strong antibody response to various LEE-encoded proteins, although the major virulence factors in EHEC are the Shiga toxins (19). In addition, only tEPEC strains possess the EPEC adherence factor plasmid, which encodes localized adhesion on cultured epithelial cells mediated by the bundle-forming pilus (Bfp) and a transcriptional activator (Per) that upregulates the expression of the *bfp* operon and the LEE genes (19, 27).

It has been shown that tEPEC flagella mediate adhesion and microcolony formation on cultured enterocytes, and they are also implicated in the virulence of EHEC and other enteropathogens (2, 4, 12, 21, 26, 44). However, the role of this structure in different aspects of aEPEC virulence, as adhesion and invasion, has not been evaluated. Invasion of eukaryotic cells results from a complex interplay between the host and the pathogen. Although aEPEC is not considered a classical invasive pathogen, the presence of some aEPEC strains inside HeLa or Caco-2 cells has been reported, but the role of flagella in this process has not been evaluated (15, 34, 37, 40).

Flagellin (FliC), the protein encoded by the *fliC* gene, is the main component of the flagella. Purified flagellin induces interleukin-8 (IL-8) production and Toll-like receptor 5 (TLR5) activation in enterocyte monolayers infected with tEPEC and enteroaggregative *E. coli* (12, 42). Rogers et al. (35) showed that flagellin H21, extracted from a Shiga toxin-producing *E. coli* (STEC) strain, stimulated IL-8 production in the enterocyte lineage HCT-8, while Zhou et al. (49) demonstrated that

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype and characteristics	Source or reference
Strains		
1711-4	aEPEC O51:H40; wild type	13
1711-4 $\Delta fliC$	<i>fliC::zeo</i> (Zeo ^r)	This study
C1P1-8	1711-4 $\Delta fliC$ carrying pZEKm <i>fliC</i> (Zeo ^r Km ^r)	This study
C1GFP	1711-4 $\Delta fliC$ carrying pZEKmGFP (Zeo ^r Km ^r)	This study
MC4160 <i>malT</i> Δ 224:: <i>zeo</i> (F ⁺)	Source of zeocin cassette	Gift from J. M. Ghigo
Plasmids		
pKOBEG- <i>Apra</i>	Unpublished derivative (<i>Apra</i> ^r) of pKOBEG plasmid carrying the λ phage red operon	5
pZE21-MCS2	Km ^r	22
pZEKm <i>fliC</i>	<i>fliC</i> gene from 1711-4 cloned into pZE21-MCS2	This study
pZEKmGFP	Green fluorescent protein-encoding gene inserted into pZE21-MCS2	Gift from J. M. Ghigo

flagellins H6 and H34 of tEPEC, H7 of STEC, and flagellin of *E. coli* K-12 stimulated IL-8 production in T84 monolayers. At least two distinct mechanisms of IL-8 induction were recognized in tEPEC and *Salmonella enterica* serovar Typhimurium: interaction of flagellin with TLR5, which is located on the basolateral surface of the intestinal epithelium, and secretion of effector proteins through the type III secretion system (11, 41). However, there is no information regarding the ability of aEPEC flagellin to elicit an inflammatory response in vitro.

In a survey undertaken in the city of São Paulo, Brazil, aEPEC strains were isolated from fecal samples of children with diarrhea. These strains were subsequently characterized for various phenotypic and genotypic properties (25, 46). Although they belonged to a large diversity of serotypes, O51:H40 was the most frequent (10.1%) (13). Unpublished experimental evidence from our group demonstrates that a motile aEPEC strain (O51:H40), but not a nonmotile aEPEC strain, elicits an intense inflammatory reaction in a rabbit ileal loop model. The aim of this work was to study the role of flagella in the virulence of aEPEC in vitro. We evaluated the aEPEC O51:H40 strain 1711-4 and its flagellin-deficient isogenic mutant in regard their ability to adhere to and invade Caco-2 and T84 intestinal cells and to elicit IL-8 production in Caco-2 monolayers.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used in this study are detailed in Table 1. Bacterial suspensions were obtained after overnight incubation in ambient air at 37°C in 5 ml of Luria-Bertani broth (LB). Strains harboring antibiotic resistance were cultivated in LB containing 50 μ g/ml of kanamycin (Km), 60 μ g/ml of zeocin (Zeo), and/or 100 μ g/ml of apramycin (*Apra*).

Cell lines and culture. Human colorectal adenocarcinoma epithelial cells (Caco-2) were cultivated in Dulbecco's minimal Eagle medium (DMEM) containing a source of glutamine (Glutamax DMEM; GIBCO-BRL) supplemented with 20% heat-inactivated fetal bovine serum (GIBCO-BRL) and 1% nonessential amino acids (32). Cells were incubated at 37°C with 10% CO₂. Human colorectal adenocarcinoma T84 cells were cultivated in DMEM-F12 1:1 Glutamax medium (GIBCO-BRL) supplemented with 10% fetal bovine serum at 37°C in a 5% CO₂-95% air atmosphere.

