INFECTION AND IMMUNITY, May 1997, p. 1644–1652 Vol. 65, No. 5
0019-9567/97/$04.00+0

Down Regulation of Intimin Expression during Attaching and Effacing Enteropathogenic *Escherichia coli* Adhesion

STUART KNUTTON,[1]* JEANNETTE ADU-BOBIE,[2] CHRISTOPHER BAIN,[1] ALAN D. PHILLIPS,[3] GORDON DOUGAN,[2] AND GAD FRANKEL[2]

Institute of Child Health, University of Birmingham, Birmingham B16 8ET,[1] Department of Biochemistry, Imperial College of Science, Technology, and Medicine, London SW7 2AZ,[2] and University Department of Paediatric Gastroenterology, Royal Free Hospital, London NW3 2QG,[3] United Kingdom

Received 27 November 1996/Returned for modification 31 December 1996/Accepted 7 February 1997

Enteropathogenic *Escherichia coli* (EPEC) produces attaching and effacing (A/E) lesions in the intestinal mucosa. The intimate bacterial adhesion associated with A/E lesion formation is promoted by intimin, a 94-kDa EPEC surface protein. Anti-intimin antisera raised in rabbits by using the purified 280-amino-acid cell binding domain of intimin as the immunogen were employed in immunofluorescence and immunoelectron microscopical studies to investigate the expression of intimin by classical EPEC strain E2348/69 (O127:H6) and defined E2348/69 derivatives during culture growth and A/E bacterium adhesion to cultured HEp-2 cells. In stationary-phase broth cultures, only a small fraction of E2348/69 bacteria expressed intimin, and of those that did, immunolabelling revealed a uniform distribution of intimin over the bacterial surface; increased numbers of bacteria expressing intimin were detected when E2348/69 was grown in tissue culture medium, an effect not seen with strain JPN15, a virulence plasmid-cured derivative of E2348/69. Strain CVD206, an *eaeA* mutant of E2348/69, did not stain with the anti-intimin antisera, but strain CVD206(pCVD438), containing a functional *eaeA* gene, stained uniformly. After a 3-h incubation of HEp-2 cells with strain E2348/69, double immunofluorescence labelling of intimin and cellular actin revealed strong intimin expression by all A/E bacteria, but after 6 h of incubation, intimin expression by most E2348/69 bacteria was greatly reduced or not detected. This effect on intimin expression was not observed with strain JPN15 but was restored for strain JPN15(pCVD450) harboring the virulence plasmid-encoded *per* genes. These results indicate that surface expression of intimin is regulated by environmental factors during bacterial growth and following A/E lesion formation and that virulence plasmid-encoded genes participate in these regulation processes.

Enteropathogenic *Escherichia coli* (EPEC) remains an important cause of severe infantile diarrheal disease in many parts of the developing world (25). EPEC colonizes the intestinal mucosa and produces attaching and effacing (A/E) lesions characterized by localized destruction of brush border microvilli, intimate bacterial adhesion, and formation of a complex cytoskeletal structure beneath intimately attached bacteria (24, 33). Accumulation of polymerized actin beneath bacteria results in the formation of cuplike pedestal structures (22). EPEC produces similar A/E lesions in a variety of tissue culture cell lines (21). All the determinants of A/E lesion formation thus far identified have been localized to a large (35-kbp) region of the EPEC chromosome termed LEE (locus of enterocyte effacement) (28). Several loci within the LEE, including *sep*, *espA*, *espB* (formerly *eaeB* [7]), and *eaeA*, have been characterized. The *sep* genes encode a type III secretion system (14), *espA* and *espB* encode secreted proteins (13, 18) involved in signal transduction to host cells (8, 19), and *eaeA* encodes a 94-kDa outer membrane protein, intimin, required for intimate bacterial adhesion and accumulation of cytoskeletal actin (5, 15); *eaeA* deletion mutants are unable to form intimate attachment or produce A/E lesions in cultured cells (4) and show reduced levels of virulence in experimental human and animal models of EPEC infection (5, 6). EPEC plasmid-encoded regulatory (*per*) genes, which activate intimin expression, have been described (12).

eaeA homologs have been found in all A/E bacteria, including human enterohemorrhagic *E. coli*, animal EPEC and enterohemorrhagic *E. coli*, and strains of *Hafnia alvei* and *Citrobacter rodentium* (formerly *Citrobacter freundii* biotype 4280) (28). Intimins show sequence homology with the invasin proteins of *Yersinia* species (16) and consist of a highly conserved N-terminal domain and a much less conserved C-terminal domain (9); intimin binding activity to eukaryotic cells resides within the 280 amino acids at the C terminus (10).

We have prepared polyclonal antisera to the 280-amino-acid C-terminal domain of intimin of an EPEC O127:H6 isolate, and in this paper we report immunofluorescence and immunoelectron microscopical studies of intimin expression by EPEC strain E2348/69 (O127:H6) and defined E2348/69 mutants during bacterial growth and during A/E bacterium adhesion to cultured HEp-2 cells.

MATERIALS AND METHODS

Bacterial strains and plasmids. The EPEC strains and plasmids used in the study are listed in Table 1. Stock cultures of the strains were subcultured in Mueller-Hinton or Luria broth and incubated aerobically for 18 h at 37°C.

Preparation of polyclonal antiserum. The DNA fragment encoding the 280-amino-acid cell binding domain of intimin was purified from the pMal c2 vector (9) after *Eco*RI/*Hin*dIII digestion and subcloned into pET28a (Novagen Biotechnology). Recombinant plasmids were transformed into *E. coli* TG1, and the His-tagged intimin domain was purified according to the manufacturer's instructions. Anti-intimin antiserum was prepared in half lop rabbits by subcutaneous immunization with 50 μg of purified intimin antigen in complete Freund's adjuvant. The rabbits received booster doses on two occasions 3 weeks apart before exsanguination. Detailed preparation and characterization of this antiserum will

* Corresponding author. Mailing address: Institute of Child Health, University of Birmingham, Francis Road, Birmingham B16 8ET, United Kingdom. Phone: 44 121 450 6026. Fax: 44 121 454 5383. E-mail: S.KNUTTON@bham.ac.uk.

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description	Reference
E2348/69	EPEC (O127:H6) isolated from an outbreak in Taunton, United Kingdom	26
JPN15	Spontaneous plasmid-cured derivative of E2348/69	16
CVD206	*eaeA* (intimin) gene deletion mutant of E2348/69	4
pCVD438	pACYC 184 vector harboring *eaeA* from E2348/69	4
pCVD450	pACYC 184 vector harboring *per* genes from E2348/69	12

be described elsewhere (1). Antibody to bundle-forming pili (BFP) was generously provided by Jorge Giron. The antiserum was prepared as previously described (11) by inoculating rabbits with purified BFP from EPEC strain B171 (O111:NM) and then refined by absorption with strain B171 grown under conditions unfavorable for BFP expression.

