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Adhesion to and Invasion of HeLa Cells by Pathogenic *Escherichia coli* Carrying the *afa-3* Gene Cluster Are Mediated by the AfaE and AfaD Proteins, Respectively

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The *afa-3* gene cluster, expressed by uropathogenic and diarrhea-associated *Escherichia coli* strains, determines the formation of an afimbrial adhesive sheath composed of the AfaD and AfaE-III adhesins. The adherence to HeLa cells by recombinant HB101 strains producing both or only one of these two adhesins was investigated. Ultrastructural analyses of the interaction and gentamicin protection assays showed adherence to HeLa cells by HB101 producing both the AfaD and AfaE-III proteins and internalization of a subpopulation of the bacteria into the cells. The interactions of HeLa cells either with HB101 mutants producing AfaD or AfaE-III or with polystyrene beads coated with purified His_6-tagged AfaD or His_6-tagged AfaE-III proteins were studied. These experiments demonstrated that AfaE-III allows binding to HeLa cells and that AfaD mediates the internalization of the adherent bacteria. Ultrastructural analyses of the interaction of His_6-AfaD–gold complexes with HeLa cells confirmed that AfaD is able to bind to the HeLa cell surface and indicated that it penetrates the cells via clathrin vesicles. These data demonstrate that the *afa* gene cluster is unique among bacteria, as alone it encodes both adhesion to and invasion of epithelial cells.

Adhesion of pathogenic *Escherichia coli* to mucosal epithelia is an important early stage in colonization and development of intestinal or urinary tract diseases. Most of the adhesins described are from pathogenic *E. coli* associated with either intestinal or urinary tract infections, and each type of adhesin is specific for a particular disease (19). The only known exceptions are the afimbrial adhesins, encoded by the *afa* gene clusters and produced by both uropathogenic and diarrhea-associated *E. coli* strains (17, 18, 20, 24–26). The *afa* determinants belong to a family of gene clusters that includes the *dra* and *daa* genes, encoding the Dr and F1845 adhesins, respectively (5, 15, 16, 25, 31). Epidemiological studies report that the prevalence of the *afa* family gene clusters in uropathogenic strains is between 11 and 32% (1, 3, 12, 31, 33). In New Caledonia, strains displaying a diffuse adherence pattern on HEp-2 and HeLa cells and expressing *afa*-related gene clusters are the major diarrhea-associated bacterial pathogens in the child population, particularly among children 2 to 6 years old (17).

afa gene clusters share a highly conserved region including the *afaA*, *afaB*, *afaC*, *afaD*, and *afaF* genes (15, 16, 23). Unlike these genes, the structural adhesin-encoding gene, designated *afaE*, is highly heterogeneous, leading to the production of antigenically distinct adhesins (AfaE-I to AfaE-IV) (16, 22). Both the AfaE-I and AfaE-III adhesins mediate a mannose-resistant hemagglutination of human erythrocytes and specifically attach to epithelial cells by recognizing the decay-accelerating factor (DAF) molecule as a receptor (15, 29, 30). The 17.5-kDa AfaD protein is also a component of the afimbrial adhesive sheath, and histidine-tagged AfaD, like AfaE, is able to bind to HeLa and Caco-2 cells (15).

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The aim of this study was to determine the roles of the two components of the afimbrial adhesive sheath during the association of *afa*-expressing strains with epithelial cells. The well-characterized and cloned *afa-3* gene cluster was used. Ultrastructural and genetic analyses of the interaction revealed that bacteria producing both AfaD and AfaE could be internalized into the HeLa cells. Then, examination of the interaction mediated by mutants and by polystyrene beads coated with either AfaD or AfaE-III demonstrated that AfaE-III and AfaD are involved in initial binding to HeLa cells and in the internalization process, respectively. Finally, the AfaD protein tagged with colloidal gold was able to penetrate HeLa cells via clathrin-associated vesicles.

MATERIALS AND METHODS

Bacterial strains, tissue culture cells, and culture conditions. *E. coli* A30 was isolated from a urine specimen from a patient with cystitis (22). *E. coli* HB101 (7) was used as a host for the recombinant plasmids listed in Table 1. These plasmids carry the *afa-3* gene cluster, whose genetic organization is shown in Fig. 1. Vectors pBR322 (6), pACYC184 (9), and pILL570 (21) were used in cloning experiments. Bacteria were grown in Luria broth (LB) without glucose (10 g of tryptone, 5 g of yeast extract, and 5 g of NaCl per liter [pH 7.0]) or on Luria plates (containing 1.5% agar) at 37°C. Antibiotics were added to growth media to the following concentrations (in milligrams per liter): carbenicillin, 100; chloramphenicol, 20; and spectinomycin, 100.

The HeLa Ohio (ECACC 84121901) human cervical cell line was maintained in minimum essential medium containing Earle's salts and L-glutamine (GIBCO Laboratories, Eragny, France) supplemented with 10% heat-inactivated fetal calf serum (GIBCO) and 1% nonessential amino acids (ICN Biomedicals, Costa Mesa, Calif.), in a 5% CO_2 atmosphere at 37°C.

DNA methodology. Standard procedures were used for restriction endonuclease digestions and other common DNA manipulations (27).

Adherence and invasion assays. Monolayers were seeded with 4×10^5 cells in 35-mm-diameter tissue culture dishes (Corning, Corning, N.Y.), incubated overnight, and washed with fresh medium. Each plate was infected with either approximately 4×10^7 to 8×10^7 bacteria, 30 μl of a suspension of protein-coated polystyrene beads, or 200 μl of a protein-colloidal gold complex preparation. The *afa*-expressing strains were grown on agar plates. The samples were then incubated for 3 to 6 h at 37°C in 1 ml of tissue culture medium containing 1% D-mannose.