Cell association and invasion assays. Monolayers were prepared by seeding six-well plates (Falcon-Becton Dickinson Labware) with 10⁵ cells in each well and then incubated for 7 to 10 days in order to allow cellular differentiation. Adherence and invasion assays were performed as previously described, with minimal modifications (17). Briefly, each well was infected with 40 μ l of an overnight static bacterial culture. In order to synchronize the infection processes, plates were immediately centrifuged for 5 min at 730 \times g. After 3 h of incubation at 37°C, supernatants were collected for IL-8 dosage, and monolayers were

washed three times with phosphate-buffered saline (pH 7.4) (PBS) to remove nonadherent bacteria. To evaluate adhesiveness, a group of monolayers were lysed with 1% Triton X-100. Cell-associated bacteria were counted by plating samples on LB agar plates. The number of internalized bacteria was evaluated by performing the same procedures and an additional treatment with 100 μ g/ml gentamicin (Sigma) for 2 hours. Monolayers were washed three times with PBS and lysed with 1% Triton X-100, and lysates were then plated on LB agar. Cell-association indices (AX) were calculated using the equation (AB/INOC) \times 100, where AB is the number of cell-associated bacteria and INOC is the number of bacteria in the initial inoculum. Invasion indices (INX) were calculated using the equation (ICB/AB) \times 100, where ICB is the number of intracellular bacteria. All tests were performed three times in triplicate.

Construction of an aEPEC 1711-4 mutant deficient in flagella. A $\Delta fliC$ derivative of 1711-4 was constructed by the one-step allelic exchange recombination method as described earlier (5, 6, 30). Primers containing a 40-bp region homologous to the 5' (*fliCH40.zeo-5*) and 3' (*fliCH40.zeo-3*) extremities of the *fliC* gene and specific sequences for the Zeo resistance-encoding gene were used to amplify the Zeo cassette (Table 2). Takara LA *Taq* DNA polymerase (Takara Bio Inc.) and a PCR thermocycler system (Applied Biosystems) were used for DNA amplification. Amplicons were purified from agarose gel using the Gene-Clean Turbo kit (Q-Biogene) and quantified (BioPhotometer; Eppendorf) before they were electroporated into competent aEPEC 1711-4 cells containing the pKOBEG-*Apra* plasmid. Recombinant bacteria were selected on Zeo-containing LB agar plates. The allelic replacement of *fliC* by the Zeo cassette was verified by PCR using the *fliCH40.ext-5* and *fliCH40.ext-3* primers (Table 2).

Complementation of the 1711-4 $\Delta fliC$ mutant. Plasmid pZE21-MCS2 (22) was digested with *Xba*I and *Xho*I, removing the polylinker and the upstream promoter. The flagellin-encoding gene *fliC* with its own promoter from the 1711-4 wild-type strain was cloned between these restriction sites, resulting in plasmid pZEKm*fliC* (Table 1). A DNA fragment was amplified using Expand high-fidelity PCR system kit (Roche Applied Science) with primers *fliCH40F* and *fliCH40R* (Table 2) and addition of 5% dimethyl sulfoxide (Sigma Aldrich). Amplification conditions were 94°C for 4 min, followed by 35 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 90 s and a final extension at 72°C for 7 min. The PCR product was digested with 1 U of both *Xba*I and *Xho*I restriction enzymes (Roche Applied Science), purified with the QIAquick gel extraction kit (Qiagen), ligated to the *Xba*I- and *Xho*I-digested vector with 1 U of T4 DNA ligase (Roche Applied Science), and transformed in *E. coli* MC1061 prior to transformation in the 1711-4 $\Delta fliC$ strain. The recombinant plasmid was named pZEKm*fliC* (Table 1). Motility restoration of the complemented strain was checked as described below. Plasmid pZEKmGFP (pZE21-MCS2 containing the green fluorescent protein gene) was used to transform the 1711-4 $\Delta fliC$ mutant. The transformant harboring this plasmid was designated C1GFP and was used as a mock control (Table 1).

Motility test. *E. coli* strains were cultivated overnight in LB without shaking. A 2- μ l sample was seeded into a thin 0.35% agar plate and incubated overnight at 37°C. Motility was evidenced by a spread of bacterial growth around the initial inoculum as evidenced by turbidity enhancement. Nonmotile strains grew only at the inoculation point.

IL-8 secretion by intestinal epithelial cell (IEC) lines. Cell culture supernatants were collected at 3 or 24 h postinfection. Monolayers used for the evaluation of IL-8 concentration at 24 h after infection were maintained in the presence of 10 μ g/ml of gentamicin. Supernatants were centrifuged for 5 min at 10,000 \times g, transferred to a new vial, and stored at -80°C. IL-8 concentrations

TABLE 2. Primers used for construction, verification, and complementation of mutations

Function and designation	Primer sequence ^a
Allelic exchange	
fliCH40.zeo-5	5'-GCCCAATAACATCAAGTTGTAATTGATAAGGAAAA GATCATGGTCATCGCTTGCAATAGAAAG
fliCH40.zeo-3	5'-AACCCCGCCGGTGGCGGGGTTTGAGCGATAAGTG TAAATTAGAATGATGCAGAGATGTAAG
Verification	
fliCH40.ext-5	5'-ATTCAGGTGCCGATACAAGG
fliCH40.ext-3	5'-CGGCATGATTATCCGTTTCT
Complementation	
PrfliCH40F	5'-AAATTTCTCGAGACCTAATTCCTTTTGATTGCAAAC
fliCH40R	5'-AAATTTCTAGACAGGGGTTATTTGGGGGTTA
<i>fliD</i> amplification and sequencing	
fliD-ext-5L	5'-CGTCAACCCTGTTATCGTCTG
fliD401	5'-CCGCCTGTTGAATGGTG
fliD333	5'-CACCACCAGAGACGATACGA
fliD1085	5'-AGTTTGTGCGCATCCAGTTC
fliD891	5'-GCATCTACGGCGGTGTAT
fliD-ext-3L	5'-AAACAGGCTCGCTCTAACCA
M13F	5'-GTAAAACGACGGCCAG
M13R	5'-CAGGAAACAGCTATGAC

^a Underlined bases correspond to XhoI or XbaI restriction enzyme recognition sequences.

were measured using the human IL-8 Duo Set enzyme-linked immunosorbent assay development system kit (R&D Systems).