Immunoblotting. Derivatives of EPEC strain E2348/69 were grown overnight in L broth (~10^9 CFU/ml), diluted 1:100 in Dulbecco's modified Eagle's medium (DMEM), and grown with shaking at 37°C for an additional 3 h. The optical densities at 600 nm of the various bacterial cultures were adjusted to yield equal absorbancies, and an equivalent of an optical density of 0.1 was taken for analysis. Following electrophoresis on sodium dodecyl sulfate–7.5% polyacrylamide gels, the whole-cell lysates were transferred to nitrocellulose membranes (0.45-μm pore size; Schleicher and Schuell) according to the method of Towbin et al. (32). Membranes were blocked overnight with 3% bovine serum albumin (BSA) in phosphate-buffered saline (PBS), pH 7.2, washed with PBS containing 0.05% Tween 20 (PBST), and then probed with anti-intimin antiserum, diluted 1:750 in 0.1% BSA-PBST, for 2 h at room temperature. Following three washes with PBST, the membranes were incubated with a 1:1,000 dilution of horseradish peroxidase-conjugated anti-rabbit immunoglobulin (DAKO), and the reaction was visualized with 3,3′-diaminobenzidine (Sigma).

HEp-2 cell adherence. Adhesion to HEp-2 cells was carried out according to the method of Cravioto et al. (2). Subconfluent cell cultures on glass coverslips or petri dishes were washed and incubated with bacteria (10 μl of bacterial broth culture/ml of tissue culture medium [DMEM–5% fetal calf serum]) for 3 and 6 h at 37°C; in 6-h assays the medium was replaced with fresh medium after 3 h. After being thoroughly washed to remove nonadhering bacteria, coverslips were fixed in 4% formalin and petri dishes were fixed in 0.1% glutaraldehyde.

Immunofluorescence. All antibody dilutions and immune reactions were carried out in PBS-BSA. Concentrated suspensions of washed bacteria or washed HEp-2 cell monolayers were incubated with suitable dilutions of the intimin or BFP antiserum (1:40) in PBS-BSA for 45 min at room temperature. After three 5-min washes in PBS, samples were stained with fluorescein isothiocyanate-conjugated goat anti-rabbit immunoglobulin G (Sigma) diluted 1:20 in PBS-BSA for 45 min. HEp-2 cell preparations were also labelled for cellular actin by simultaneously staining coverslips with a 5-μg/ml solution of tetramethyl rhodamine isothiocyanate-phalloidin (Sigma). Preparations were washed a further three times in PBS and mounted in glycerol-PBS. Specimens were examined by incident-light fluorescence with a Leitz Dialux microscope, and fluorescence and phase-contrast images of the same field were recorded.

Electron microscopy. For immunolabelling of bacteria, 10-μl samples of washed bacterial suspensions were applied to carbon-coated grids for 5 min, excess liquid was removed, and the grids were immediately placed facedown on drops of intimin antiserum (1:40 dilution) for 30 min. After being washed, grids were placed for 30 min on drops of goat anti-rabbit sera (1:20 dilution) (British BioCell International) labelled with 10-nm-diameter gold beads. After a further wash in PBS and distilled water, grids were air dried.

For immunogold labelling of cell-associated bacteria, HEp-2 cell monolayers were briefly fixed for 10 min in 0.1% glutaraldehyde, washed, and incubated with intimin antiserum for 2 h at room temperature. Cells were washed and incubated with gold-labelled goat anti-rabbit serum for 12 h at 4°C. After a further thorough wash, cells were fixed in 3% buffered glutaraldehyde and processed for thin-section electron microscopy by standard procedures (20). Samples were examined in a JEOL 1200EX electron microscope operated at 80 kV.

RESULTS

Intimin expression during bacterial growth. Western blot analysis of the EPEC strains probed with the anti-intimin polyclonal antiserum revealed that intimin expression was dependent on both the growth medium and the phase of growth. Intimin expression in strain E2348/69 was low in stationary-phase cultures but was induced during logarithmic-phase growth; intimin expression was considerably greater when bacteria were grown in DMEM tissue culture medium than in L broth. Interestingly, intimin expression by the plasmid-cured derivative JPN15 appeared higher than that of the wild type, E2348/69, in stationary-phase cultures, but unlike intimin expression by strain E2348/69, which was significantly enhanced during the logarithmic phase of growth, intimin expression in JPN15 appeared to be unchanged. The anti-intimin antiserum did not react with the *eaeA* gene deletion mutant strain CVD206, whereas strain CVD206(pCVD438) (CVD206 expressing intimin encoded by a cloned *eaeA* gene) behaved like the parent strain, although intimin expression appeared to be less efficient (Fig. 1).

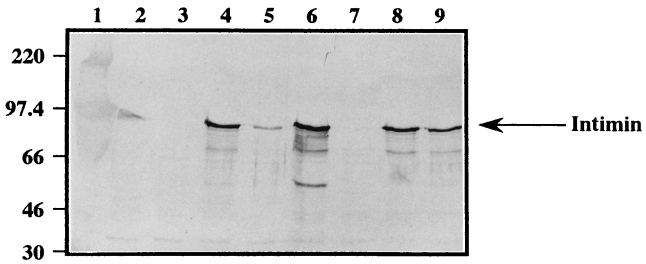

FIG. 1. Western blot of whole-cell extracts probed with the anti-intimin polyclonal antiserum. Low-level intimin expression was detected in stationary-phase L broth cultures of E2348/69 and CVD206(pCVD438) (lanes 2 and 5, respectively), while a higher intimin expression level was detected in the plasmid-cured derivative (JPN15) under the same experimental conditions (lane 4). Intimin expression was induced during logarithmic growth in DMEM in both E2348/69 and CVD206(pCVD438) (lanes 6 and 9, respectively), although the former appeared to express the polypeptide more efficiently. In contrast, during logarithmic growth intimin expression in JPN15 appeared to be unchanged (lane 8). The anti-intimin antibody did not react with CVD206 grown in either L broth or DMEM (lanes 3 and 7, respectively). Molecular weight markers (lane 1) are given in thousands on the left.

Similar results were obtained when surface intimin expression was examined by immunofluorescence and immunoelectron microscopy. In stationary-phase broth culture, only a fraction of E2348/69 (Fig. 2a), JPN15, and CVD206(pCVD438) bacteria stained with the anti-intimin antisera, and for those that did, both immunofluorescence (Fig. 2) and immunogold

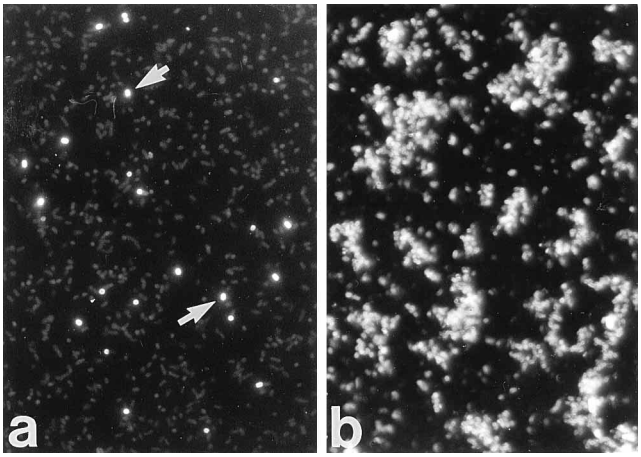

FIG. 2. Intimin fluorescence micrographs of strain E2348/69 grown overnight in broth culture (a) and after 3 h of growth in DMEM (b). In stationary-phase broth culture only a small percentage of bacteria expressed intimin (a [arrows]); unlabelled bacteria can also be seen. Bacterial aggregates produced following growth in DMEM all expressed intimin (b). Magnification, ×304.

