Late Establishment of the Attaching and Effacing Lesion Caused by Atypical Enteropathogenic *Escherichia coli* Depends on Protein Expression Regulated by Per

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Enteropathogenic *Escherichia coli* (EPEC) is classified as typical (tEPEC) or atypical (aEPEC) based on the presence or absence of the *E. coli* adherence factor plasmid (pEAF), respectively. The hallmark of EPEC infection is the formation of the attaching and effacing (A/E) lesions on the gut mucosa. We compared the kinetics of A/E lesion formation induced by aEPEC and tEPEC. The examination of infected HEp-2 cells clearly demonstrated delayed A/E lesion formation by aEPEC in comparison to tEPEC. This delay was associated with the expression of locus of enterocyte effacement (LEE)-encoded virulence factors (i.e., intimin and EspD). Indeed, the insertion of a plasmid containing *perABC*, a transcriptional regulator of virulence factors involved in A/E formation, into aEPEC strains increased and accelerated the formation of A/E lesions. Interestingly, the enhanced expression and translocation of LEE-encoded proteins, such as those expressed in LEE5 (intimin) and LEE4 (EspD), in aEPEC (*perABC*) was independent of bacterial adhesion. The secretion kinetics of these two proteins representing LEE5 and LEE4 expression correlated with A/E lesion formation. We conclude that the lack of Per in the regulation network of virulence genes is one of the main factors that delay the establishment of A/E lesions induced by aEPEC strains.

Enteropathogenic *Escherichia coli* (EPEC) is one of the main causative agents of infantile acute diarrhea in developing countries (1–4). The hallmark of EPEC pathogenesis is the formation of a histopathological lesion in the intestinal epithelium known as attaching and effacing (A/E) (5). Distinctive features of A/E lesions are intimate attachment of the bacterium to the intestinal epithelial surface, effacement of microvilli, and reorganization of actin filaments in the intestinal cell just beneath the site of attachment, which leads to the formation of pedestal-like structures (5, 6). The proteins involved in A/E lesion formation are encoded by chromosomal genes located in a 35-kb pathogenicity island known as the locus of enterocyte effacement (LEE) (7). These proteins have a crucial and well-established role in EPEC pathogenesis. For example, intimin is responsible for the intimate adherence of the bacterium to the host cell, Tir acts as a translocated receptor for intimin, and EspA is the main constituent of a filamentous structure that establishes contact between the bacterium and the host cell, by which secreted and effector proteins such as EspB, EspD, and Tir are translocated (8, 9).

EPEC strains are currently classified as typical (tEPEC) if they harbor the *E. coli* adherence plasmid (pEAF) or atypical (aEPEC) if they lack that plasmid (10, 11). pEAF contains the *bfp* operon, encoding the bundle-forming pilus (BFP), and *per* (plasmid-encoded regulator) operon, which encodes the regulator proteins PerA, PerB, and PerC, involved in the regulation of BFP, LEE genes, and *per* itself (12–14).

In recent years, tEPEC strains have rarely been isolated as agents of acute childhood diarrhea, and the predominance of aEPEC strains has been observed in both industrialized and developing countries (reviewed in references 3 and 11). Also, some studies have implicated aEPEC strains as agents of persistent diarrhea (15, 16). Strains of tEPEC adhere to cultured epithelial cells in a localized adherence (LA) pattern (17), in which the bacteria initially form compact clusters, due to expression of BFP and EspA

filament, followed by the intimate adhesion of the bacteria on the surface of the epithelial cell, mediated by intimin and its translocated receptor Tir (18). On the other hand, aEPEC strains frequently display a localized adherence-like (LAL) pattern, in which the compact clusters are not observed (19). In contrast to the LA pattern, which can be observed after 3 h of bacterium-eukaryotic cell contact (17), the LAL pattern is expressed after 6 h of interaction (11). Consequently, the A/E lesions can be detected in tEPEC after 3 h of interaction, while in aEPEC this phenotype is delayed and not detected until after 6 h (20–24).

The precise cause of such delay is unknown. aEPEC strains do not harbor pEAF, and it has been established that the LA phenotype is dependent on the presence of this plasmid (25), mainly because of BFP expression, involved in the initial steps of intestinal colonization (18). Moreover, the absence of the Per regulator, also pEAF encoded, could delay the expression of some LEE genes involved in adhesion and the formation of A/E lesions in aEPEC, yet this has not been investigated. Therefore, the aim of this work

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was to characterize EPEC factors involved in early or late A/E lesion formation that distinguish between aEPEC and tEPEC infection.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The typical and atypical EPEC strains selected for this study belong to the classical EPEC serogroups. We selected 10 aEPEC strains (two O26:H11, four O55:H7, one O111ab:H9, one O111:HNM, and two O119:H2) characterized by the *eae*⁺ *EAF*[−] genetic profile and expression of LAL in HEp-2 cells (6-h adherence assay) in previous studies (19, 22, 26–29). For comparison, three tEPEC strains (*eae*⁺ *EAF*⁺) belonging to the classical serogroups (O55:H6, O111:H2, and O119:H6) were also studied (19, 26, 27). All strains were kept in Luria-Bertani (LB) broth supplemented with 15% glycerol at −80°C. Bacteria were grown overnight in LB broth to the mid-logarithmic phase at 37°C without shaking.