For the adherence assay, after incubation, infected monolayers were washed

TABLE 1. Recombinant plasmids carrying the *afa-3* gene cluster used in this study

Plasmid	Description	Reference
pILL1101	pILL570 carrying an 11.6-kb *Sau*3A fragment containing the *afa-3* gene cluster from A30	16
pILL1168	pBR322 carrying a 10.5-kb *Bam*HI fragment of pILL1101 in which the *afaD* gene was replaced by an amplification product in which a stop codon was created	15
pILL1169	pBR322 carrying a 10.5-kb *Bam*HI fragment of pILL1101 containing a *cat* cartridge introduced into the *afaE* gene	15
pILL1189	Insert of pILL1168 in pACYC184	This study

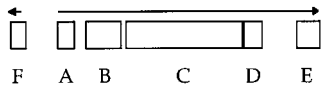

FIG. 1. Genetic organization of the *afa-3* gene cluster.

three times with phosphate-buffered saline (pH 7.4), fixed with methanol, stained with 10% Giemsa stain, and examined under a light microscope. Alternatively, cells were prepared for electron microscopy.

The bacterial invasion of epithelial cells was measured as protection against gentamicin, a bactericidal antibiotic. The minimal bactericidal concentration that reduced the bacterial count by 99.9% (MBC) of the antibiotic for the bacterial strains was determined in tissue culture medium, and the drug was first used at 10- to 100-fold the MBC (MBC, <1 μg/ml). Since similar results were obtained independent of the gentamicin concentration, a final concentration of 50 μg/ml was used in subsequent experiments. After incubation of the infected monolayers, samples were taken from the medium and the number of extracellular nonadherent bacteria was determined. Infected monolayers were then washed six times with minimum essential medium, and fresh medium containing gentamicin was added to kill extracellular bacteria. After incubation for 2 h at 37°C, monolayers were washed six times, 1 ml of cold sterile water was added, the monolayers were incubated at 4°C for 45 min, and then the cells were lysed by vigorous pipetting. Samples were removed, diluted, and plated on LB agar with appropriate antibiotics to determine the number of CFU per monolayer.

Cell-associated bacteria (adherent and intracellular bacteria) were similarly counted, except that the HeLa cells were lysed after the 3-h incubation. The total number of bacteria present per monolayer was estimated as the sum of the number of extracellular, nonadherent bacteria and the number of cell-associated bacteria. Each strain was tested in triplicate.

Proteins. Bacteria were grown overnight in LB with vigorous shaking at 37°C and then pelleted (2,830 × *g* at 4°C). The culture supernatant was removed, and proteins from the supernatant were precipitated by addition of trichloracetic acid to a final concentration of 10% (wt/vol). After incubation at 4°C for 4 h, precipitated proteins were pelleted (2,830 × *g* at 4°C) and rinsed sequentially twice with an acetone-chloridric acid solution (200:1, vol/vol) and then with pure acetone. Pellets were suspended in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer so as to concentrate supernatant proteins 100 times. Bacterial pellets containing cell-associated proteins were suspended in one-fifth of the culture volume in SDS-PAGE loading buffer. SDS-PAGE and Western blotting were performed as previously described (15). The AfaD and AfaE-III products encoded by the *afa-3* gene cluster were previously purified as histidine-tagged recombinant fusion proteins (rAfaD and rAfaE-III) (15).

Antibodies. Polyclonal rabbit anti-rAfaE-III and anti-rAfaD antibodies were obtained previously (15).

Coating of carboxylated polystyrene beads with proteins. Carboxylated microspheres 1 μm in diameter were coated with either rAfaE-III, rAfaD, or bovine serum albumin (BSA) as recommended by the supplier (Polysciences, Inc., Warrington, Pa.). The concentration of the coated beads was approximately 1.8×10^{10} beads/ml, and the amounts of rAfaE-III, rAfaD, and BSA bound to each microsphere were estimated as 20, 15, and 12 fg, respectively. By varying the amount of protein to be coupled, we also obtained rAfaD beads each coated with 0.04, 0.2, 0.4, or 1 fg of protein.

Protein-colloidal gold conjugate preparation. Proteins were conjugated to 10-nm-diameter gold particles (British BioCell International) as previously described (8) by adding 600 μl of a 0.6-mg/ml solution of rAfaE-III or rAfaD, or 500 μl of 1% BSA, to 10 ml of the colloidal gold.

Microscopic examination. For scanning electron microscopy, glass coverslips of infected HeLa cells were processed as previously described (28) and examined with a Jeol JSM 35CF electron microscope.

Alternatively, infected monolayers were fixed in tissue culture dishes with 1.6% glutaraldehyde (Merck) in 0.1 M phosphate buffer (pH 7.4), postfixed in 1% osmium tetroxide in same buffer, and dehydrated through a series of ascending concentrated ethanol washes. The cell monolayers were pelleted as described by Arnold and Boor (2) by addition of butyl-2,3-epoxy-propyl ether. The cells which floated free from the plastic surface were transferred to an Eppendorf tube, centrifuged, washed with epoxy-1,2-propane, and then embedded in epoxy resin. Ultrathin sections (80 nm) were cut with a diamond knife in a Reichert Ultracut S microtome, placed onto 200-mesh copper grids, stained with uranyl acetate and lead citrate, and examined with a Jeol 1010 transmission electron microscope operating at 80 kV. Semithin sections (2 μm) were stained with toluidine blue and azur II in borax buffer and examined under a light microscope.