SEM. Confluent Caco-2 monolayers were infected as described for adhesion and invasion assays. Cells were rinsed twice in 1× PBS buffer, fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) overnight at 4°C or 30 min at room temperature, washed three times in 0.2 M cacodylate buffer for 5 min each, postfixed for 1 h in 1% (wt/vol) osmium in 0.2 M cacodylate buffer, and finally rinsed with distilled water. Samples were dehydrated through washing with a graded series of ethanol solutions (25, 50, 75, 95, and 100%) (5 min each), followed by critical-point drying with CO₂ in a CPD Baltec apparatus. The dried specimens were mounted on stubs with carbon tape and ions sputtered with 15-nm gold-palladium using a Gatan 681 high-resolution ion beam coat. Analysis of the secondary electron image was performed on a JSM 6700F JEOL microscope with a field emission gun operating at 5 kV. For flagellum identification, immunogold labeling was performed with rabbit anti-*E. coli* flagellum H40. Three hours after infection, monolayers were rinsed with PBS, fixed with 4% formaldehyde–0.1% glutaraldehyde for 15 min, and then washed with PBS. Monolayers were incubated in 50 mM NH₄Cl and then with 0.1% bovine serum albumin in PBS. Preparations were incubated with a 1:100 dilution of anti-flagellin H40 antibody in PBS overnight at 4°C and washed with PBS before addition of 10-nm gold bead-coupled suspension. Unbound beads were removed by rinsing with PBS before monolayers were processed for scanning electron microscopy (SEM) as described above, except that they were sputtered with a 15-nm layer of carbon and observed with a backscattered electron detector.

Transmission electron microscopy. Confluent Caco-2 cells were infected as described for adhesion and invasion assays. Preparations were rinsed in 1× PBS buffer, fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2), and kept overnight at 4°C. After being rinsed with 0.1 M cacodylate buffer, preparations were postfixed in 1% OsO₄, dehydrated through a series of graded ethanol solutions and propylene oxide, and embedded in araldite resin. Blocks were polymerized at 60°C for 48 h. Ultrathin sections were stained with 2.0% uranyl acetate aqueous solution and 2.5% lead citrate and examined under a transmission electron microscope (LEO 906E; Zeiss) at 80 kV.

Sequencing of the *fliD* gene. The *fliD* gene was amplified with primers fliD-ext-5L, fliD401, fliD333, fliD1085, fliD891, and fliD-ext-3L (Table 2). A total of 5 μl of genomic DNA suspension (30 μg/ml) was added to 45 μl of a PCR mixture containing 50 mM KCl, 20 mM Tris-HCl (pH 8.4), 2.5 mM MgCl₂, 200 μM each 2'-deoxynucleoside 5'-triphosphate, 1 μM each primer, and 1.0 U of Platinum *Taq* DNA polymerase (Invitrogen). The PCR mixture was heated at 95°C for 1 min and then subjected to 35 cycles of denaturation at 94°C for 30 s, 55°C for 30 s, and 72°C for 60 s, with a final single step of 72°C for 5 min. PCR products were separated by 1.5% agarose gel electrophoresis, bands were cut from the gel, and DNA was extracted and ligated into pCR-XL-TOPO vector (Invitrogen). Cloning reaction products were used to transform chemically competent TOP10 *E. coli* (Invitrogen). Transformants were selected on LB agar containing 50 μg/ml

of Km. Inserts were amplified and sequenced with primers M13F and M13R (Invitrogen). Amplicons were purified with GFX PCR DNA and a Gel Band purification kit (GE) and sequenced in an ABI PRISM 3100 sequencer with a BigDye Terminator cycle sequencing kit (Applied Biosystems). Sequences were assembled with DNA Baser program version 2.75 (Heracle Software) and then compared with those deposited in the GenBank database using BLAST (Basic Local Alignment Tool) (<http://www.ncbi.nlm.nih.gov/BLAST>).

Statistical analysis. The Shapiro-Wilk test was applied to determine if variables fit in a normal distribution. The Kruskal-Wallis test and Bonferroni error protection were applied to verify the statistical significance of differences in AX and IN VX among strains. Analysis of variance (ANOVA) and Tukey's post hoc test were applied to evaluate the differences between IL-8 results. Data were reported as mean ± standard deviation (SD) or median and interquartile range (IQR). A statistical significance of 5% was applied ($\alpha = 0.05$). All tests were performed with Analyze-it for Microsoft Excel version 2.07 (Analyze-it Software, Ltd.).

Nucleotide sequence accession number. The GenBank database accession number generated in this study is FJ598057 (*fliD*).