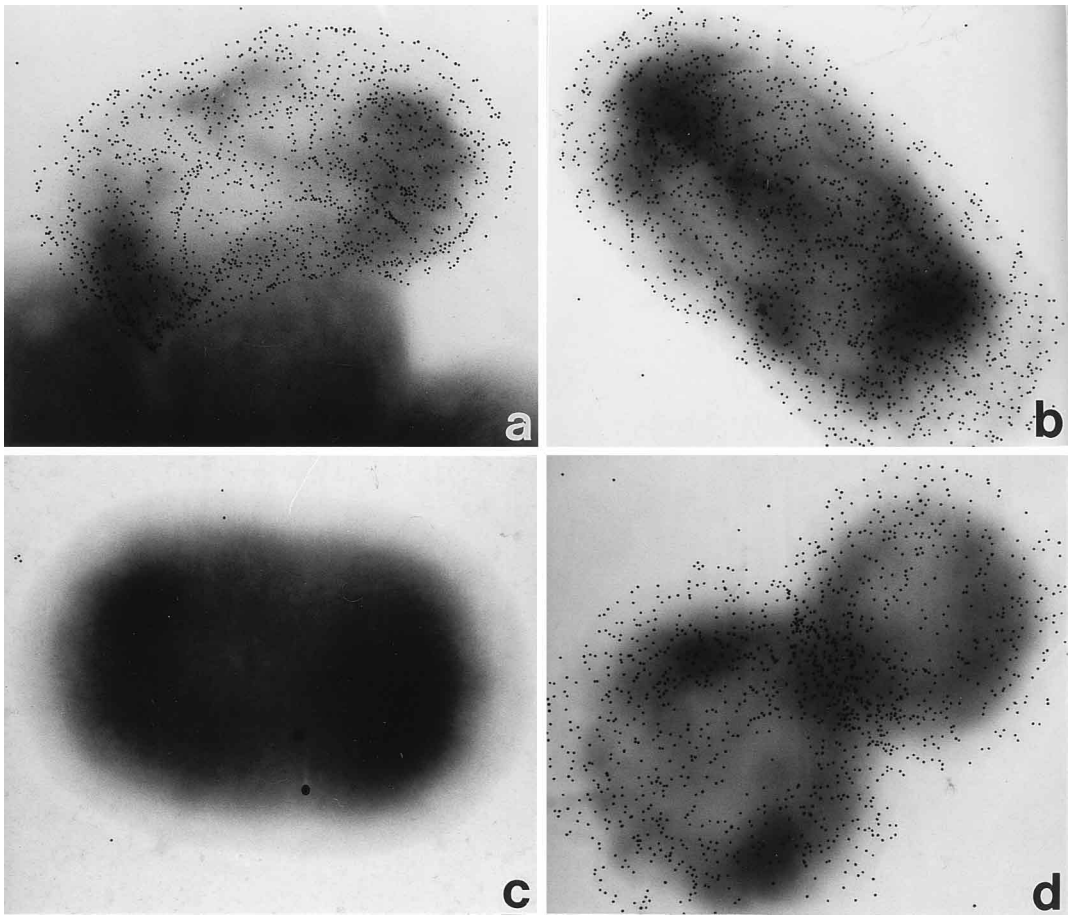

FIG. 3. Immunogold labelling of intimin. A uniform distribution of intimin over the bacterial surface was seen with strains E2348/69 (a), JPN15 (b), and CVD206(pCVD438) (d); strain CVD206 did not stain with the anti-intimin antiserum (c). Magnification, ×42,500.

labelling (Fig. 3) revealed a uniform distribution of intimin over the bacterial surface; strain CVD206 did not stain with the antibody. Based on the intensity of fluorescence, intimin expression by JPN15 appeared to be somewhat greater and that by CVD206(pCVD438) somewhat less than that of the parent strain, E2348/69, which is consistent with the Western blot data. This difference in fluorescence intensity between strains E2348/69, JPN15, and CVD206(pCVD438) is illustrated in Fig. 4a, d, and j, showing these strains adhering to HEp-2 cells (see below). Induction of intimin expression following the growth of E2348/69 in DMEM appeared to result predominantly from an increase in the numbers of bacteria expressing intimin, since there was a severalfold increase in the number of bacteria expressing intimin and there did not appear to be any significant increase in fluorescence intensity or density of gold labelling. Growth of E2348/69 in DMEM resulted in autoaggregation of some bacteria due to expression of BFP (11); all bacteria within these aggregates also expressed intimin (Fig. 2b). When JPN15 was grown in DMEM, no further induction of intimin expression by that strain was detected (data not shown).

Intimin expression during adhesion by A/E bacteria. E2348/69 adheres to cultured HEp-2 cells in localized microcolonies (the localized-adherence [LA] pattern) and produce A/E lesions which can be identified by intense spots of actin fluorescence that appear beneath intimately attached bacteria in the fluorescence actin staining (FAS) test (22). After a 3-h incubation of HEp-2 cells with EPEC strain E2348/69, bacteria adhered in localized microcolonies (Fig. 4c) and had a positive FAS test result (Fig. 4b), and all A/E bacteria expressed intimin (Fig. 4a). A similar pattern of adhesion and of actin and intimin fluorescence was seen with strains JPN15 (Fig. 4d through f) and CVD206(pCVD438) (Fig. 4j through l), although strain JPN15 adhered to HEp-2 cells in much smaller numbers than the parent strain. Strain CVD206 did not stain with the intimin antibody (Fig. 4g), although this strain did adhere to HEp-2 cells in localized colonies (Fig. 4i) and caused some localized, disorganized rearrangement of cellular actin, the so-called shadow FAS pattern (3) (Fig. 4h). Characteristic A/E lesions with bacteria intimately attached to pedestal-like structures were seen by transmission electron microscopy of HEp-2 cells infected with E2348/69 and JPN15. Immunogold labelling revealed intimin uniformly distributed over the bacterial surface, except at the site of intimate bacterial attachment to the HEp-2 cell surface, where there was no intimin labelling (see Fig. 6a and c).