RESULTS

Electron microscopy study of *afa*-expressing *E. coli* strains interacting with HeLa cells. The recombinant plasmid pILL1101 carries the *afa-3* gene cluster from isolate A30; it codes for an afimbrial adhesive sheath composed of AfaD and AfaE-III (15). Scanning electron microscopy of cultured HeLa cells incubated for 3 h with HB101(pILL1101) showed classical diffuse adherence (25) (data not shown). By transmission electron microscopy (TEM), adherent bacteria were observed surrounded by microvillar extensions of the cellular membrane without other major modifications of the cellular surface. In some cases, bacteria were totally enwrapped by elongated microvilli (Fig. 2A). These observations were similar to those for isolate A30 infecting HeLa cells (data not shown).

TEM revealed that some of the HB101(pILL1101) bacteria were intracellular (Fig. 2A). Internalized bacteria were inside membrane-bound vesicles, some of which were close to the nucleus (Fig. 2A). Cellular material assumed to be remnants of microvillar extensions of the HeLa cell was visible in some vacuoles containing the bacteria (Fig. 2C). After 3 and 6 h of infection or after a prolonged incubation (15 h) of infected HeLa cells in fresh tissue culture medium, no more than one bacterium per vacuole was found, and no bacteria were free within the cytoplasm of the epithelial cells. Presumably, therefore, the bacteria did not multiply within the cells.

The recombinant plasmid pILL1168 carries a mutant *afaD* gene, and consequently, the HB101(pILL1168) strain produces an afimbrial adhesive sheath composed of only the AfaE-III protein. By Western blotting analysis of equivalent amounts of whole-cell extracts, the levels of AfaE-III produced by HB101(pILL1168) and HB101(pILL1101) were previously shown to be similar (15). By TEM, this AfaD-negative mutant showed an pattern of adherence to HeLa cells that was similar to that of HB101(pILL1101), i.e., a tight adherence to the cell and the induction of microvillar extensions of the cell surface that enwrapped the bacteria. However, no bacteria of strain HB101(pILL1168) were found inside HeLa cells (Fig. 2B), even after a 6-h incubation.

Finally, HeLa cells infected with the HB101(pILL1169) strain, which produces AfaD but not AfaE-III, were examined by TEM. This AfaE-deficient mutant did not adhere to or penetrate cultured HeLa cells (data not shown).

Adherence and invasion assays. (i) Determining parameters for adherence assays. The time course of adhesion of the AfaE-III- and AfaD-producing strain HB101(pILL1101) to HeLa cell monolayers was studied. An adhesion index (the mean number of bacteria per cell) was estimated at every 15 min for 3 h of incubation. Adherence increased regularly to a maximum of approximately 90 after 3 h. Moreover, at this