FAS assay. All atypical and typical EPEC strains were evaluated by the fluorescent actin staining (FAS) assay under the same conditions and in triplicate. HEp-2 cells were cultivated in 8-well Lab-Tek chamber slides (Nalgene Nunc, USA) in Dulbecco modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum, for 24 h at 37°C in 5% CO₂, to reach 70 to 80% confluence. Before the infection, HEp-2 cells were washed three times with DMEM, and fresh DMEM with 1% D-mannose was added. Bacterial strains were grown overnight in LB broth without shaking at 37°C. Afterwards, the cultures were diluted 1:20 in DMEM with 1% D-mannose and incubated at 37°C for 2 h. HEp-2 cell monolayers were infected with these bacterial cultures at a multiplicity of infection (MOI) of 0.5 for tEPEC and 5 for aEPEC. Infections were allowed to proceed for 1, 4.5, and 6 h. Following infection, the coverslips were washed four times with phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde in PBS. The monolayers were washed three times with PBS, and the cells were permeabilized with 0.1% Triton X-100 in PBS for 5 min. For the FAS assay (30), preparations were washed 7 times with PBS and stained for 40 min with phalloidin-rhodamine (1:80) (Molecular Probes, USA) for actin and with TO-PRO-3 (1:500) (Molecular Probes, USA) for nucleic acids. After washing 5 times with PBS and twice with water, the cells were mounted in Vectashield (Vector Laboratories, USA) and visualized with a Leica TCS SP2 and TCS SP5 confocal tandem microscope (Leica, Germany).

Immunofluorescence assays for intimin and EspD detection. Analysis of intimin and EspD expression in HEp-2 cells incubated with typical and atypical EPEC was performed by immunofluorescence. HEp-2 cells were infected as indicated for FAS assays (see above). Following infection, the coverslips were washed four times with PBS and fixed with 4% paraformaldehyde in PBS. The monolayers were washed three times with PBS, and the cells were permeabilized with 0.1% Triton X-100 in PBS for 5 min. Cells were washed 7 times with PBS and blocked with 1% bovine albumin serum in PBS for 30 min. The monolayers were probed with rabbit anti-intimin (1:250) or anti-EspD (1:450) antisera for 1 h at room temperature, washed 7 times with PBS, and incubated with 1:350 fluorescein isothiocyanate (FITC)-anti-rabbit antibody (Invitrogen, USA) for 1 h at room temperature. Preparations were then washed 7 times with PBS and stained for 40 min with phalloidin-rhodamine (1:80) (Molecular Probes, USA) for actin and with TO-PRO-3 (1:500) (Molecular Probes, USA) for nucleic acids. After washing 5 times with PBS and twice with water, the cells were mounted in Vectashield (Vector Laboratories, USA) and visualized with a Leica TCS SP2 and TCS SP5 confocal tandem microscope (Leica, Germany).

CFU. HEp-2 cells were infected as in the immunofluorescence assays but using 48-well microplates. After 4.5 h of infection, the wells were washed with PBS, and the adhered bacterial cells were collected by scraping them into PBS; serial dilutions were plated onto LB agar plates and incubated overnight. The number of adherent bacteria was determined by counting the resulting colonies in triplicate. Data were analyzed by using

an unpaired two-tailed *t* test (GraphPad Prism 5.01). *P* values of <0.0001 were considered statistically significant.

Quantification of pedestals on HEp-2 cells. To quantify the pedestals on HEp-2 cells induced by infection with aEPEC, tEPEC, or Per-expressing aEPEC, three photos from each condition were taken and pedestals were quantified on 5 randomly chosen cells (4.5 h postinfection). Any accumulation of phalloidin staining (polymerized actin) at the site of bacterial adhesion was considered to indicate the presence of a pedestal. In all cases, values are the means ± standard errors from three independent assays. Statistical analyses were performed by using an unpaired two-tailed *t* test (GraphPad Prism 5.01). *P* values of <0.001 were considered statistically significant.

Expression and secretion of proteins. Bacterial strains were grown overnight in LB broth without shaking at 37°C. Afterwards, the cultures were diluted 1:50 in DMEM (3 ml) and incubated at 37°C in a shaking incubator until reaching an optical density at 600 nm of 0.7. Bacterial cultures were centrifuged at 15,000 × *g* for 15 min at 4°C to obtain pellets and supernatants. Supernatants were concentrated with 20% trichloroacetic acid. Secreted proteins were obtained by centrifugation at 15,000 × *g* for 25 min. The pellets and supernatant proteins were resuspended in equal volumes of SDS-PAGE loading buffer and analyzed by SDS-PAGE and Western blotting using anti-intimin and anti-EspD antibodies.