After a 6-h incubation of HEp-2 cells and E2348/69 bacteria, the surfaces of the cells were covered with bacteria producing A/E lesions (Fig. 5b and c), but intimin staining was virtually absent (Fig. 5a) and only a small number of bacteria still showed positive staining with the antibody (Fig. 5a); the residual punctate fluorescence seen in Fig. 5a does not represent intimin staining but results from the very strong actin rhodamine fluorescence signal transmitted through the fluorescein

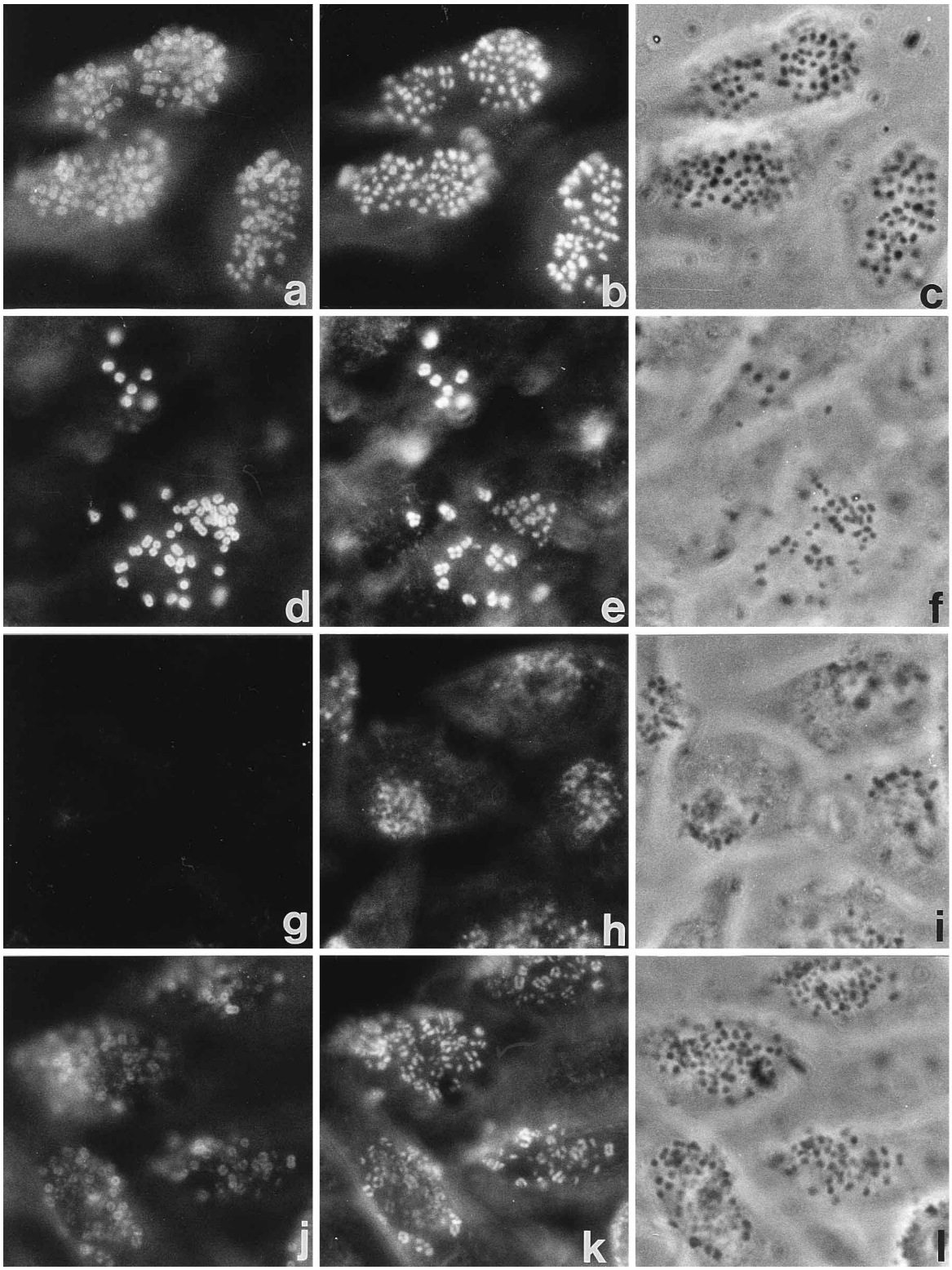

FIG. 4. Corresponding intimin fluorescence, actin fluorescence, and phase-contrast micrographs of HEp-2 cells incubated with EPEC strains E2348/69 (a through c), JPN15 (d through f), CVD206 (g through i), and CVD206(pCVD438) (j through l) at 37°C for 3 h. Strains E2348/69, JPN15, and CVD206(pCVD438) displayed an LA pattern (c, f, and l), had a positive FAS test result (b, e, and k), and expressed surface intimin; strain CVD206 showed LA (i) and a shadow pattern of actin accumulation (h) but did not stain with the anti-intimin antibody (g). Magnification, ×720.