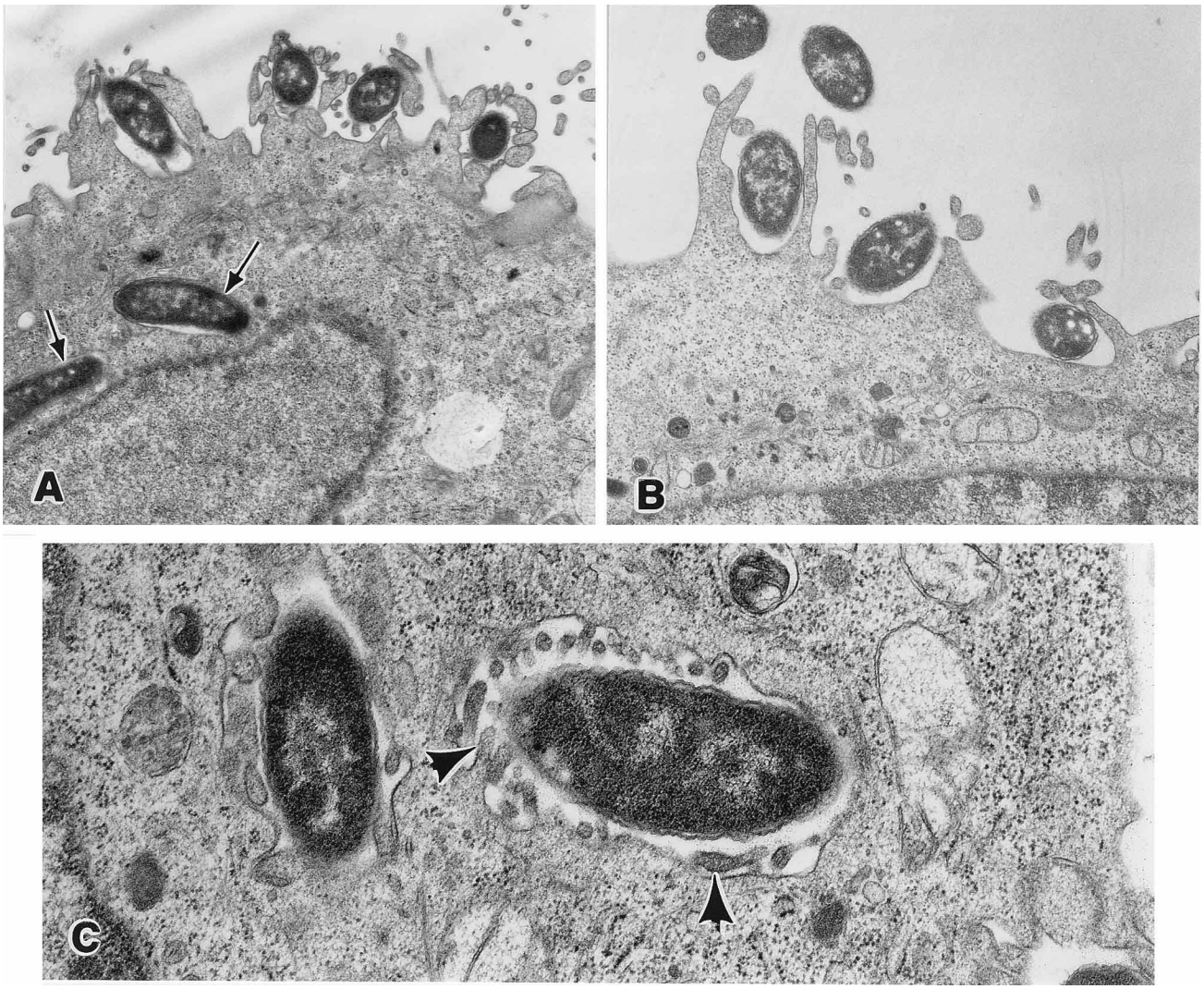

FIG. 2. Transmission electron micrographs of *afa-3*-expressing strains interacting with HeLa cells 3 h postinfection. (A and C) HB101(pILL1101), producing both AfaD and AfaE-III. Bacteria were embedded in microvillar extensions of the HeLa cell. Intracellular bacteria (arrows) were observed near the nucleus in membrane-bound vacuoles. Remains of microvillar extensions (arrowheads) were frequently visualized within the vacuoles containing bacteria. (B) HB101(pILL1168), producing AfaE-III but not AfaD. No intracellular bacteria were visualized. Magnifications, ×10,000 (A), ×12,000 (B), and ×30,000 (C).

incubation time all of the eukaryotic cells were associated with bacteria. A 3-h incubation time was used for subsequent cell infection experiments.

(ii) The *afa-3* gene cluster mediates adherence to and invasion of HeLa cells. HB101(pILL1101) efficiently adhered to (50.5% of the bacteria were associated with the cells at 3 h of infection) and invaded (1.1% of total bacteria) the HeLa cells, whereas HB101(pILL570) did not adhere to or invade these cells (Table 2). Two percent of cell-associated HB101(pILL1101) bacteria were thus internalized.

(iii) Roles of AfaD and AfaE. As observed by TEM, the AfaE-negative HB101 mutant carrying pILL1169 neither associated with nor invaded the epithelial cells (Table 2). Thus, AfaE-III is needed for adherence, and although AfaE-III is not needed for invasion, the bacteria must adhere before invasion can occur.

The association of the AfaD-deficient HB101(pILL1168) to the HeLa cells was similar to that of the AfaE-III- and AfaD-producing HB101 strain. However, the relative level of invasion of HB101(pILL1168) was significantly lower than that of HB101(pILL1101) (Table 2). Therefore, AfaD is necessary for internalization of bacteria into HeLa cells. Although low, the level of invasion of HB101(pILL1168) was not zero, and this invasion was independent of the gentamicin concentration (Table 2). Examination by TEM of HeLa cells infected with HB101(pILL1168) after gentamicin treatment showed the presence of some bacteria enwrapped in elongated microvilli but no intracellular bacteria. This indicates that extracellular adherent HB101 bacteria surrounded by membrane extensions were protected from the gentamicin.

(iv) Complementation experiments. To test for complementation of the deficiency for invasion of the AfaD-negative HB101 mutant, HeLa cell monolayers were coinfected with both HB101(pILL1189), which produces only AfaE-III, and HB101(pILL1169), which produces only AfaD (Table 2). Coinfection did not enhance adherence of HB101(pILL1169) or invasion of HB101(pILL1189). In contrast, when plasmids pILL1189 and pILL1169 were introduced into the same bacterium, complementation occurred: the recombinant HB101 (pILL1189, pILL1169) showed levels of adherence and inva-

TABLE 2. Adherence to and invasion of HeLa cells by *E. coli* HB101 producing AfaD and/or AfaE-III

Strain	Production[a] of:		% Association[b] (mean ± SD)	% Relative association[c]	% Invasion[d] (mean ± SD)	% Relative invasion[e]
	AfaD	AfaE				
HB101(pILL1101)	+	+[f]	50.5 ± 6.1	100	1.10 ± 0.15	100
HB101(pILL1168)	−	+[f]	45.3 ± 3.6	89.7	0.20 ± 0.02	18.1
HB101(pILL1169)	+	−	0.5 ± 0.3	1.0	<0.005	<0.5
HB101(pILL570)	−	−	1.2 ± 0.7	2.3	<0.005	<0.5
HB101(pILL1189, pILL1169)	+	+	54.2 ± 4.5	107	2.47 ± 0.26	224
HB101(pILL1189)[g]	−	+	38.9 ± 8.8	77.1	0.27 ± 0.06	24.5
HB101(pILL1169)[g]	+	−	3.1 ± 1.0	6.0	<0.005	<0.5

[a] The production of AfaD or AfaE was confirmed by Western blotting.
[b] Bacteria associated with washed monolayers as a percentage of total bacteria at 3 h postinfection.
[c] Association relative to that of *E. coli* HB101(pILL1101), which was defined as 100%.
[d] Bacteria resisting treatment with gentamicin as a percentage of total bacteria at 3 h postinfection.
[e] Invasion relative to that of *E. coli* HB101(pILL1101), which was defined as 100%.
[f] The levels of AfaE produced by HB101(pILL1101) and HB101(pILL1168) were estimated to be equivalent by Western blotting, as previously reported (15).
[g] Results are for coinfection with HB101(pILL1189) and HB101(pILL1169).

sion similar or even greater than those of HB101(pILL1101) (Table 2). Therefore, AfaD and AfaE-III must be produced by the same bacterium to promote internalization.

