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Role of Plasmid-Encoded Adherence Factors in Adhesion of Enteropathogenic *Escherichia coli* to HEp-2 Cells

STUART KNUTTON,[1]* MARY M. BALDINI,[2] JAMES B. KAPER,[2] AND ALEXANDER S. McNEISH[1]

Institute of Child Health, University of Birmingham, Birmingham B16 8ET, United Kingdom,[1] and Center for Vaccine Development, University of Maryland, Baltimore, Maryland[2]

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Plasmid-encoded adherence factors have been shown to be important for the full expression of enteropathogenic *Escherichia coli* (EPEC) pathogenicity and for EPEC adhesion to cultured HEp-2 cells. EPEC strain E2348 (O127) shows localized HEp-2 cell adhesion and possesses a 60-megadalton plasmid, pMAR2. When E2348 is cured of pMAR2 it loses the ability to adhere to HEp-2 cells, while nonadherent *E. coli* K-12 strains P678-54 and HB101 acquire HEp-2 adhesiveness after they gain the plasmid. By electron microscopy, E2348 was seen to adhere to HEp-2 cells in a manner that closely resembled EPEC adhesion to intestinal mucosa; bacteria were intimately attached to projections of the apical HEp-2 cell membrane and caused localized destruction of microvilli. The plasmid-containing K-12 strains, on the other hand, did not show intimate attachment and there was no modification of cell surface architecture. It is concluded that plasmid pMAR2 codes for an adhesin, possibly fimbrial in nature, that promotes HEp-2 adhesion but that other chromosomally encoded factors are required for EPEC to achieve the characteristic mode of intimate cell attachment.

Enteropathogenic *Escherichia coli* (EPEC), the first diarrheic *E. coli* isolate to be described, remains a major cause of infant gastroenteritis in many parts of the developing world (9). The pathogenic mechanisms of EPEC, however, have yet to be established. Mucosal adhesion is now recognized as a reproducible feature of intestinal colonization by EPEC (13, 16, 17, 22), and recent studies have shown that plasmid-encoded adherence factors are required for full expression of pathogenicity (10). Several in vitro assays have been developed to study adherence properties of EPEC. McNeish et al. (12) and Knutton et al. (7) used fetal and adult human intestinal epithelial cells, respectively. Cravioto et al. (4) described an adherence assay in which they used tissue culture cells after they observed that most strains of EPEC causing an outbreak of diarrhea adhered to cultured HEp-2 cells, whereas adhesion of non-EPEC strains was rare. Localized (LA) and diffuse (DA) patterns of adherence have now been described (18). EPEC showing LA adhere in discrete microcolonies to localized areas of the HEp-2 cell surface, whereas those showing DA adhere to the entire cell surface. Different plasmid-encoded adherence factors appear to be responsible for LA and DA (15). Baldini et al. (1) showed that a 60-megadalton (MDa) plasmid, pMAR2, of EPEC strain E2348 codes for LA, but the gene product responsible for HEp-2 adhesion and designated EPEC adhesion factor (14) has yet to be identified.

EPEC causes a very striking and characteristic intestinal histopathological lesion. By electron microscopy, bacteria are seen to adhere intimately to cuplike projections of the apical enterocyte membrane and to cause localized destruction of brush border microvilli (16, 17, 20, 22). The electron microscopic appearance of EPEC adhesion to cultured cells, however, has not been reported. In this report we show that EPEC strain E2348 adheres to HEp-2 cells in a manner that closely resembles EPEC adhesion to intestinal mucosa, whereas *E. coli* K-12 strains possessing E2348 plasmid pMAR2 neither show intimate attachment nor cause any disruption of cell surface architecture.

* Corresponding author.

MATERIALS AND METHODS

Bacterial strains. EPEC strain E2348 (O127:H6), originally isolated from an outbreak of infant gastroenteritis in a nursery in Taunton, United Kingdom, was kindly provided by B. Rowe, Central Public Health Laboratory, Colindale, United Kingdom. The 60-MDa plasmid pMAR2 in strain E2348, which confers localized HEp-2 adhesion, was cured as described previously (1). Insertion of ampicillin transposon Tn*801* into pMAR2 gave rise to pMAR7 and pMAR8, which retain HEp-2 adhesiveness (1). pMAR15 is a derivative of pMAR7 in which regions that are nonessential for HEp-2 adhesiveness have been deleted. The construction of this derivative has been described previously (2). HB101 and P678-54, nonadhesive *E. coli* K-12 strains, were transformed with these derivatives as described previously (1, 2). HEp-2-positive derivatives P678-54(pMAR8) and HB101 (pMAR15) were used in this study. Stock cultures of the strains were subcultured into Mueller-Hinton broth (Oxoid Ltd., Basingstoke, United Kingdom) with or without ampicillin and incubated aerobically for 18 h at 37°C.