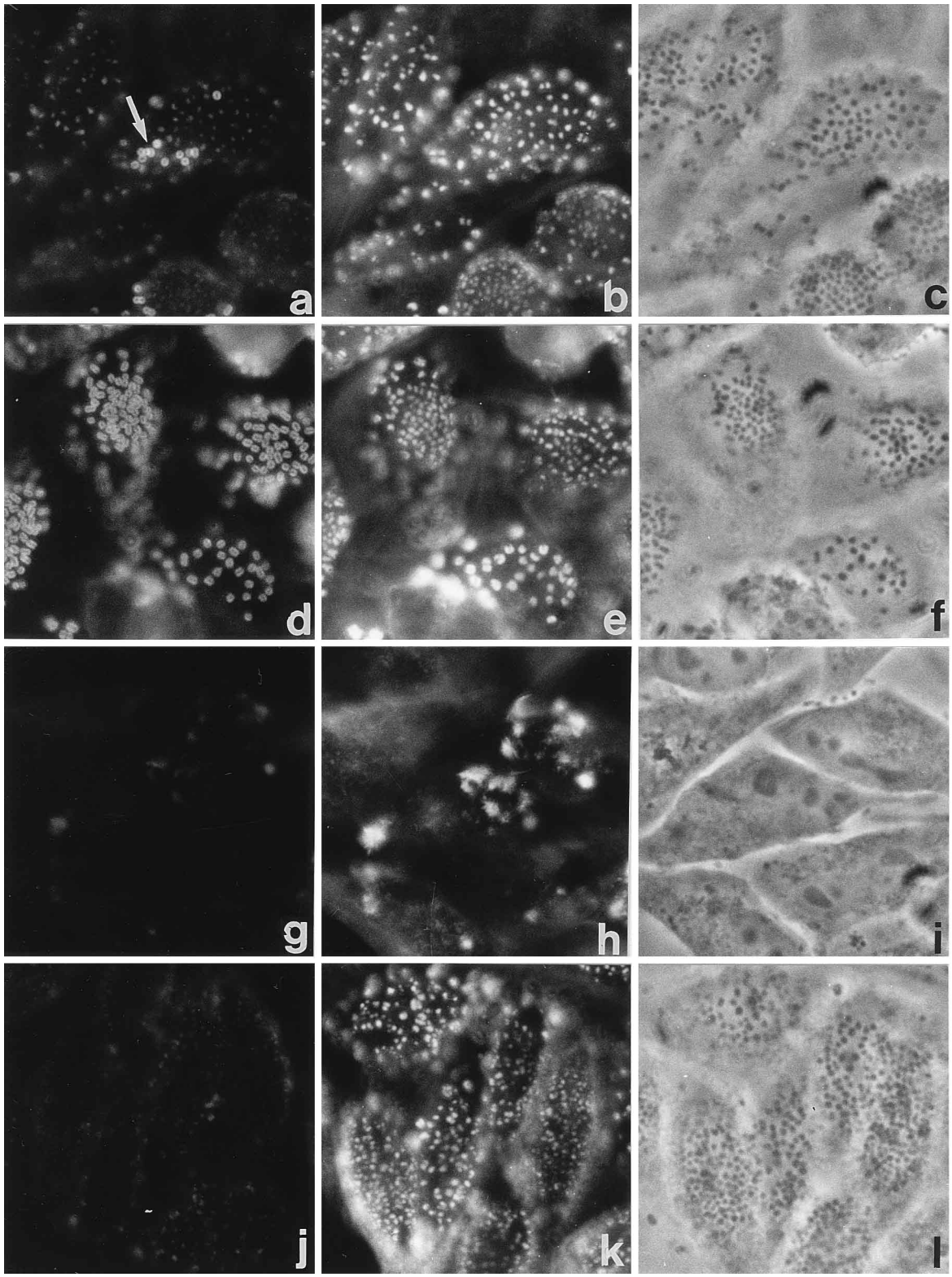

FIG. 5. Corresponding intimin fluorescence, actin fluorescence, and phase-contrast micrographs of HEp-2 cells incubated with EPEC strains E2348/69 (a through c), JPN15 (d through f), CVD206 (g through i), and CVD206(pCVD438) (j through l) at 37°C for 6 h. Strains E2348/69, JPN15, and CVD206(pCVD438) displayed LA (c, f, and l) and had a positive FAS test result (b, e, and k), but there were marked differences in intimin expression; most JPN15 bacteria still expressed intimin strongly (d), whereas very few E2348/69 (a [arrow]) or CVD206(pCVD438) (j) bacteria stained with the antibody. No CVD206 bacteria remained attached to HEp-2 cells (i), although a disorganized pattern of actin accretion remained (h). Magnification, ×720.

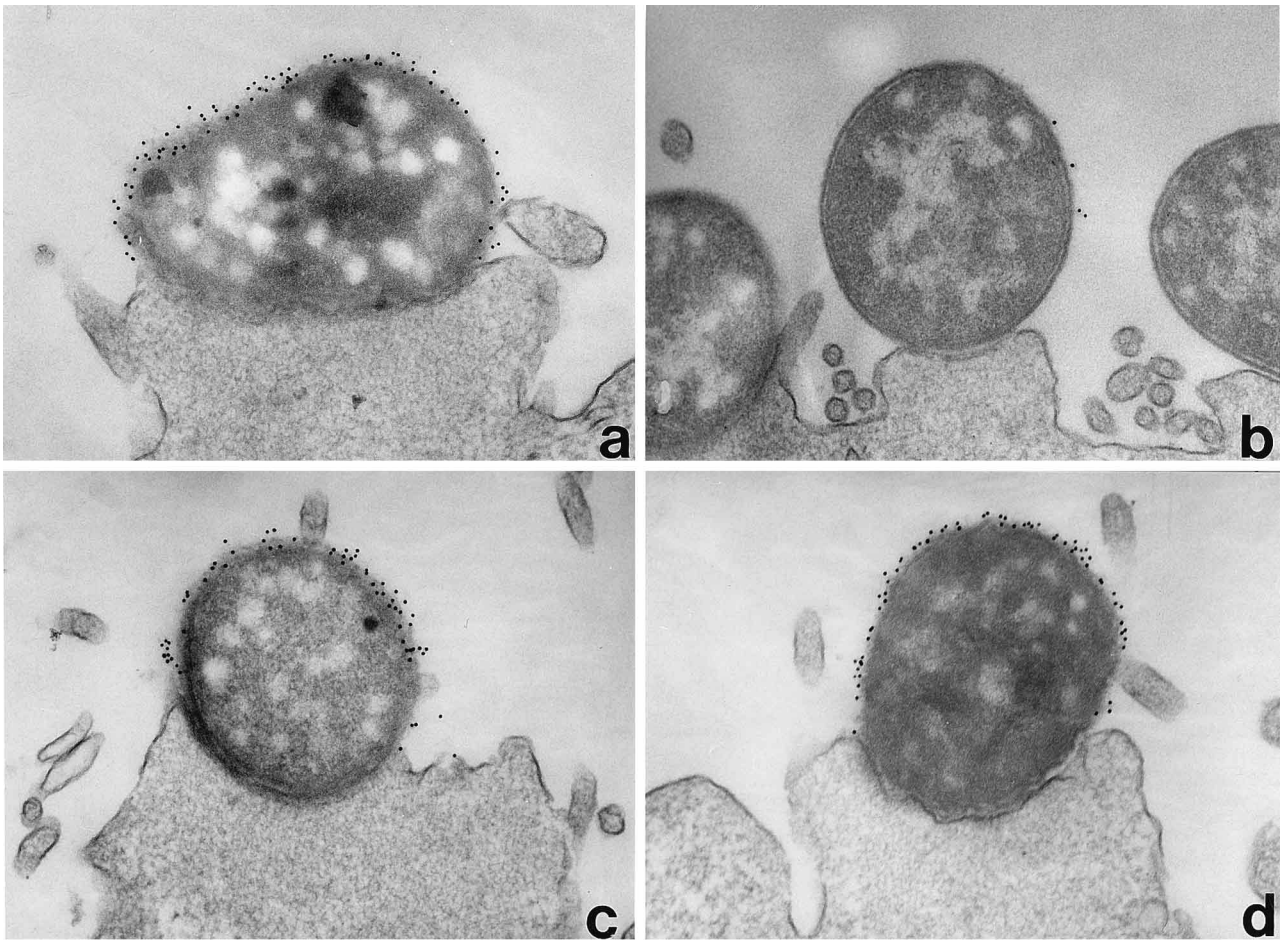

FIG. 6. Immunogold labelling of intimin following incubation of strains E2348/69 (a and b) and JPN15 (c and d) with HEp-2 cells for 3 h (a and c) and 6 h (b and d). Adherent bacteria showed typical A/E lesions, and after 3 h both strains showed uniform surface expression of intimin, except at the site of intimate adhesion (a and c); after 6 h JPN15 still showed surface expression of intimin (d) but E2348/69 showed little or no intimin staining (b). Magnification, ×55,000.

isothiocyanate filter. A loss of intimin fluorescence after 6 h also occurred with strain CVD206(pCVD438) (Fig. 5j), but in contrast, most JPN15 bacteria retained a strong intimin fluorescence (Fig. 5d). After 6 h no CVD206 bacteria remained attached to HEp-2 cells (Fig. 5i), although regions of disorganized actin accretion, presumably where bacterial microcolonies had been attached, were still apparent (Fig. 5h). The greatly reduced surface expression of intimin by strain E2348/69 but not by strain JPN15 after a 6-h incubation with HEp-2 cells was confirmed by immunogold labelling (Fig. 6b and d).