Microscopic studies of AfaD- or AfaE-coated beads interacting with HeLa cells. Carboxylated polystyrene beads were covalently coupled to either rAfaE-III (20 fg/bead), rAfaD (15 fg/bead), or BSA (12 fg/bead) and incubated for 3 h with HeLa cells (Fig. 3). The rAfaE-III beads, like the bacteria producing only AfaE-III, adhered to the HeLa cells by interacting with elongated microvilli and were never seen internalized (Fig. 3a). The rAfaD beads also interacted with the HeLa cells (Fig. 3b). However, in contrast to the bacteria producing AfaD but not AfaE-III, rAfaD beads were observed both interacting with the cell surface and internalized into the HeLa cells. Moreover, like internalized HB101(pILL1101) bacteria, the internalized beads were found in membrane-bound vacuoles (Fig. 3b, inset). Beads coated with BSA were used as a control and did not associate with HeLa cells (Fig. 3c). These data confirmed that AfaE-III and AfaD are involved in adhesion and internalization, respectively.

Carboxylated polystyrene beads covalently coupled to various amounts of rAfaD (0.04 to 1 fg of protein/bead) were also incubated for 3 h with HeLa cells. The percentage of cells associated with beads as well as the numbers of adherent and internalized beads were estimated by direct counting on semithin sections (2 μm) examined under a light microscope (Fig. 4). From these data, it appeared that the number of cells associated with beads increased proportionally with the amount of rAfaD bound to the beads (Fig. 4A). Moreover, both the number of beads associated with cells and the fraction of internalized beads also increased with the amount of rAfaD per bead (Fig. 4B). The variation in the amount of rAfaD from 0.4 to 1 fg per bead promoted the highest increase (more than threefold) in the number of associated beads per HeLa cell.

Locations of the AfaE and AfaD proteins during the interaction between bacteria and epithelial cells. (i) Immunolocalization of AfaE-III and AfaD on bacteria. It was previously demonstrated by immunogold staining of whole-mount bacteria that AfaE-III and AfaD are both exposed on the bacterial surface (15). The medium and cell pellet of broth cultures of strain HB101(pILL1101) were analyzed by SDS-PAGE and Western blotting with anti-rAfaE-III (Fig. 5A) and anti-rAfaD (Fig. 5B) sera. AfaE-III was mostly associated with the bacterial pellet (Fig. 5A), whereas AfaD protein was detected in the culture supernatant (Fig. 5B), suggesting that it was released from the bacteria.

The concentration of AfaD in the supernatant of a broth culture of strain HB101(pILL1101) was estimated on the basis of the relative intensities of the signal produced on immunoblots by serial dilutions of the extract and of the purified rAfaD protein. Approximately, 60 to 120 ng of AfaD was estimated to be present in 1 ml of medium of a broth culture containing 10^8 bacteria. Since no AfaD was detected in a bacterial pellet corresponding to 10^8 bacteria, it was possible to extrapolate that less than 1 fg of AfaD was present at the surface of each bacterium.

(ii) Electron microscopic study of AfaD- or AfaE-gold complexes interacting with HeLa cells. To study the mechanism by which AfaD penetrates, HeLa cells were incubated for 6 h with either rAfaE-III–, rAfaD–, or BSA-colloidal gold preparations and examined by TEM (Fig. 6). rAfaE-III–gold complexes were found only at the outer surface of the plasma membrane; the cytosol was devoid of particles (Fig. 6a). At higher magnification, the AfaE-III–gold particles could be observed scattered on the microvillar extensions of the HeLa cells (Fig. 6b). rAfaD-gold complexes were detected both at the cell surface and in the cytosol (Fig. 6c and d). rAfaD-gold complexes were observed associated with invaginations (coated pits) of the cell surface pinching off from the plasma membrane (Fig. 6c) and with intracellular clathrin vesicles (Fig. 6d). No BSA-gold particles were observed associated with the HeLa cells (data not shown).

DISCUSSION

The *afa-3* gene cluster determines the formation of an afimbrial adhesive sheath that is produced by both uropathogenic and diarrhea-associated *E. coli* strains. This adhesive sheath is composed of two proteins, AfaD and AfaE-III, and analysis of AfaD- or AfaE-III-negative mutants indicated that the biogenesis of one protein did not require production of the other one (15). We examined ultrastructurally HeLa cells incubated with laboratory strains producing AfaD and/or AfaE-III and with beads coated with one of the two adhesins. We demonstrated that the bacterium-cell interaction involves two steps in which AfaE-III is required for adhesion and AfaD contributes to internalization.