Immunodetection of intimin and EspD in HEp-2 cells infected with different EPEC strains. For the immunodetection assays, HEp-2 cells were cultivated in 60-mm cell culture dishes (Corning, USA) in DMEM supplemented with 10% fetal bovine serum and incubated for 48 h at 37°C in 5% CO₂ until 90 to 100% confluence was obtained. The monolayers were infected with aEPEC, tEPEC, and Per-expressing aEPEC, as described for the immunofluorescence assays. After 1, 3, 4.5, and 6 h of infection, the cells were washed five times with PBS and fresh DMEM with ampicillin (4 mg/ml) was added followed by 1 h of incubation at 37°C in 5% CO₂. The monolayers were washed five times and harvested with 250 μl of radioimmunoprecipitation assay (RIPA) buffer (Sigma-Aldrich, USA). The samples were sonicated with 3 pulses of 30 s at 40% amplitude and centrifuged at 12,000 rpm for 15 min at 4°C. The total protein content in the supernatant was quantified by the Bradford method.

For tEPEC, 35 μg of total proteins was analyzed by 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). For aEPEC, 175 μg of total proteins was used, since aEPEC infected fewer cells than did tEPEC, resulting in a lower bacterial/eukaryotic protein ratio. The proteins were transferred to activated polyvinylidene fluoride (PVDF) membranes (Immobilon-P; 0.45 μm; Millipore, USA) for 110 min at 4°C under 320 mA. The membranes were blocked at room temperature with 5% skim milk in PBS-Tween 20 (0.05%) for 1 h. Next, the membranes were incubated with anti-intimin (2 μg/ml) in PBS-Tween 20 overnight at 4°C or with anti-EspD (1:10,000) in PBS-Tween 20 for 1 h at room temperature. After three washes with PBS-Tween 20 for 10 min, the membranes were incubated with horseradish peroxidase (HRP)-goat anti-rabbit IgG (Invitrogen, USA) at 1:20,000 for 1 h at room temperature and washed 5 to 10 times for 10 min. The transferred proteins were revealed by autoradiography, using the Immobilon Western chemiluminescent HRP substrate (Merck Millipore, USA), and analyzed by densitometry. Statistical analyses were performed by using an unpaired two-tailed *t* test (GraphPad Prism 5.01). *P* values of <0.005 were considered statistically significant.

Construction of an aEPEC strain harboring *perABC*. aEPEC strains LB-7 (O55:H7), BA-4147 (O55:H7), and LB-12 (O119:H2) were selected for the introduction of a *perABC*-containing plasmid (pJLM161), which is a clone that carries a 3.9-kb insert containing the entire *perABC* operon (31). DNA extraction, transformation, and DNA agarose electrophoresis were performed according to standard methods (32). Plasmid was introduced into aEPEC strains by electroporation of competent cells using the *E. coli* pulser apparatus (Bio-Rad Laboratories, USA). Transformants were selected on LB agar containing 30 μg/ml chloramphenicol. The presence of *per* in transformants LB-7(pJLM161), BA-4147(pJLM161), and LB-12(pJLM161) was confirmed by PCR with *perA*-specific primers (*perA*

forward, AACAAAGAGGAGAATTTAGCG; *perA* reverse, CTTGTGTAAT AGAATAAACGC) based on the available sequence (GenBank accession number Z48561). PCRs were performed as described elsewhere (33), employing the following amplification cycle: 30 cycles of 1 min at 94°C, 1 min at 56°C, and 1 min at 72°C and then 8 min at 72°C. Transformed strains were thereafter evaluated for adherence characteristics using the HEp-2 cell adherence assay and FAS assay, as well as for intimin and EspD detection by immunofluorescence and immunoblotting.

RESULTS

aEPEC adherence to epithelial cells and induction of A/E lesion are delayed in relation to tEPEC. To discern the role of bacterial adherence and the adherence pattern in the beginning of A/E lesion establishment in distinguishing between tEPEC and aEPEC, adherence assays at 1-, 3-, 4.5-, and 6-h interaction periods were carried out, simultaneously with the fluorescent actin staining (FAS) assay on HEp-2 cells (30). Based on previous work, and considering that tEPEC adheres and multiplies quickly on the epithelial cells (after 4 h of incubation, the cell monolayers are overcolonized and most of the cells are dying), we have found that MOIs of 5 for aEPEC and 0.5 for tEPEC are optimal conditions to compensate for this differential adhesion and to guarantee that we have preparations containing similar quantities of adhered bacteria. All 10 aEPEC strains tested belonging to serogroups O26, O55, O111, and O119 displayed a LAL pattern, whereas the three tEPEC strains of similar serogroups (O55, O111, and O119) displayed a LA pattern. Kinetics of pedestal formation induced by typical (LB-28, O55:H6) and atypical (LB-7, O55:H7) EPEC on HEp-2 cells showed that pedestal formation was faster in cells infected by tEPEC than in cells infected by aEPEC (Fig. 1). In tEPEC-infected cells, actin accumulation (red in figure) at the site of bacterial adherence (blue) could be visualized after 1 h of infection and these interactions increased with incubation time (3, 4.5, and 6 h). Thus, pedestal formation increased until bacteria formed compact clusters (Fig. 1). In contrast, in aEPEC, actin accumulation could be clearly observed after 4.5 h, becoming intense after 6 h of interaction, when it began to be comparable to those pedestals formed by tEPEC, but bacteria were unable to form clusters (Fig. 1).

These data suggest that bacterial adherence and/or adherence pattern influences the pedestal formation process.