RESULTS

Flagella play a role in adhesion of aEPEC 1711-4 to IEC monolayers. The ability of aEPEC 1711-4 to adhere to IECs was investigated. The median (IQR) AX, which represents the percentage of inoculated bacteria that adhered to and/or invaded eukaryotic cells until 3 hours after infection, was significantly higher in T84 (4.7% [4.1 to 5.8%]) than in Caco-2 (0.3% [0.2 to 1.1%]) monolayers ($P < 0.001$ [main value and Bonferroni contrast result]). To determine whether flagella played a role in such adhesion, an isogenic aEPEC 1711-4 mutant deficient in flagella (1711-4 Δ *fliC*) was constructed. Lack of flagellum expression in this mutant was confirmed by SEM (Fig. 1C) and by motility testing in semisolid agar (Fig. 2B). In contrast to the results obtained with the wild-type aEPEC 1711-4 strain, no statistically significant difference was observed when comparing the median (IQR) AXs of 1711-4 Δ *fliC* in Caco-2 (0.6% [0.4 to 0.8%]) and T84 (0.8% [0.4 to 1.3%]) monolayers ($P = 1.00$). An increase in the median AX was observed in the interaction of the *fliC*-deficient mutant with Caco-2 monolayers compared to the wild-type strain. This difference was not statistically significant ($P = 1.00$). In contrast,