We showed that the adhesion mediated by AfaE-III was

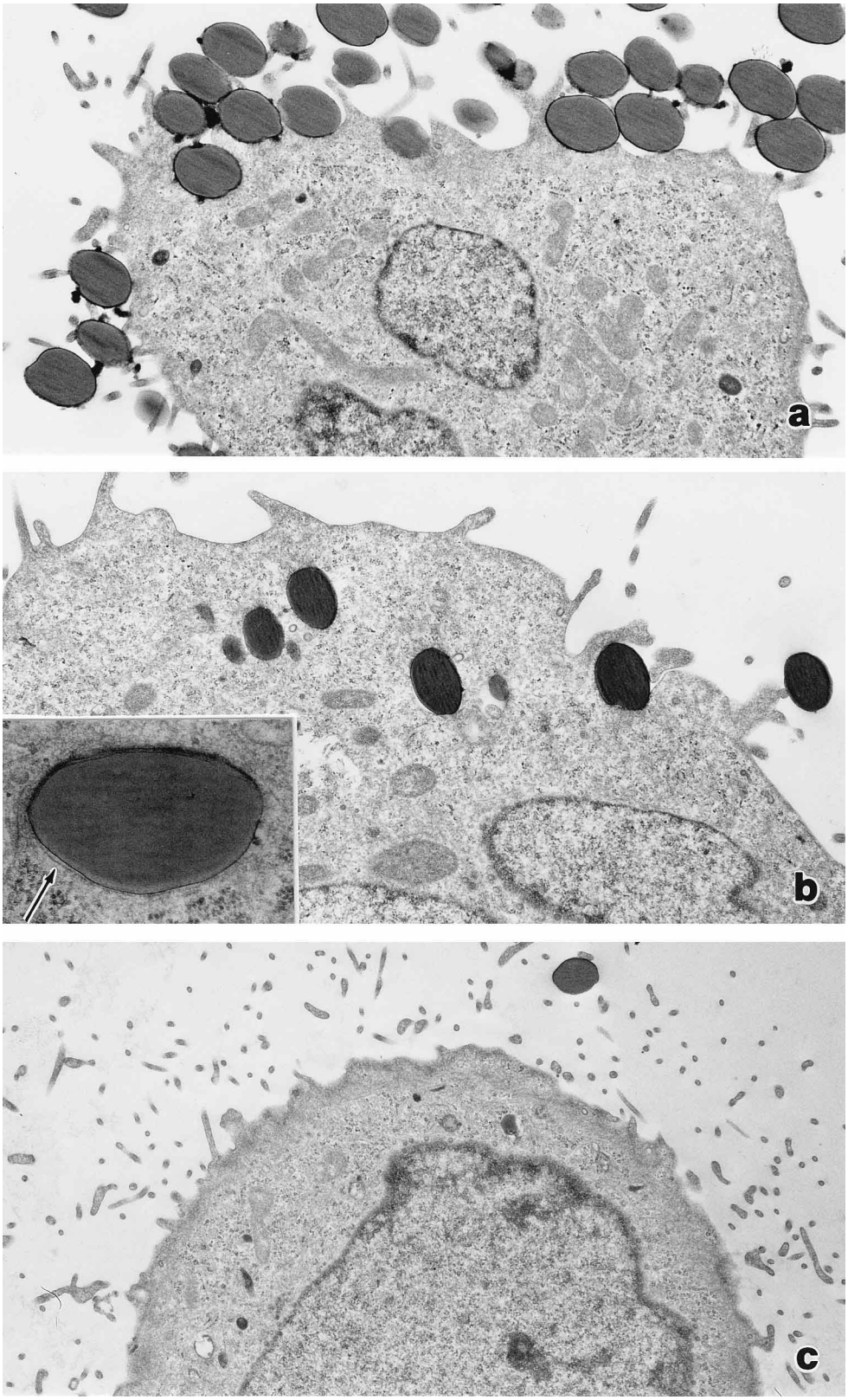

FIG. 3. Transmission electron micrographs of HeLa cells incubated for 3 h with polystyrene beads coated with either rAfaE-III, rAfaD, or BSA. (a) rAfaE-III-coated beads attached to the HeLa cell surface but were not internalized into the cells. (b) rAfaD-coated beads were observed principally inside the HeLa cells within membrane-bounded vacuoles, as indicated by an arrow in the inset. (c) BSA-coated beads did not interact with the HeLa cells. Magnifications, ×10,000 (a and b), ×40,000 (inset), and ×8,000 (c).

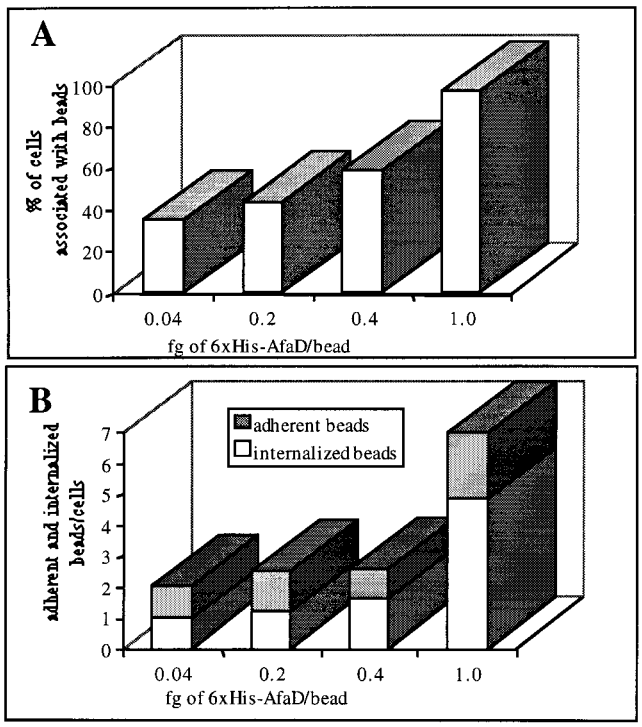

FIG. 4. Association of HeLa cells with beads coated with various amounts of rAfaD. HeLa cells were incubated with beads each coated with either 0.04, 0.2, 0.4, or 1 fg of rAfaD and embedded in epoxy resin. One hundred cells on semithin sections were examined under a light microscope. (A) Effect of the amount of rAfaD per bead on the percentage of cells associated with beads. (B) Effect of the amount of rAfaD per bead on the numbers of adherent and internalized beads per cell. For the experiment with beads coated with 1 fg of rAfaD, the counting was performed for only 67 of the 97 cells associated with beads. For the 30 other cells associated with beads, the numbers of adherent and internalized beads could not be estimated because beads were aggregated.