Introduction of *Per*-expressing plasmid in aEPEC allows earlier formation of A/E lesion. To further determine if pedestal formation is influenced by bacterial adherence and/or adherence pattern, a *perABC*-expressing plasmid (pJLM161), a regulator that could increase the expression of LEE proteins but not the initial adhesion (since aEPEC does not harbor the *bfp* genes), was introduced in aEPEC strain LB-7 (O55:H6). The adherence assay on HEp-2 cells showed that the LAL pattern was maintained by the *per*-transformed aEPEC strain and that it was observed in a shorter period of interaction (after 3 h) than in the wild-type aEPEC strain (after 6 h). Pedestal formation induced by the transformed aEPEC (pJLM161) strain was also evaluated by FAS. The strain supplied with *perABC* was able to cause defined pedestals at 3 h postinfection, and the pedestals increased with incubation time (4.5 and 6 h). However, aEPEC was unable to form clusters, in contrast to those cells infected by tEPEC (Fig. 1). To evaluate the difference between tEPEC, aEPEC (pJLM161), and aEPEC in their capabilities to form pedestals, we determined the difference in pedestal formation between these strains in 15 cells at 4.5 h of interaction. Pedestal quantification in the FAS assay showed an average of 50 pedestals/cell induced by tEPEC and 16 pedestals/cell by

FIG 1 Time course of A/E lesion formation in tEPEC (strain LB-28), aEPEC (strain LB-7), and *Per*-expressing aEPEC [LB-7(pJLM161)], evaluated at the postinfection times indicated by the FAS assay using confocal microscopy. Cells were double labeled with FITC-rhodamine for actin staining (red) and with TO-PRO-3 for bacterial and HEp-2 cell DNA (blue). Magnification, $\times 100$; bar, 20 μ m. Arrows point to actin pedestals.

aEPEC, while aEPEC (pJLM161) was able to induce an average of 32 pedestals/cell, which was twice as high as those induced by aEPEC but still lower than those induced by tEPEC (Fig. 2A).

All these data indicate that *Per* plays a relevant role in pedestal formation by allowing early expression of the A/E lesion in the absence of any additional adherence factor (such as BFP) and does not modify the adherence pattern.

To investigate if *Per* regulation is required for pedestal formation without affecting initial adherence, we compensated for the difference at initial adherence by increasing the MOI of aEPEC 10 times (MOI = 5) with respect to tEPEC (MOI = 0.5), and CFU were quantified after 4.5 h of EPEC-epithelial cell interaction. Interestingly, tEPEC with an MOI of 0.5 produced around 20 times more CFU than did aEPEC with an MOI of 5, and surprisingly, aEPEC (pJLM161) produced fewer CFU than did its parental aEPEC strain (Fig. 2B). Thus, even though the *perABC*-expressing aEPEC was less efficient in adhering to epithelial cells, it was more efficient at inducing pedestal formation than the wild-type aEPEC (Fig. 1 and 2A).

Delayed formation of A/E lesion induced by aEPEC is related to late expression of LEE-encoded virulence factors. To better understand the role of the *Per* regulator in aEPEC, we compared the expressions of LEE proteins among the aEPEC, aEPEC (pJLM161), and tEPEC strains. Thus, the secretion of two proteins encoded in LEE, representing the LEE5 (intimin) and LEE4 (EspD) operons, was detected in bacterial cultures by Western blotting with anti-intimin and anti-EspD antibodies. The amount of intimin in the bacterial pellet from aEPEC was near the limit of

FIG 2 Role of Per in pedestal formation and bacterial adherence. (A) Pedestal formation. Quantification of pedestals on HEp-2 cells induced by tEPEC (strain LB-28), aEPEC (strain LB-7), and Per-expressing aEPEC LB-7(pJLM161) after 4.5 h of infection. In all cases, values are means $\pm$ standard deviations of three independent experiments (error bars). ***, *P* values of <0.0001 were considered statistically significant. (B) Bacterial adherence. HEp-2 cells were infected with the different strains as indicated above. After the wells were washed with PBS, the cell-adherent bacteria in the wells were collected by scraping and serial dilutions were plated onto LB agar plates. The number of adherent bacteria was determined by counting the resulting colonies in triplicate.

detection, whereas tEPEC intimin was strongly detected (around 12-fold more than in aEPEC). Interestingly, *perABC*-expressing aEPEC showed increased levels of intimin of about 7-fold more than did the wild-type aEPEC. Thus, aEPEC (pJLM161) expressed an intermediate level of intimin between aEPEC and tEPEC. On the other hand, tEPEC secreted about 2-fold (densitometrically) more EspD to the supernatant than did aEPEC, but *perABC*-expressing aEPEC showed 3-fold-higher secretion of EspD (Fig. 3). As expected, there was no detection of intimin in the supernatant of any strain, and the levels of EspD expression in the pellets were similar in all strains (Fig. 3).