Adhesion assay. Adhesion to HEp-2 cells was tested essentially by the method of Cravioto et al. (4). Overnight cultures of HEp-2 cells on glass cover slips or in petri dishes were washed and suspended in 2 to 3 ml of modified Eagle medium containing 2% fetal calf serum and 0.5% D-mannose. A total of 25 μl of a bacterial broth culture was added to each dish, and the cells were cultured at 37°C for an additional 3 h. After a thorough washing to remove nonadhering bacteria, cells were either fixed in 70% methanol and stained with Giemsa for light microscope observations or fixed in glutaraldehyde and processed for electron microscopy.

Electron microscopy. For transmission electron microscopy, cell monolayers were fixed in 3% phosphate-buffered

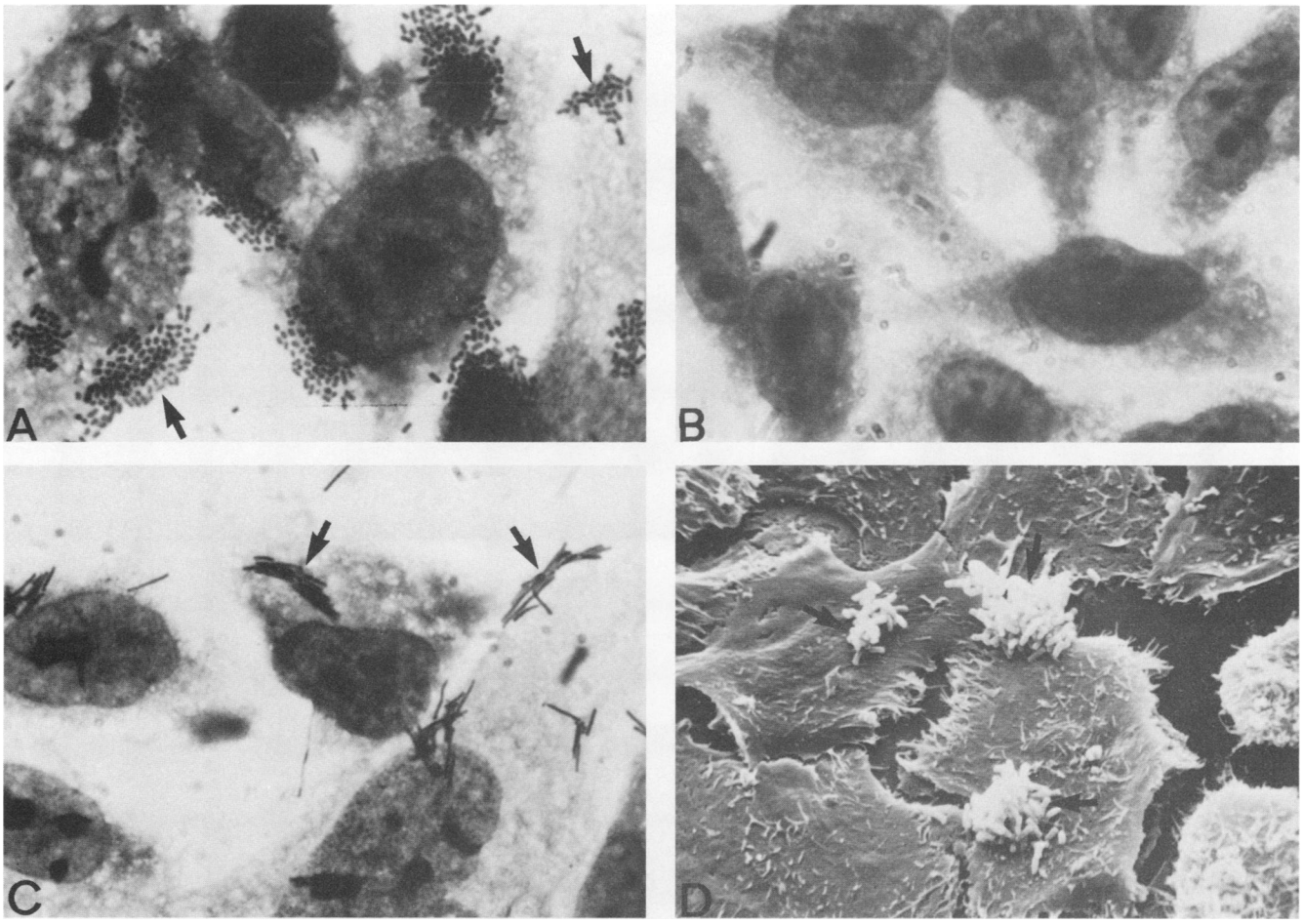


FIG. 1. Light (A to C) and scanning electron (D) micrographs showing E2348 (A), P678-54(pMAR8) (C), and HB101(pMAR15) (D) adhering to HEp-2 cells in microcolonies (arrows). Plasmid-cured E2348 was nonadherent (B). Magnification: A to C, ×850; D, ×1,700.

glutaraldehyde, postfixed in 1% osmium tetroxide, dehydrated through graded ethanol solutions, and embedded in Epon. For ruthenium red staining, phosphate buffer was replaced with cacodylate buffer and ruthenium red (concentration, 0.075%) was added to both fixative and wash solutions. Ultrathin sections were stained in aqueous uranyl and lead salts and examined in an electron microscope (EM301; Philips).