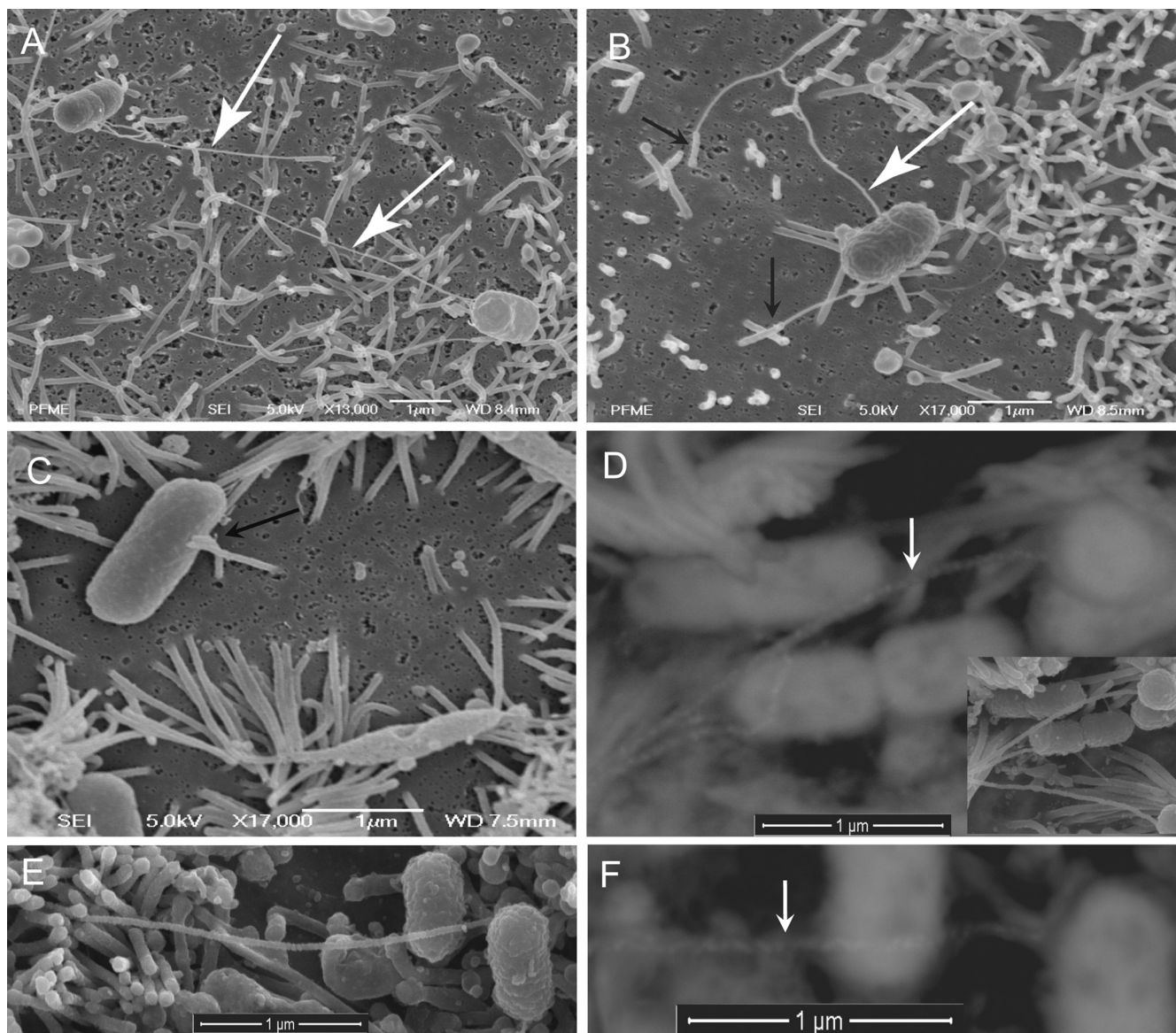


FIG. 1. Interaction of aEPEC wild-type 1711-4, the *FliC*-deficient mutant, or the C1P1-8 complemented mutant with Caco-2 monolayers. (A and B) SEM of monolayers infected with wild-type aEPEC 1711-4, evidencing interaction between the flagella (white arrows) and microvillus (black arrows). Note interaction of the microvillus with the tips of flagella. The microvillus also interacts with the bacterial cell body. (C) SEM showing interaction of isogenic mutant 1711-4 $\Delta fliC$ with Caco-2 cells. Note the absence of flagella and interaction of the microvillus tip with the bacterial cell body (black arrow). (D) SEM with backscattered electron detection of an immunogold preparation with rabbit anti-*E. coli* H40 serum. Caco-2 cells were infected with wild-type aEPEC 1711-4. Note labeled flagella (white arrow). On the lower right is a secondary electron image of the same microscopic field. (E) SEM showing interaction of the C1P1-8 complemented isogenic mutant with Caco-2 cells. (F) SEM with backscattered electron detection of an immunogold preparation with rabbit anti-*E. coli* H40 serum. Caco-2 cells were infected with the complemented mutant C1P1-8. Note labeled flagella (white arrow).

the 1711-4 $\Delta fliC$ mutant showed a significant reduction in median AX in T84 monolayers ($P = 0.003$) compared to the wild-type strain, suggesting a role of flagella in the adhesion of this aEPEC strain to this cell lineage.

As the median AXs of the 1711-4 $\Delta fliC$ mutant strain were similar in both cell lineages used, SEM of infected monolayers was performed only with the Caco-2 cells. Monolayers infected with the wild-type aEPEC 1711-4 strain evidenced direct contact between the bacterial cell body and the microvillus tip (Fig. 1A and B), forming a microvillus cluster (Fig. 1B). Of

note, SEM also evidenced interaction of the wild-type aEPEC 1711-4 flagellum cap and the tip of the microvillus (Fig. 1A and B). In SEM immunogold assays with rabbit anti-*E. coli* H40 serum, these structures were clearly labeled, confirming that they are in fact flagella (Fig. 1D). In contrast, only direct interaction of the microvillus tip with the bacterial cell body was observed with the *fliC*-deficient mutant (Fig. 1C).

The flagellum-deficient aEPEC mutant 1711-4 has a decreased ability to invade IECs. Since wild-type aEPEC 1711-4 adhered to IECs, the capacity of this strain to invade Caco-2

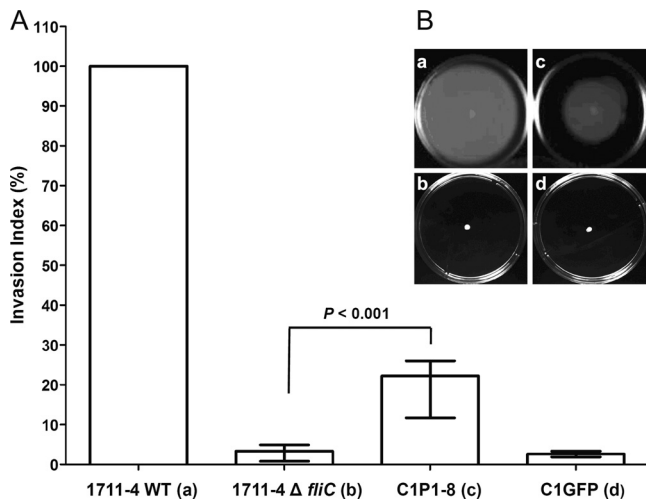


FIG. 2. Intact flagella are required for efficient invasion of Caco-2 cells. (A) Relative median INVX (error bars represent IQRs) of aEPEC wild-type (WT) 1711-4, the *fliC*-deficient mutant 1711-4 $\Delta fliC$, and isogenic deletion strains complemented with pZEK*m**fliC* (C1P1-8) or pZEK*m*GFP (C1GFP). The wild-type strain INVX was assumed to be 100%. See text for exact values. (B) Motility test in nutrient agar with 0.35% agarose. Note the absence of motility in strains 1711-4 $\Delta fliC$ (b) and C1GFP (d) and restoration of motility in strain C1P1-8 (c).

and T84 cell monolayers was investigated using the gentamicin protection assay. Both cell lineages were invaded by aEPEC 1711-4. With the wild-type strain, the median (IQR) INVX obtained in assays with T84 cells (10.7% [9.9 to 15.4%]) was similar to that observed with Caco-2 cells (15.1% [9.9 to 20.8%]) ($P = 1.00$). When the 1711-4 $\Delta fliC$ strain was tested, the lowest median (IQR) INVX (0.08% [0.06 to 0.14%]) was observed with Caco-2 cells. Compared to the median INVX of the wild-type strain, this difference was statistically significant ($P < 0.001$). The median (IQR) INVX of the 1711-4 $\Delta fliC$ strain (2.2% [1.9 to 4.8%]) in assays with T84 cells was significantly lower than that observed for the wild-type strain ($P = 0.02$). These data strongly evidenced a role of flagella in internalization of the wild-type 1711-4 strain into both Caco-2 and T84 cells.