the initial step of the bacterium-cell interaction; an AfaD-negative mutant exhibited a diffuse adherence pattern similar to that displayed by *E. coli* strains [wild-type A30 and HB101(pILL1101)] producing both AfaD and AfaE-III, and an AfaE-negative mutant failed to associate with epithelial cells. The intimate adherence of bacteria promoted by AfaE-III is associated with the elongation of the microvillar extensions of HeLa cells, leading to total enwrapment of adherent bacteria. Consistent with earlier reports (10, 14), no evidence for AfaE-III-mediated perturbation of actin polymerization was found (data not shown). Comparisons of HeLa cells incubated with either rAfaE-III-, rAfaD-, or BSA-coated beads indicated that the microvillus elongation might be triggered by the AfaE-III protein. Thus, the induction or lack of induction of microvillus elongation may be correlated to the known heterogeneity of the *afaE* genes (22, 25, 30). The AfaE-III adhesin recognizes as a receptor DAF, a membrane-associated glycoprotein which was reported to be able to transduce signals (29, 32). The nature of the signal(s) transmitted by the AfaE-III-linked DAF is unknown.

Strains producing an AfaE-III adhesin were previously described as noninvasive because they do not cause keratoconjunctivitis in guinea pigs (Sereny test), a property associated with typical invasive enterobacteria such as *Shigella* spp. or enteroinvasive *E. coli* (25). We established by both electron microscopy examination and gentamicin protection assays that *E. coli* carrying the *afa-3* gene cluster can invade HeLa cells. Unlike true intracellular pathogens, but similarly to enteropathogenic *E. coli* (EPEC), *afa-3*-expressing strains do not appear to multiply intracellularly or escape from endocytic vacuoles (13). Interestingly, the morphology of the vacuoles was unusual. They included material that possibly was remnants of microvilli internalized with the bacteria. Moreover, like the EPEC strains, the number of *afa-3*-expressing bacteria entering each HeLa cell was low; a small proportion of the adherent HB101(pILL1101) (2 to 3%) were found to be internalized, and only when the AfaD protein was produced. However, there is no similarity between the genetic determinants responsible for the invasion process of EPEC and those of *afa-3*-expressing strains.

We found that AfaD, previously described as an adhesin, was required for the entry of bacteria into HeLa cells. The Inv, Ail, and YadA proteins of *Yersinia* spp. similarly have both properties (11), and thus AfaD may be an invasin. Our results demonstrated that the AfaD concentration is important for the invasion process. Even though beads could be internalized when coupled with approximately 0.04 fg of rAfaD, they were more efficiently internalized when coated with 1 fg of protein. Immunolabelling of cell-associated bacteria confirmed that AfaD was exposed at the bacterial surface (data not shown). However, we also demonstrated that AfaD was able to detach from the bacterial surface, and we estimated that the average amount of AfaD at the surface of a bacterium grown in LB was less than 1 fg. All of these data might explain the low level of bacterial internalization observed in our experiments; i.e., only bacteria having at their surface the critical amount of AfaD necessary for internalization might enter the HeLa cells.

How AfaD mediates the entry of the bacteria into HeLa cells is not clearly understood. Gentamicin protection assays showed that expression of the *afaD* gene in *trans* complemented the defective entry phenotype of the AfaD-negative mutant, but neither the presence of the rAfaD protein in the tissue culture medium (data not shown) nor coinfection with an AfaE-negative mutant producing AfaD allowed entry of the AfaD-negative mutant into cells. These observations suggest that only the AfaD molecules associated with the bacterial surface are implicated in the invasion process and confirm that the local concentration of AfaD at the interaction site is critical for the bacterial internalization. Additionally, rAfaD-gold complexes were shown to enter HeLa cells via clathrin vesicles, suggesting that the AfaD protein enters the HeLa cells by

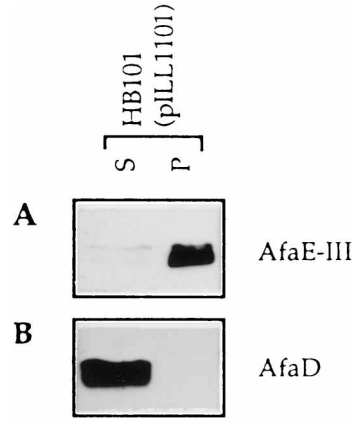

FIG. 5. Western blot analysis of HB101(pILL1101) broth cultures with antisera against rAfaE-III and rAfaD. Supernatant proteins (S) and proteins from the bacterial pellet (P) were visualized by consecutive immunoblotting with antibodies directed against rAfaE-III (A) and rAfaD (B) diluted 1:100,000 and 1:10,000, respectively.