To monitor the levels of EspD and intimin during infection of epithelial cells by tEPEC, aEPEC, and aEPEC (pJLM161), the temporal expression of both proteins was evaluated after 1, 4.5, and 6 h of bacterium–HEp-2 cell interaction (using an MOI of 0.5 for tEPEC and an MOI of 5 for aEPEC) by immunofluorescence and immunoblotting after killing the bacteria. The immunofluorescence assays were carried out using anti-intimin and anti-EspD antisera and FITC-secondary antibody, giving a green fluorescence to the labeled proteins. In tEPEC-infected cells, intimin was strongly detected (through its binding to Tir, since the bacteria

FIG 3 Detection of intimin and EspD in bacterial cultures from tEPEC (strain LB-28), aEPEC (strain LB-7), and Per-expressing aEPEC [LB-7 (pJLM161)]. Cultures were diluted 1:50 in DMEM (3 ml) and incubated at 37°C in a shaking incubator until reaching an optical density at 600 nm of 0.7. Bacterial cultures were centrifuged to obtain pellets, and supernatants were concentrated by the addition of trichloroacetic acid. Secreted proteins were obtained by centrifugation. The pellets and supernatant (SN) proteins were resuspended in equal volumes of SDS-PAGE loading buffer and analyzed by SDS-PAGE and Western blotting using anti-intimin and anti-EspD antibodies.

were killed before staining) at 4.5 h of infection, but not at 1 h (it was initially detected starting at 2 h [data not shown]), and the fluorescence increased at 6 h postinfection. In contrast, in aEPEC-infected cells, we were unable to detect intimin; no green fluorescence was detected at any time tested. Interestingly, the *perABC*-expressing aEPEC strain was able to express more intimin when in contact with epithelial cells than was the wild-type aEPEC. However, this intimin expression by aEPEC (pJLM161) did not reach the levels reached by tEPEC (Fig. 4).

Immunoblot detection was performed to quantify the temporal expression of intimin in cells infected by the tEPEC, aEPEC, and aEPEC (pJLM161) strains. As shown in Fig. 5A, intimin detection on the epithelial cells was also higher and earlier in cells infected by tEPEC (at 3 h) than in those infected by aEPEC (at 6 h). Interestingly, by using the same MOI as with wild-type aEPEC, intimin in cells infected by aEPEC (pJLM161) at 4.5 h was higher than in those infected by aEPEC but considerably less than in cells infected by tEPEC. Intimin levels increased with time (i.e., at 6 h), but the same difference between the three strains persisted. Relative detection levels of intimin after 4.5 and 6 h of interaction from three independent immunoblot assays were quantified and normalized against actin expression as indicative of intimin detection in a similar amount of cells. Figure 5B shows that intimin levels in cells infected by tEPEC were 18 times higher than in those infected by aEPEC after 4.5 h of interaction and 11 times higher after 6 h of interaction. Interestingly, intimin detection in cells infected by aEPEC (pJLM161) at 4.5 and 6 h was four times greater for both times than in those infected by aEPEC and only seven times less than in cells infected by tEPEC at 6 h.

Subsequently, detection of the secreted protein EspD, which is inserted into the plasma membrane of infected cells, was evaluated after 1, 4.5, and 6 h of bacterium–cell interaction by immunofluorescence. As shown in Fig. 6, EspD in cells infected by tEPEC was weakly detected within the first hour of infection, and expression gradually increased until it was more intense after 6 h, while in aEPEC-infected cells, fluorescence intensity for EspD was very

FIG 4 Temporal expression of intimin during infection by tEPEC (strain LB-28), aEPEC (strain LB-7), and aEPEC (pJLM161), evaluated at 1, 4.5, and 6 h postinfection by immunofluorescence and confocal microscopy. Anti-intimin was detected with FITC-anti-mouse (green), nucleic acids were stained with TO-PRO-3 (blue), and actin was labeled with phalloidin-rhodamine (red). Magnification, $\times 100$; bar, 50 μm .

low. Even when the fluorescence intensity was almost imperceptible, EspD expression also increased with time. Remarkably, in cells infected by the *perABC*-expressing aEPEC strain, EspD signals were clearly higher than in cells infected by the wild-type

aEPEC and almost at the same levels detected in cells infected by tEPEC.

Immunoblot detection was also performed to evaluate the temporal detection of EspD in cells infected by the same strains. As

FIG 5 Immunoblot analysis of intimin expression in HEp-2 cells infected for 1, 3, 4.5, and 6 h with tEPEC (strain LB-28), aEPEC (strain LB-7), and Per-expressing aEPEC [LB-7(pJLM161)]. Cells were solubilized in RIPA lysis buffer, and whole-cell lysates were harvested for immunoblotting. (A) Blots were probed with anti-intimin antibody, stripped, and reprobed with antiactin antibodies to demonstrate equal loading of infected cells. Strips were revealed by autoradiography using the Immobilon Western chemiluminescent HRP substrate. (B) Relative expression levels from three independent immunoblot assays ($n = 3$) were quantified by densitometry, and band intensities were normalized using actin. To quantify intimin increment, the values of intimin expression from aEPEC were used as a reference and arbitrarily given the value of 1. P values of < 0.005 were considered statistically significant. *, $P < 0.005$; **, $P < 0.001$. A typical immunoblot is shown.