Complementation of the 1711-4 $\Delta fliC$ mutant for flagellin production partially restores its capacity to invade Caco-2 cells. Since the most clear significant reduction in the invasion capacity of the 1711-4 $\Delta fliC$ mutant was observed in assays with Caco-2 monolayers, subsequent investigations were performed only with this cell lineage. The 1711-4 $\Delta fliC$ mutant was complemented with a plasmid carrying the complete *fliC* gene (coding for flagellin H40) or a mock plasmid and then compared to the wild-type strain. Motility was restored in the 1711-4 $\Delta fliC$ mutant complemented with *fliC* (strain C1P1-8) (Fig. 2B) but not in the same strain containing the mock plasmid (strain C1GFP). Transmission electron microscopy analysis of monolayers infected with wild-type aEPEC 1711-4 or C1P1-8 confirmed that both strains invaded Caco-2 cells (Fig. 3). Although the intracellular localization of the 1711-4 $\Delta fliC$ mutant was detected with the gentamicin protection assay, this strain could not be visualized within Caco-2 cells, probably due to the low number of intracellular bacteria (Fig. 3).

In gentamicin protection assays, the complemented strain C1P1-8 showed a partial but statistically significant restoration of the ability to invade Caco-2 monolayers, since the median (IQR) INVX was 5.8% (3.1 to 6.8%) ($P < 0.001$), while the strain containing the mock plasmid (C1GFP) showed no increase in median (IQR) INVX (0.7% [0.5 to 0.9%]) ($P = 1.00$) compared to strain 1711-4 $\Delta fliC$. Assuming the median INVX obtained with the wild-type strain to be 100%, the median (IQR) INVX obtained with 1711-4 $\Delta fliC$, C1P1-8, and C1GFP strains would be 3% (0.8 to 4.9%), 22% (11.7 to 26.0%), and 2% (1.9 to 3.3%), respectively (Fig. 2A).

The *fliC*-deficient mutant has a reduced ability to stimulate IL-8 production only in the early stage of infection of Caco-2 cells. In order to evaluate the role of flagella in the stimulation of IL-8 production during infection of Caco-2 cells, the concentration of this inflammatory mediator in supernatants was determined at 3 and 24 h after infection with wild-type aEPEC 1711-4, 1711-4 $\Delta fliC$, C1P1-8, or C1GFP. Three hours after infection, a significant decrease ($P < 0.001$; 95% confidence interval [CI] = 17.9 to 52.7 pg/ml) in the mean (\pm SD) IL-8 production was noted with the *fliC*-deficient mutant (12 ± 2.6 pg/ml) compared to the wild-type strain (47 ± 6.9 pg/ml) (Fig.

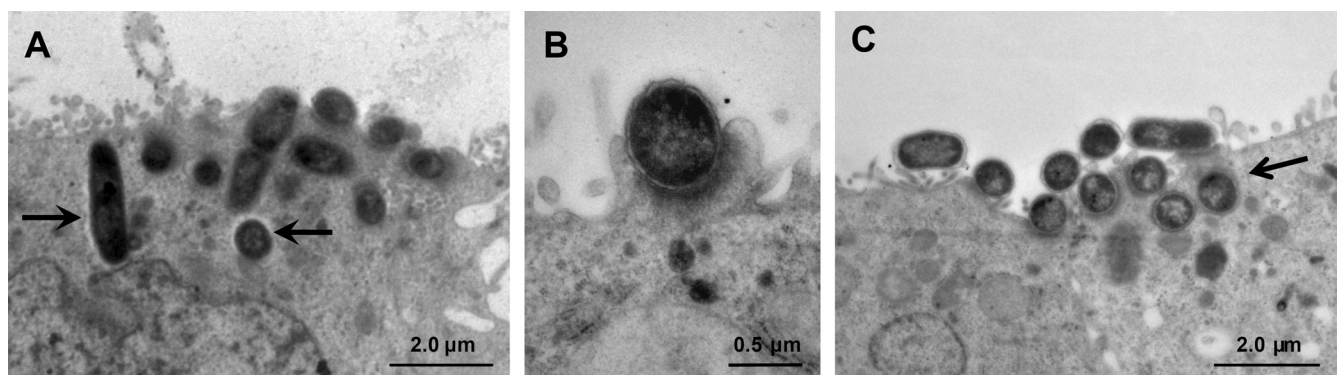


FIG. 3. Transmission electron microscopy of Caco-2 cells infected with wild-type aEPEC 1711-4 (A), 1711-4 $\Delta fliC$ (B), or C1P1-8 (C). Both the wild type and the complemented mutant (C1P1-8) were observed in the intracellular compartment (A and C, arrows). The *fliC*-deficient mutant was not detected in the intracellular compartment. Note that both the *fliC* mutant and the complemented strain retain the ability of the parental strain to produce AE lesions as revealed by pedestal formation (B).