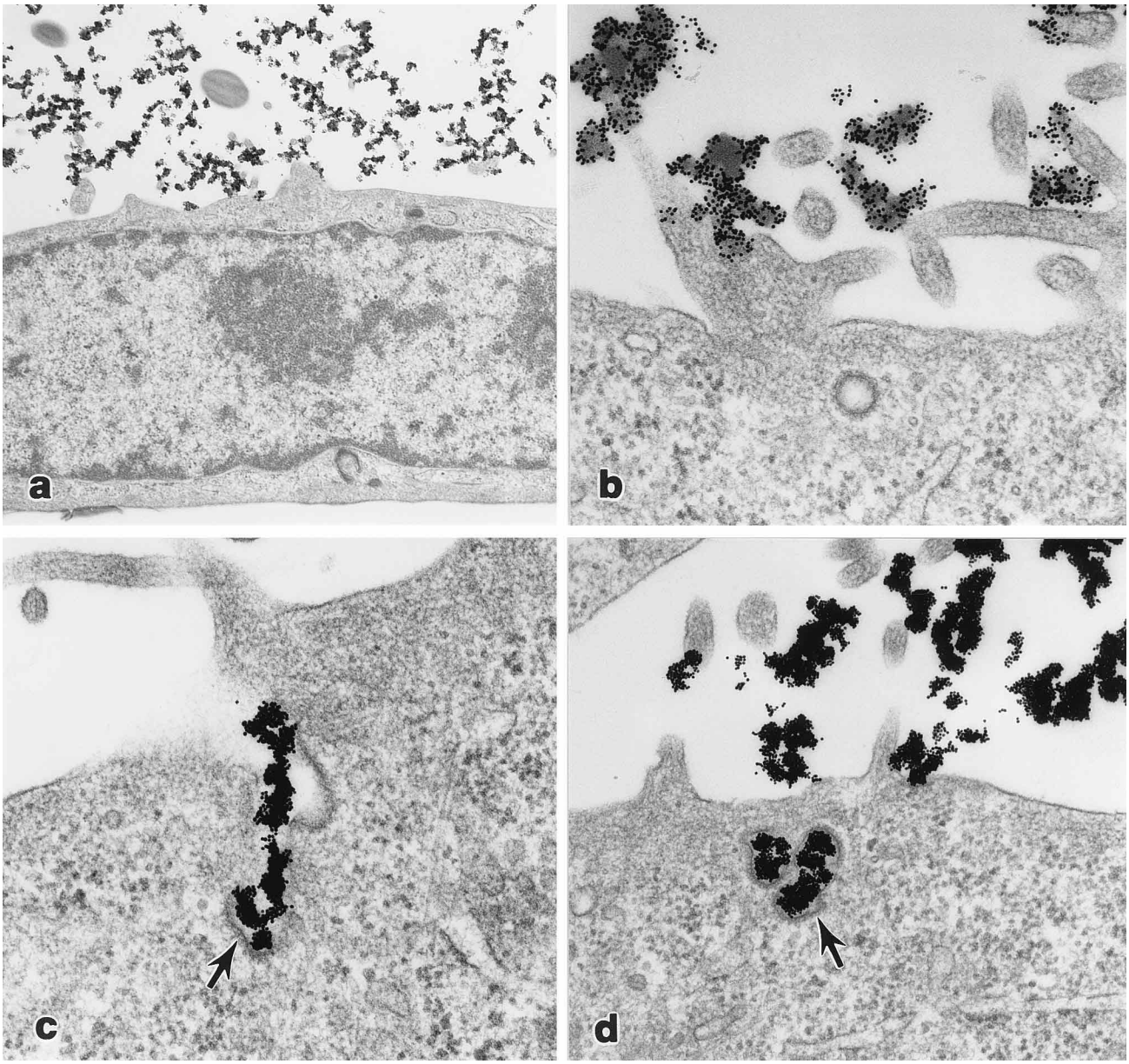

FIG. 6. Transmission electron micrographs of HeLa cells incubated for 6 h with protein-gold complexes. (a and b) rAfaE-III–gold complexes adhered to but did not enter the HeLa cells. (c and d) rAfaD-gold complexes were localized in clathrin-coated pits formed at the plasma membrane (arrows indicate clathrin). Magnifications, ×12,000 (a) and ×60,000 (b, c, and d).

receptor-mediated endocytosis. Whether the bacteria are internalized by the same pathway remains to be clarified.

Since the AfaE-negative mutant was unable to enter HeLa cells, the adhesion of the bacteria to the cell appears to be a requirement for the internalization process. Introduction in *trans* in the AfaE-negative mutant (producing the AfaD protein) of determinants encoding the AIDA adhesin, which mediates a diffuse adherence pattern on HeLa cells (4), did not allow internalization of the bacteria (data not shown). These data indicate that bacterial internalization may be dependent on an adhesion step mediated specifically by the AfaE adhesin.

Strains carrying an *afa* gene cluster are associated with diarrhea and urinary tract infections (17, 24, 25). The role, if any, of invasion of epithelial cells in the pathogenesis of these diseases remains to be established. Possibly epithelial cell penetration provides a protected niche for the organism such that it can survive and persist within the host.

In conclusion, the *afa* gene cluster is unusual in that a single operon encodes both adhesion of the bacteria to the epithelial cell and invasion of these cells. Consequently, the *afa* gene clusters are good models for studying bacterium-cell interaction mechanisms. Analyses of signals transduced to the epithelial cells mediated either by AfaE-DAF interactions or by penetration into cells promoted by AfaD should allow better understanding of the pathogenicity of strains producing an afimbrial adhesive sheath.

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