In order to investigate the possible role of host cell factors in the observed down regulation in intimin expression following A/E bacterium adhesion, we investigated whether such down regulation occurred following adhesion of E2348/69 to nonviable formalin-fixed HEp-2 cells. After both 3- and 6-h (Fig. 7) incubations, E2348/69 adhered to fixed cells in localized microcolonies (Fig. 7c) and did not produce A/E lesions (Fig. 7b), and all adherent bacteria expressed intimin (Fig. 7a).

EPEC *per* genes, which increase the expression of intimin (12), and plasmid-encoded *bfpTVW* genes, which increase expression of BFP (31), have been characterized, and based on sequence comparisons, these two sets of regulatory genes appear to be the same. In order to investigate the possible role of *per* genes in the observed changes in intimin expression following A/E bacterium adhesion, we examined a derivative of strain JPN15 possessing cloned *per* genes [strain JPN15 (pCVD450)]. After a 3-h incubation with HEp-2 cells, strain JPN15(pCVD450) appeared to be similar to strain JPN15, with all A/E bacteria expressing intimin (data not shown), whereas after 6 h more than 50% of the bacteria no longer stained with the intimin antiserum (Fig. 7d through f). In order to investigate whether BFP expression was regulated in a similar manner following A/E bacterium adhesion, we performed immunofluorescence studies of BFP expression by wild-type strain E2348/69 following 3- and 6-h incubations with HEp-2 cells. BFP was expressed after both 3 and 6 h but in greater amounts after 6 h (Fig. 7g and h).

DISCUSSION

Intimin is essential for A/E lesion formation induced by EPEC both in vivo and in vitro and for full expression of EPEC virulence in volunteers (5, 6). Antisera raised against the 280-amino-acid carboxy-terminal region of intimin of an EPEC O127:H6 isolate specifically stained strain E2348/69 (O127:H6) intimin expressed at the bacterial surface, thus allowing us to examine directly the expression of this essential EPEC virulence factor during bacterial growth and A/E bacterium adhesion to cultured cells. The increased expression of intimin

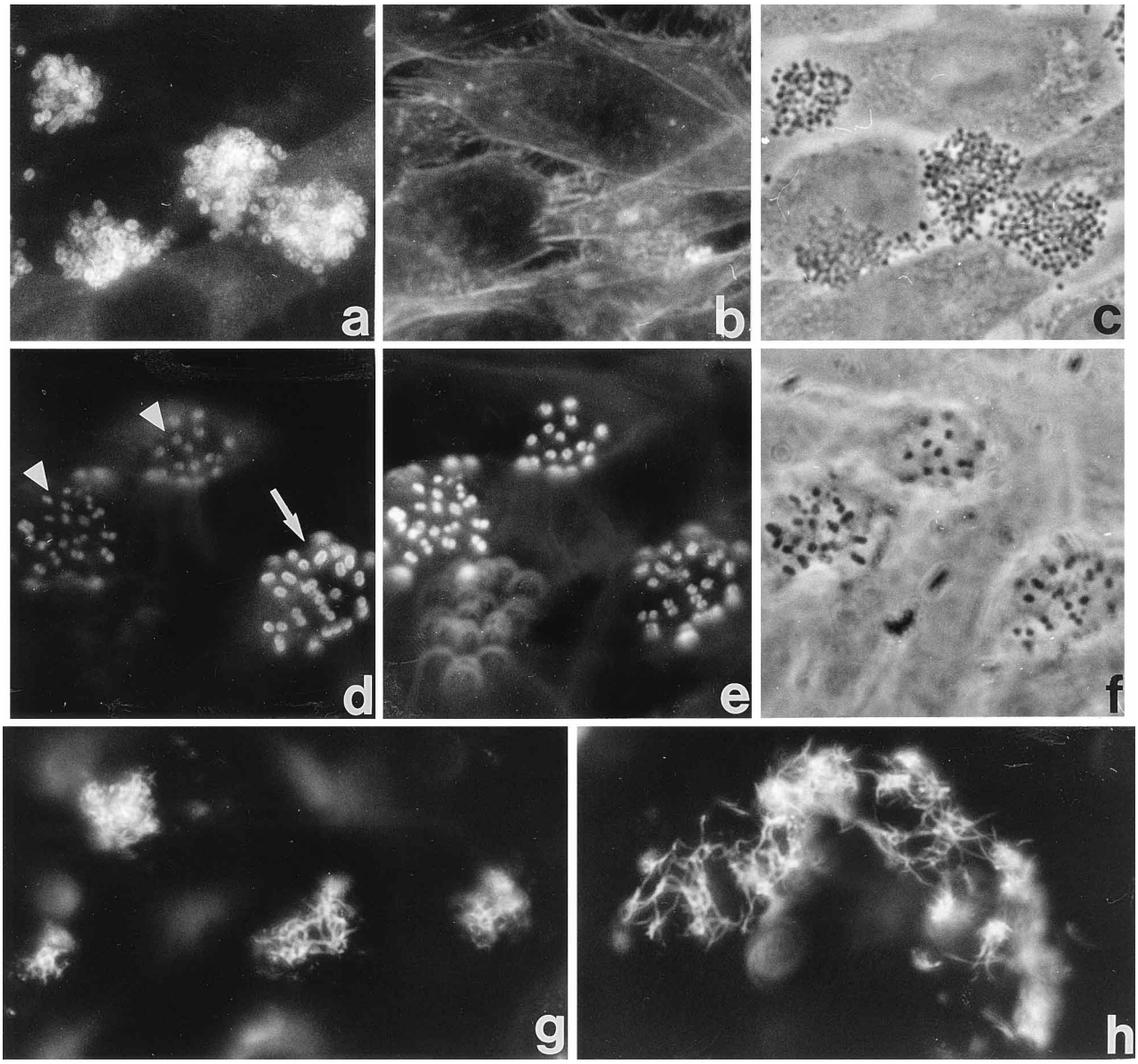

FIG. 7. Corresponding intimin fluorescence (a and d), actin fluorescence (b and e), and phase-contrast micrographs (c and f) of formalin-fixed HEp-2 cells incubated with strain E2348/69 (a through c) and HEp-2 cells incubated with strain JPN15(pCVD450) for 6 h (d through f) and fluorescence micrographs of HEp-2 cells incubated with E2348/69 for 3 and 6 h (g and h, respectively) and stained for BFP. E2348/69 adhered to fixed cells in localized microcolonies (c) and did not induce actin accumulation (b), but after 6 h all bacteria expressed intimin (a). JPN15(pCVD450) displayed LA (f) and had a positive FAS test result (e), but fewer than half of the colonies stained for intimin (d [arrow]); the residual fluorescence in some microcolonies (d [arrowheads]) is actin fluorescence. E2348/69 microcolonies expressed BFP, and increased expression of fibrillar BFP at 6 h (h) compared to that at 3 h (g) was seen. Magnification, ×800.

during exponential-phase growth of EPEC and the subsequent decreased expression following A/E bacterium adhesion to HEp-2 cells reported in this study indicate that intimin expression is regulated by environmental factors during bacterial growth and possibly in response to host cell factors following A/E bacterium adhesion; *per* genes appear to be involved in both regulatory mechanisms.