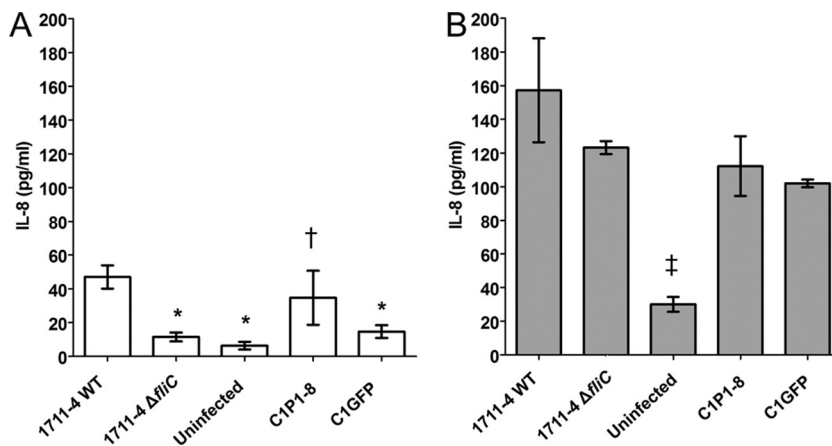


FIG. 4. IL-8 concentration in supernatants of Caco-2 cell cultures infected with wild-type (WT) aEPEC 1711-4, its isogenic *fliC* mutant, and the complemented strains at 3 h (A) and 24 h (B) after infection or with no infection. Data are expressed as the mean \pm SD from triplicate experiments. *, statistically significant difference compared to the WT strain 1711-4 ($P < 0.001$ by ANOVA). †, statistically significant difference compared to the 1711-4 Δ *fliC* strain (95% CI, 4.3 to 41.8 pg/ml). ‡, statistically significant difference compared to each infected monolayer ($P < 0.001$ by ANOVA).

4). Complementation of this mutant with a plasmid encoding FliC partially restored its ability to stimulate IL-8 production (35 ± 16.1 pg/ml), and this difference was statistically significant (95% CI = 4.3 to 41.8 pg/ml). In contrast, no significant increase (95% CI = -22.2 to 15.4 pg/ml) was observed when strain C1GFP (*fliC*-deficient mutant complemented with a mock plasmid) was tested (15 ± 3.8 pg/ml). Twenty-four hours after infection, all strains induced average levels of IL-8 that were 2 to 5 times higher than that observed in uninfected monolayers (30 ± 4.5 pg/ml). These differences were statistically significant ($P < 0.001$). However, there was no statistically significant difference (95% CI = -1.3 to 69.6 pg/ml) between average IL-8 levels induced by the wild-type strain (157 ± 30.9 pg/ml) and 1711-4 Δ *fliC* (123 ± 4.0 pg/ml). Although strains C1P1-8 and C1GFP induced average IL-8 levels that were 29% and 35%, respectively, lower than that induced by the wild-type strain, these differences were not statistically significant. In fact, the average induced IL-8 levels of strains C1P1-8 and C1GFP were, respectively, 112 pg/ml (95% CI = 47.1 to 118.0 pg/ml) and 102 pg/ml (95% CI = 36.8 to 107.7 pg/ml). These levels were significantly higher than that observed in uninfected monolayers. These data suggest a predominantly flagellin-independent stimulation of IL-8 production at late stages after infection (24 h).

The FliD proteins from aEPEC 1711-4 and the nonpathogenic strain *E. coli* K-12 share the same amino acid sequence. When comparing the nucleotide sequence of the *fliD* gene from aEPEC strain 1711-4 to those available at GenBank, the highest similarity index, 99.6% (1401/1407), was obtained with sequences pertaining to *E. coli* K-12 (accession numbers CP000948.1, AP009048.1, and U00096.2). Their deduced amino acid sequences were identical.

DISCUSSION

In this study we showed that aEPEC 1711-4 (serotype O51:H40) was able to adhere in vitro to two different enterocyte lineages (Caco-2 and T84 cells). Interestingly, this strain ad-

hered more efficiently to T84 cells than to Caco-2 cells. In contrast, Eaves-Pyles et al. (8) showed that an adherent invasive *E. coli* O83:H1 strain adhered better in Caco-2 cells than in T84 cells. As suggested by Girón et al. (12), this difference could be due to antigenic heterogeneity and, consequently, distinct adhesive properties of flagella and/or to the presence of other adherence factors.

Girón et al. (12) also demonstrated that H2 and H6 flagella purified from tEPEC but not H7 flagella purified from STEC (O157:H7) bound to HeLa cells. It has also been demonstrated that flagella of *Salmonella enterica* (7), *Yersinia enterocolitica* (48), *E. coli* strains pathogenic for birds (20), and adherent invasive *E. coli* strains (8) are involved in enterocyte adhesion in vitro. Likewise, we showed that flagella of aEPEC 1711-4 contribute to bacterial adhesion to IECs as evidenced by the lower AX of the aEPEC 1711-4 Δ *fliC* mutant in T84 monolayers. In addition, our SEM images suggest that interaction of aEPEC 1711-4 flagella occurred preferentially between the flagellum cap and the microvillus tip (Fig. 1A and B). Considering the small surface area of both the flagellum cap and microvillus tip compared to the entire Caco-2 cell surface, there is probably a strong affinity between the two structures. These findings are in accordance with those previously reported by Tasteyre et al. (44), who demonstrated that radiolabeled cultured Vero cells bound to a high degree to recombinant FliD and weakly to crude flagellar preparations or recombinant FliC from *Clostridium difficile*. The flagellum cap protein, FliD, is essential for flagellum assembly, and its central portion, which forms a lid, is exposed at the tip of the flagellum (23, 47). Mutants deficient in FliC, as is the case in our work, are expected to express the flagellar hook with the FliD protein at its end exposed at the bacterial wall. This may explain our SEM findings evidencing the microvillus tip in close contact with the *fliC*-deficient derivative cell body in a topography that could correspond to the flagellar hook. Although our SEM data could suggest that FliD rather than FliC would play a role in the interaction of aEPEC 1711-4 with IECs, the finding that the FliD protein produced by this strain

has the same amino acid sequence found in *E. coli* K-12, a nonvirulent strain, does not support this hypothesis. We cannot exclude the possibility that adhesion of the flagellar cap to the microvilli tip could also be mediated by an adhesin secreted by a two-partner secretion pathway, as is the case for EtpA, found in enterotoxigenic *E. coli* (38). EtpA is plasmid mediated and to date has been described only in enterotoxigenic *E. coli*, although no aEPEC strains have been evaluated regarding the presence of this protein (9).