FIG 6 Temporal expression of EspD during infection by tEPEC (strain LB-28), aEPEC (strain LB-7), and aEPEC (pJLM161) evaluated at 1, 4.5, and 6 h postinfection by immunofluorescence and confocal microscopy. Anti-EspD was detected with FITC-anti-mouse (green), nucleic acids were stained with TO-PRO-3 (blue), and actin was labeled with phalloidin-rhodamine (red). Magnification, $\times 100$; bar, 50 μm .

shown in Fig. 7A, EspD was detected after 3 h in cells infected by both typical and atypical EPEC strains, increasing gradually until 6 h. However, the labeling of this protein was remarkably more intense in cells infected by tEPEC at all incubation times, while in

cells infected by aEPEC (pJLM161), EspD was detected at an intermediate level compared to aEPEC and tEPEC (Fig. 7A). Relative expression levels of EspD after 3, 4.5, and 6 h of interaction from three independent immunoblotting assays were also quan-

FIG 7 Immunoblot analysis of EspD expression in HEP-2 cells infected for 1, 3, 4.5, and 6 h with tEPEC (strain LB-28), aEPEC (strain LB-7), and Per-expressing aEPEC [LB-7(pJLM161)]. Cells were solubilized in RIPA lysis buffer, and whole-cell lysates were harvested for immunoblotting. (A) Blots were probed with anti-EspD antibody, stripped, and reprobed with antiactin antibodies to demonstrate equal loading of infected cells. Strips were revealed by autoradiography using the Immobilon Western chemiluminescent HRP substrate. (B) Relative expression levels from three independent immunoblot assays ($n = 3$) were quantified by densitometry, and band intensities were normalized using actin. To quantify EspD increment, the values of EspD expression from aEPEC were used as a reference and arbitrarily given the value of 1. P values of <0.005 were considered statistically significant. *, $P < 0.005$; **, $P < 0.001$; ***, $P < 0.0001$. Results from a typical immunoblot assay are shown.

FIG 8 Other Per-expressing aEPEC strains increase intimin and EspD expression and early pedestal formation, independently of bacterial adhesion. (A) Increase in bacterial adhesion in pJLM161-transformed aEPEC strains. HEP-2 cells were infected by BA-4147, BA-4147(pJLM161), LB-12, LB-12(pJLM161), and a tEPEC strain (LB-28), and the number of adherent bacteria was determined as indicated for Fig. 2B. (B) Pedestal formation. Quantification of pedestals on HEP-2 cells induced by the different strains (see above) after 4.5 h of infection was performed as indicated for Fig. 2A. (C) Detection of intimin and EspD in bacterial cultures. Bacterial cultures of the different strains (see above) were fractionated, and the detection of EspD in supernatants (SN) and intimin (pellet) was performed as indicated for Fig. 3. (D) Temporal expression of intimin and EspD in epithelial cells infected by the different aEPEC strains. Intimin and EspD expression in cells infected by BA-4147, BA-4147(pJLM161), LB-12, LB-12(pJLM161), and a tEPEC strain (LB-28) were analyzed by immunoblotting as indicated for Fig. 5. (E and F) Relative expression levels of intimin (E) and EspD (F) from three independent immunoblot assays ($n = 3$) were quantified by densitometry, and band intensities were normalized using actin. To quantify intimin and EspD increment, the values of intimin and EspD expression from aEPEC were used as references and arbitrarily given the value of 1. P values of <0.005 were considered statistically significant. *, $P < 0.005$; **, $P < 0.001$; ***, $P < 0.0001$. Results from a typical immunoblot assay are shown.

tified and normalized against actin expression, as for intimin detection in the infected cells. Figure 7B shows that EspD in cells infected by tEPEC was nine times higher than in cells infected by aEPEC at 3 h postinfection, 14 times higher at 4.5 h, and four times higher at 6 h postinfection. EspD in cells infected by aEPEC (pJLM161) was three times higher than in cells infected by aEPEC at 3 h and 4.5 h and twice as high at 6 h postinfection (Fig. 7B).

All these data suggest that delayed formation of A/E lesions induced by aEPEC is related to late expression of LEE-encoded virulence factors, which can be expressed early by the insertion of the Per regulator.

Relevance of the expression of LEE-encoded proteins to bacterial adherence. To reproduce the data in other aEPEC strains and to compare the roles of LEE-encoded protein expression in bacterial adhesion in the pedestal formation process, two other aEPEC strains were analyzed. aEPEC (BA-4147) of serotype O55:H7 (same as the LB-7 strain shown above) and aEPEC (LB-12) of serotype O119:H2 were transformed with the Per-express-

ing plasmid (pJLM161). Intimin and EspD production and secretion were analyzed in bacterial culture and infected cells, as well as their bacterial adhesion on infected cells. These two aEPEC strains were selected because they displayed similar adhesion on epithelial cells as measured by CFU; BA-4147 recovered from infected cells was able to produce around 4.6×10^4 CFU, and LB-12 produced around 5.8×10^4 CFU, which differed from that produced by tEPEC (1.3×10^6 CFU). Interestingly, in these cases, the transformation of both strains with the Per-expressing plasmid (pJLM161) increased bacterial adhesion to 3×10^5 and 3.1×10^5 CFU for BA-4147 and LB-12, respectively (Fig. 8A). The expression of intimin in bacterial membranes clearly increased in LB-12(pJLM161) cultures of 4.5 h and was almost imperceptible in BA-4147(pJLM161), whereas in both transformed strains, EspD detected in the supernatants increased in a variable way in comparison to their respective aEPEC wild-type strains (Fig. 8C).