We and others have previously shown that the A/E activity of EPEC depends on bacterial growth phase. In contrast to the long (1- to 2-h) lag period in A/E activity seen following infection of epithelial cells with stationary-phase bacterial cultures, infection with tissue culture medium-grown exponential-phase cultures results in strong A/E activity within 10 to 15 min (23, 30). Growth phase regulation of expression of both EspA and EspB (secreted LEE-encoded proteins involved in signal transduction to host cells and essential for A/E lesion formation) and of BFP (important for the LA phenotype and thought to be important for initial EPEC attachment to epithelial cells) has also been reported (13, 18, 29). Together with evidence for the growth phase-regulated expression of intimin, this suggests a tightly regulated and coordinately expressed set of virulence determinants of EPEC pathogenicity.

per genes, which increase expression of intimin, have been characterized. In the study by Gómez-Duarte and Kaper (12), increased intimin expression was assessed by increased alkaline phosphatase activity of an *eaeA*::Tn*phoA* gene fusion, increased expression of the intimin protein, and increased production of *eaeA* mRNA (12). The present study indicated that

regulation of surface expression of intimin primarily affects the numbers of bacteria expressing the protein, since there did not appear to be a significant difference in fluorescence intensity or density of gold particles between stationary- and logarithmic-phase bacteria that were expressing intimin, whereas there was a significant increase in the numbers of E2348/69 bacteria expressing intimin. Interestingly, strain JPN15 appeared to express intimin more strongly than the parent (E2348/69) strain, but this strain, which does not contain *per* genes, lacked growth phase regulation of intimin expression. It has been known for some time that plasmid-cured EPEC is capable of forming A/E lesions but that it does so much less efficiently than wild-type EPEC (21, 24). However, the observed lack of increased expression of intimin during exponential growth in tissue culture medium is likely to be only one factor involved, since strain JPN15 also lacks BFP expression and possibly other plasmid-encoded factors required for full virulence.

The environmental signals involved in intimin regulation have not been characterized, although calcium and ammonium ions may be involved, since recently characterized plasmid-encoded *bfpTVW* genes for the transcriptional activation of BFP (31) appear, from sequence comparisons, to be identical to the *per* genes described by Gómez-Duarte and Kaper (12). The observed intimin expression by all E2348/69 bacteria expressing BFP (bacterial aggregates) when grown in DMEM and when adhering to fixed cells is further evidence for coordinate expression of BFP and intimin. Carbon dioxide-regulated expression of EspB (EaeB) secretion, another coordinately expressed LEE-encoded protein, has also been reported (13).

All A/E bacteria which adhered to HEp-2 cells expressed intimin, although intimin was not detected at the actual site of intimate adhesion. However, we presume that intimin is present but not labelled either because the antibody is prevented from penetrating this region of intimate contact between intimin and its receptor or because of the conformational changes that intimin underwent during receptor binding that changed the antigenicity of the polypeptide. An aspect of intimin regulation not previously reported is the observed down regulation of intimin expression following A/E lesion formation. Whereas after a 3-h incubation of cells and bacteria all E2348/69 bacteria expressed intimin, after 6 h little or no intimin could be detected. The fact that down regulation of intimin expression was not seen to any significant degree after a 6-h incubation of HEp-2 cells with strain JPN15 but did occur to a significant extent with strain JPN15 expressing the cloned *per* genes from pCVD450 suggests the additional involvement of EPEC *per* genes in down regulation of intimin expression following A/E lesion formation; interestingly, BFP expression was not down regulated in the same time frame following A/E bacterium adhesion.

Since the observed down regulation of intimin expression by E2348/69 does not occur in DMEM in the absence of cells (data not shown) or following adhesion to fixed cells, it most likely occurs in response to host cell factors. The nature of the signals transduced from infected host cells to adherent bacteria is not known. Also, other issues yet to be investigated include the following: how the *per* genes are able to act as activators of surface intimin expression during exponential growth but as repressors during stationary phase and A/E bacterium adherence; the nature of the bacterial sensor apparatus; the mechanism by which *per* function is modulated; and the level at which the decreased expression of intimin during intimate adherence is regulated, whether during transcription or translation or posttranslation (for example, by increased turnover of intimin not involved in A/E lesion formation). At present we cannot exclude the possibility that the *per* genes induce lateral mobility of intimin within the plane of the membrane so that intimin becomes concentrated in the region of bacterium-cell interaction.

If the observed down regulation of intimin expression following A/E lesion formation occurs in vivo, it could be an important EPEC regulatory mechanism for overcoming host immune responses, since intimin is known to be highly immunogenic and patients with EPEC infections develop immune responses to this protein (27). The lack of such down regulation by strain JPN15 and possibly by other atypical EPEC strains which lack EPEC adherence factor virulence plasmid and *per* genes (17) could be an additional factor contributing to the reduced virulence of these strains compared to that of classical EPEC.

ACKNOWLEDGMENTS

We are grateful to Jim Kaper, Center for Vaccine Development, University of Maryland, for providing the E2348/69 derivative strains and plasmids used in this study and to Jorge Giron, Universidad Autonoma de Puebla, Puebla, Mexico, for the BFP antiserum.

This study was supported by the Wellcome Trust.