In this study we also demonstrated that the aEPEC 1711-4 Δ *fliC* mutant invaded IECs significantly less than the wild-type aEPEC strain. Anchoring mediated by flagella probably is also required for efficient enterocyte invasion by aEPEC 1711-4, since addition of *fliC* to the *fliC*-deficient mutant partially restored its invasion capability. This partial restoration is most probably due to low efficiency of *fliC* expression when located in the plasmid under the control of its own promoter and not to *fliC* overexpression due to plasmid copy number, since wild-type strains and their derivatives have the same growth rate (data not shown). These findings thus suggest that wild-type aEPEC 1711-4 flagella are necessary for efficient adhesion to and invasion of Caco-2 and T84 cells. Likewise, flagella were shown to be necessary for both association and invasion of human brain microvascular endothelial cells by a meningitis-associated *E. coli* O18:K1:H7 strain (29). Similarly, the ability of *E. coli* O83:H1 to adhere to and invade Caco-2BBE or T84 monolayers was shown to be dependent on these structures (8). Flagella have also been involved in the internalization of uropathogenic *E. coli* into collecting duct cells in vitro (30).

In this work we found that the aEPEC 1711-4 Δ *fliC* mutant induced lower levels of IL-8 production in Caco-2 cell culture supernatants than the wild-type strain at the earlier stage of infection (3 hours). These levels were partially restored in the 1711-4 Δ *fliC* mutant complemented with *fliC* (C1P1-8 strain) but not in the same mutant complemented with the mock plasmid (C1GFP strain). However, the *fliC* mutant still induced IL-8 levels at least 2 times higher than that of uninfected monolayers at 24 h postinfection. Therefore, wild-type aEPEC 1711-4 may stimulate IL-8 production in a flagellin-dependent manner at an earlier stage of infection and by a flagellin-independent pathway at a later stage of infection (24 h). However, when interpreting results from experimental procedures that evaluate the IL-8 concentration in cells cultivated in vitro, one might consider which portion of the cell monolayer was directly exposed to the bacterial suspension. Gewirtz et al. (11) demonstrated that TLR5, the main receptor for flagellin, is expressed on the basolateral rather than the apical surface of polarized T84 cells. Moreover, they showed that flagellin added at the apical cell surface achieves TLR5 receptors after translocation through the cytoplasm. Although to our knowledge there is no report in the literature on the topology of TLR5 in Caco-2 cells, Ruchaud-Sparagano et al. (39) demonstrated that the apical infection of Caco-2 cells by tEPEC strain E2348/69 induced IL-8 secretion to a lesser extent than the basolateral infection. IL-8 concentrations reported by these authors at 2, 3, 4, and 6 hours of infection with flagellated tEPEC are similar to those that we obtained with wild-type aEPEC 1711-4 at 3 hours after infection. Our findings of similar IL-8 levels in Caco-2 cells infected with the wild-type strain or *fliC*-deficient mutant after 24 h of infection could be due to

the interaction of eukaryotic cell membrane receptor TLR4 and the bacterial lipopolysaccharide. Huang et al. (16) described that IL-8 levels in Caco-2 culture supernatants stimulated with lipopolysaccharide were shown to be irrelevant at 3 h but significant after 24 h of infection, with mean levels of 75 pg/ml. However, the data of that study may not be comparable to ours, since those authors used 14- to 15-day-old Caco-2 cultures while we used 7- to 10-day-old cultures. Caco-2 monolayers are known to achieve confluence on day 6 and stationary phase on day 9 after subculture (31). Since we obtained higher mean IL-8 levels (102 to 157 pg/ml) and our experiments were performed with 7- to 10-day-old monolayers, we cannot exclude the possibility that other factors may have stimulated IL-8 secretion. One such factor could be DNA CpG from dead bacteria inside late endosomes, recognized by TLR3 or TLR9 (18, 43). As these receptors are able to identify diverse chemical structures, another possible explanation of our findings could be that an effector protein, secreted by wild-type aEPEC 1711-4 in the intracellular compartment, would interact with TLR3 or TLR9 and stimulate IL-8 production also independently of flagella.

The data presented in this study strongly suggest a role for flagella in both adhesion to and invasion of aEPEC 1711-4 into IECs and in early but not late stimulation of IL-8 production after Caco-2 cell infection. Even though differentiated Caco-2 cells are often used as a model for human small intestinal cells, the in vivo significance of our findings remains to be established. In the complex intestinal environment, flagella could contribute not only to traverse the mucus layer and reach the epithelial surface but also in the early stages of interaction with enterocytes, acting as an anchoring device. Moreover, flagella could contribute in vivo to entrance into enterocytes, favoring aEPEC escape from the immune response.

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