Kinetics of intimin and EspD expression during infection of epithelial cells also increased in a time-dependent manner (Fig. 8D).

Intimin was undetectable at 3 h of infection in any aEPEC strain, transformed or not, unlike the tEPEC strain (LB-28), whereas EspD expression increased only in the transformed aEPEC strains [BA-4147(pJLM161), 18-fold, and LB-12(pJLM161), 13-fold compared with the wild-type strains] and tEPEC (Fig. 8C and E). At 4.5 h of infection, intimin expression was detected only in cells infected with the transformed strains [increased 12-fold with BA-4147(pJLM161) and 9-fold with LB-12(pJLM161)] (Fig. 8D). EspD expression was detected only in cells infected with the transformed strain [BA-4147(pJLM161)] but not in the wild-type aEPEC strain (BA-4147), and LB-12(pJLM161) increased 2-fold over the wild-type aEPEC strain (LB-12). At 6 h of infection, intimin detection increased in cells infected with the transformed aEPEC strains [BA-4147(pJLM161), 18-fold, and LB-12(pJLM161), 7-fold compared with those cells infected with the wild-type strains, where detection was almost imperceptible] and at levels similar to those expressed by the tEPEC strain (Fig. 8C). EspD expression increased in cells infected with the transformed aEPEC strains (BA-4147, 3-fold, and LB-12, 1.5-fold) compared with the wild-type aEPEC strains and even more than with the wild-type tEPEC. It is worth noting that at 6 h postinfection the difference in virulence factor levels induced by transformed strains and wild-type strains was low.

Remarkably, these data for two aEPEC strains of different serogroups support those obtained above with the LB-7 strain, which was studied in detail. Moreover, the transformation of both aEPEC strains (BA-4147 and LB-12), as described above, increased bacterial adherence to the same extent (Fig. 8A), but they showed different expression of intimin and EspD (Fig. 8E and F), which correlated with their difference in pedestal formation (Fig. 8B).

DISCUSSION

The central mechanism of EPEC pathogenesis is the A/E lesion on the gut mucosa (5, 34, 35). tEPEC strains adhere to epithelial cells in the characteristic LA pattern, while aEPEC, especially the strains belonging to classical EPEC serogroups (36), frequently displays the LAL pattern, which lacks compact clusters and occurs at a later stage of infection than does LA (17, 19, 20, 22). It has been shown that the actin-rich pedestals, characteristic of A/E lesions, can be easily visualized by the FAS assay and that tEPEC strains are FAS positive after 3 h of infection *in vitro* (30).

Since the LAL pattern shown by aEPEC can be observed only after 6 h of infection (21–24), we sought to analyze the FAS assay after 1, 3, 4.5, and 6 h of interaction, to study some factors involved in the early or late A/E lesion establishment. We compared the observed results between tEPEC and aEPEC strains, belonging to the same serogroups (O55, O111, and O119). The results clearly demonstrated a delay in bacterial adhesion and formation of actin-rich pedestals in aEPEC, in comparison to that observed with tEPEC strains. There are some factors that could explain these results, such as the absence of BFP and Per, both located in pEAF (11). In fact, all aEPEC strains of this study lack the *perABC* sequences (data not shown). BFP is involved in initial bacterial adhesion to the eukaryotic cell and, more precisely, in the formation of microcolonies (18). It has been demonstrated that the LA phenotype relies mainly on BFP expression. Jerse et al. (37) demonstrated that the JPN15 strain (the prototype tEPEC strain E2348/69 cured from pEAF) is FAS positive after 6 h of incubation and does not express the LA phenotype. In addition, when pEAF of E2348/69(pMAR2) was reintroduced in this strain, the LA pattern was observed, and the strain was FAS positive after 3 h of

bacterial interaction with the host cell. Moreover, the absence of *perABC* regulators could delay the expression of some LEE genes involved in A/E lesion formation, since Per activates not only BFP expression but LEE operons as well (14). Interestingly, we demonstrated here that in an aEPEC strain transformed with a *perABC*-expressing plasmid (pJLM161), which could increase the expression of LEE proteins but not the initial adhesion, the LAL pattern was maintained but pedestal formation occurred earlier, after 3 h (Fig. 1). Furthermore, the *perABC*-expressing aEPEC strain was less efficient in adhering to the epithelial cells but it was more efficient in inducing pedestal formation than the wild-type aEPEC strain, suggesting that other factors are decisive for contributing to increasing pedestal formation (Fig. 2).