REFERENCES

1. **Adu-Bobie, J., G. Frankel, S. Knutton, G. Douce, and G. Dougan.** Unpublished data.
2. **Cravioto, A., R. J. Gross, S. M. Scotland, and B. Rowe.** 1979. An adhesive factor found in strains of *Escherichia coli* belonging to the traditional infantile enteropathogenic serogroups. Curr. Microbiol. **3:**95–99.
3. **Donnenberg, M. S., S. B. Calderwood, A. Donohue-Rolfe, G. T. Keusch, and J. B. Kaper.** 1990. Construction and analysis of Tn*phoA* mutants of enteropathogenic *Escherichia coli* unable to invade HEp-2 cells. Infect. Immun. **58:**1565–1571.
4. **Donnenberg, M. S., and J. B. Kaper.** 1991. Construction of an *eae* deletion mutant of enteropathogenic *Escherichia coli* by using a positive-selection suicide vector. Infect. Immun. **59:**4310–4317.
5. **Donnenberg, M. S., and J. B. Kaper.** 1992. Enteropathogenic *Escherichia coli*. Infect. Immun. **60:**3953–3961.
6. **Donnenberg, M. S., C. O. Tacket, S. P. James, G. Losonsky, J. B. Nataro, S. S. Wasserman, J. B. Kaper, and M. M. Levine.** 1993. Role of the *eaeA* gene in experimental enteropathogenic *Escherichia coli* infection. J. Clin. Invest. **92:**1412–1417.
7. **Donnenberg, M. S., J. Yu, and J. B. Kaper.** 1993. A second chromosomal gene necessary for intimate attachment of enteropathogenic *Escherichia coli* to epithelial cells. J. Bacteriol. **175:**4670–4680.
8. **Foubister, V., I. Rosenshine, M. S. Donnenberg, and B. B. Finlay.** 1994. The *eaeB* gene of enteropathogenic *Escherichia coli* is necessary for signal transduction in epithelial cells. Infect. Immun. **62:**3038–3040.
9. **Frankel, G., D. C. A. Candy, P. Everest, and G. Dougan.** 1994. Characterization of the C-terminal domains of intimin-like proteins of enteropathogenic and enterohemorrhagic *Escherichia coli*, *Citrobacter freundii*, and *Hafnia alvei*. Infect. Immun. **62:**1835–1842.
10. **Frankel, G., D. C. A. Candy, E. Fabiani, J. Adu-Bobie, S. Gil, M. Novakova, A. D. Phillips, and G. Dougan.** 1995. Molecular characterization of a carboxy-terminal eukaryotic-cell-binding domain of intimin from enteropathogenic *Escherichia coli*. Infect. Immun. **63:**4323–4328.
11. **Girón, J. A., S. Y. Ho, and G. K. Schoolnik.** 1991. An inducible bundle-forming pilus of enteropathogenic *Escherichia coli*. Science **254:**710–713.
12. **Gómez-Duarte, O. G., and J. B. Kaper.** 1995. A plasmid-encoded regulatory region activates chromosomal *eaeA* expression in enteropathogenic *Escherichia coli*. Infect. Immun. **63:**1767–1776.
13. **Haigh, R., T. J. Baldwin, S. Knutton, and P. H. Williams.** 1995. Carbon dioxide regulated secretion of the EaeB protein of enteropathogenic *Escherichia coli*. FEMS Microbiol. Lett. **129:**63–68.
14. **Jarvis, K. G., J. A. Giron, A. E. Jerse, T. K. McDaniel, M. S. Donnenberg, and J. B. Kaper.** 1995. Enteropathogenic *Escherichia coli* contains a specialised secretion system necessary for the export of proteins involved in attaching and effacing lesion formation. Proc. Natl. Acad. Sci. USA **92:**7996–8000.
15. **Jerse, A. E., and J. B. Kaper.** 1991. The *eae* gene of enteropathogenic *Escherichia coli* encodes a 94-kilodalton membrane protein, the expression of which is influenced by the EAF plasmid. Infect. Immun. **59:**4302–4309.
16. **Jerse, A. E., J. Yu, B. D. Tall, and J. B. Kaper.** 1990. A genetic locus of enteropathogenic *Escherichia coli* necessary for the production of attaching and effacing lesions on tissue culture cells. Proc. Natl. Acad. Sci. USA **87:**7839–7843.

17. **Kaper, J. B.** 1996. Defining EPEC. Rev. Microbiol. Sao Paulo **27**(Suppl.)**:** 130–133.
18. **Kenny, B., and B. B. Finlay.** 1995. Protein secretion by enteropathogenic *Escherichia coli* is essential for transducing signals in epithelial cells. Proc. Natl. Acad. Sci. USA **92:**7991–7995.
19. **Kenny, B., L. C. Lai, B. B. Finlay, and M. S. Donnenberg.** 1996. EspA, a protein secreted by enteropathogenic *Escherichia coli* (EPEC) is required to induce signals in epithelial cells. Mol. Microbiol. **20:**313–323.
20. **Knutton, S.** 1995. Electron microscopical methods in adhesion. Methods Enzymol. **253:**145–158.
21. **Knutton, S., M. M. Baldini, J. B. Kaper, and A. S. McNeish.** 1987. Role of plasmid-encoded adherence factors in adhesion of enteropathogenic *Escherichia coli* to HEp-2 cells. Infect. Immun. **55:**78–85.
22. **Knutton, S., T. Baldwin, P. H. Williams, and A. S. McNeish.** 1989. Actin accumulation at sites of bacterial adhesion to tissue culture cells: basis of a new diagnostic test for enteropathogenic and enterohemorrhagic *Escherichia coli*. Infect. Immun. **57:**1290–1298.
23. **Knutton, S., G. K. Collington, T. J. Baldwin, R. D. Haigh, and P. H. Williams.** 1996. Cellular responses to EPEC infection. Rev. Microbiol. Sao Paulo **27**(Suppl.)**:**89–94.
24. **Knutton, S., D. R. Lloyd, and A. S. McNeish.** 1987. Adhesion of enteropathogenic *Escherichia coli* to human intestinal enterocytes and cultured human intestinal mucosa. Infect. Immun. **55:**69–77.
25. **Levine, M. M.** 1987. *Escherichia coli* that cause diarrhoea: enterotoxigenic, enteropathogenic, enteroinvasive, enterohaemorrhagic, and enteroadherent. J. Infect. Dis. **155:**377–389.
26. **Levine, M. M., E. J. Berquist, D. R. Nalin, D. H. Waterman, R. B. Hornick, C. R. Young, S. Stoman, and B. Rowe.** 1978. *Escherichia coli* strains that cause diarrhoea but do not produce heat-labile or heat-stable enterotoxins and are non-invasive. Lancet **i:**119–122.
27. **Levine, M. M., J. P. Nataro, H. Karch, M. M. Baldini, J. B. Kaper, R. E. Black, M. L. Clements, and A. D. O'Brien.** 1985. The diarrhoeal response of humans to some classic serotypes of enteropathogenic *Escherichia coli* is dependent on a plasmid encoding an enteroadhesiveness factor. J. Infect. Dis. **152:**550–559.
28. **McDaniel, T. K., K. G. Jarvis, M. S. Donnenberg, and J. B. Kaper.** 1995. A genetic locus of enterocyte effacement conserved among diverse enterobacterial pathogens. Proc. Natl. Acad. Sci. USA **92:**1664–1668.
29. **Puente, J. L., D. Beiber, S. W. Ramer, W. Murray, and G. K. Schoolnik.** 1996. The bundle-forming pili of enteropathogenic *Escherichia coli*: transcriptional regulation by environmental signals. Mol. Microbiol. **20:**87–100.
30. **Rosenshine, I., S. Ruschowski, and B. B. Finlay.** 1996. Expression of attaching/effacing activity by enteropathogenic *Escherichia coli* depends on growth phase, temperature, and protein synthesis upon contact with epithelial cells. Infect. Immun. **64:**966–973.
31. **Tobe, T., G. K. Schoolnik, I. Sohel, V. H. Bustamante, and J. L. Puente.** 1996. Cloning and characterisation of *bfpTVW*, genes required for the transcriptional activation of *bfpA* in enteropathogenic *Escherichia coli*. Mol. Microbiol. **21:**963–975.
32. **Towbin, H., T. Staehelin, and J. Gordon.** 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA **76:**4350–4354.
33. **Ulshen, M. H., and J. L. Rollo.** 1980. Pathogenesis of *Escherichia coli* gastroenteritis in man—another mechanism. N. Engl. J. Med. **302:**99–101.

Editor: A. O'Brien