Since the kinetics of A/E lesion formation by aEPEC demonstrated an evident delay in comparison to that by tEPEC, we hypothesize that the expression of the proteins involved with this lesion by this pathotype also occurs at a later stage of infection. Thus, we compared the temporal expressions of intimin (an outer membrane protein) and EspD (a secreted protein) by typical and atypical EPEC strains at different times of HEP-2 cell infection (Fig. 4 to 7). Leverton and Kaper (38) used real-time PCR to analyze the transcription of LEE and *bfp* operons of tEPEC (E2348/69), after 10 min, 3 h, and 5 h of infection in HEP-2 cells. Their results showed that after 10 min of infection, the transcription from operons LEE3, LEE4, and LEE5 as well as *bfp* was already detectable and increased at 3 h. In the present study, we showed by immunoblotting that in tEPEC, intimin (LEE5) expression occurred at 3 h of interaction, increasing gradually until 6 h. These results were expected, since tEPEC is capable of causing an A/E lesion after 3 h of infection, whereas in aEPEC, intimin was weakly detected in the initial 4.5 h and became more evident only after 6 h of bacterium-epithelial cell interaction. On the other hand, the expression of EspD (LEE4) was detected at 3 h in tEPEC and aEPEC, but it was more intense in tEPEC. Afterwards, EspD expression increased with time for both strains, but with their differences in intensity maintained. It is worth mentioning that the detection of intimin and EspD by immunoblotting was more evident than in the immunofluorescence assays because the proteins are enriched in the cell lysates, since we used more infected cells than those seen in a field under microscopy. Our results with tEPEC corroborate those described by Leverton and Kaper (38), where the expression levels of intimin, Tir, and EspA reached a maximum after 3 h and remained constant during the remaining period of infection. On the other hand, the findings described here for aEPEC demonstrate that the delay of A/E lesion formation by this pathotype is directly associated with the delayed expression of its LEE-encoded virulence factors.

The delayed formation of the A/E lesion by aEPEC strains could be explained by the absence of the Per regulator, encoded by the *perABC* operon located on pEAF of tEPEC (12) and absent in aEPEC (1). As mentioned before, the *perABC* operon activates not only itself but also the transcription of BFP and LEE. PerA belongs to the AraC family of transcriptional activators (12) and activates the transcription of the *eae* and *bfp* operons (12, 13). PerC activates directly the transcription of *ler*, the first gene on the LEE1 operon, which in turn activates an expression cascade of other LEE genes, among them *eae*, *tir*, and *espD* (14, 39, 40). In an attempt to elucidate the delayed A/E lesion formation observed in aEPEC, three aEPEC strains (LB-7, BA-4147, and LB-12), lacking *perABC*, were transformed with a plasmid expressing the *perABC* operon

(31) and studied regarding A/E formation kinetics and temporal expression of intimin and the secreted protein EspD. As clearly seen in Fig. 1, the LAL pattern was maintained in aEPEC harboring *perABC*, and as expected, this adhesion pattern could be observed after 4.5 h of infection. Moreover, actin accumulation at the site of bacterial adhesion observed by the FAS assay was also accelerated. The temporal expression of intimin and EspD during cell infection with the aEPEC strains transformed with *perABC* (Fig. 4 to 8) demonstrated that the expression of these virulence factors was higher and also earlier, compared with the results of the wild-type strains (aEPEC). These findings could explain the fact that the FAS assay was positive in a shorter period of infection and also that the formation of the LAL pattern was evident after only 4.5 h. Taken together, these results support the hypothesis that the absence of *perABC* contributes to the delay of host cell adhesion and the formation of the LAL pattern observed in aEPEC, since its presence enabled the earlier establishment of infection. However, it must be pointed out that the presence of *perABC* was not capable of providing the tEPEC phenotype in the transformed aEPEC strain and did not enable the formation of compact bacterial clusters, as seen in the LA pattern. It has been demonstrated that other factors are involved in the formation of compact microcolonies, such as BFP, which is absent in aEPEC and whose participation in the formation of LA has been previously described (34). Zahavi et al. (41) showed that in the *bfp* operon, *bfpF* promotes pilus retraction, which could provide a tight bacterium-bacterium interaction, leading to microcolony formation, and could also bring the attached bacteria closer to the epithelial cell, facilitating the translocation of secreted effector proteins. Moreover, other factors such as flagellum-mediated adhesion and quorum sensing were also related to LA formation and regulation of LEE genes in tEPEC (42–45). Independently of these previous findings, our data showed that Per does not increase bacterial adhesion in an aEPEC background but that it does increase LEE-encoded proteins, which appear to be decisive for streamlining pedestal formation.

In summary, our results clearly show a delay in the adherence of aEPEC to epithelial cells and in the formation of A/E lesions compared with tEPEC strains. In aEPEC, the expression of some of the main virulence factors (intimin and EspD) is also delayed, compared with tEPEC. The expression of the Per regulator in aEPEC strains was able to accelerate the expression of intimin and EspD, as well as the formation of A/E lesions by aEPEC. Interestingly, our data showed that early EspD (LEE4) expression correlated more with early pedestal formation, suggesting that the translocator/effectors injected play a relevant role, since the LEE4 operon encodes SepL, EspA, EspD, EspB, CesD2, EscG, and EspF.

We conclude that the main reason for the delay in the establishment of A/E lesions induced by aEPEC is a transcription regulation matter. The regulation of the A/E phenotype is extremely complex and involves several regulatory proteins and environmental signals as well. As mentioned before, quorum sensing regulation is involved in the A/E phenotype, especially through the coordination of Ler regulation (46). The lack of BFP in aEPEC, which provides a tight bacterium-bacterium interaction through the formation of microcolonies, could diminish bacterial communication via quorum sensing, since the number of adherent bacteria is smaller in LAL phenotype strains, and the absence of the Per regulon, which acts directly on Ler, delays the transcription of the main proteins involved in the A/E phenotype.

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