For freeze-fracture electron microscopy, glutaraldehyde-fixed cell monolayers were scraped from petri dishes and infiltrated with 25% glycerol. Cell pellets were rapidly frozen in liquid Freon 22 (E. I. du Pont de Nemours & Co., Inc., Wilmington, Del.), fractured at −110°C in a freeze-fracture device (Denton), and replicated with platinum and carbon. Replicas were cleaned in 5% sodium hypochlorite and washed in distilled water prior to examination.

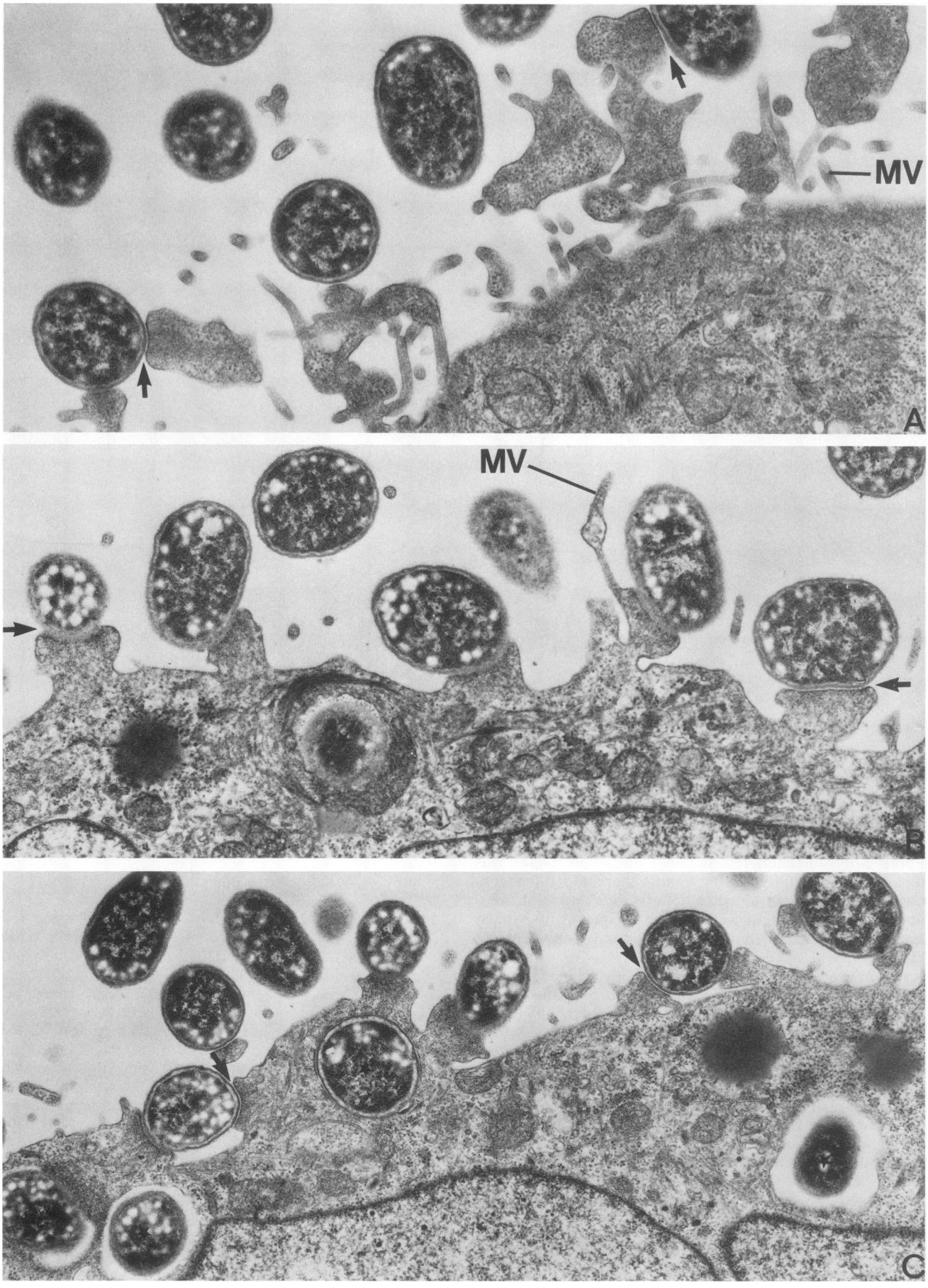
For scanning electron microscopy, cell monolayers fixed with glutaraldhyde and osmium on glass cover slips were dehydrated through graded acetone solutions and dried to the critical point. Cover slips were mounted on stubs, sputter coated with gold, and examined in a scanning electron microscope (S4; Cambridge).

For negative staining, 10 μl of a washed bacterial suspension was mixed with equal volumes of bacitracin (150 μg/ml) and ammonium molybdate (2%, pH 7.0). A total of 10 μl was applied to carbon-coated copper grids for 30 s, and the excess liquid was removed with filter paper.

RESULTS

Strain E2348 exhibited localized HEp-2 adhesion (Fig. 1A), whereas E2348 cured of its 60-MDa plasmid, pMAR2, lost the ability to adhere to HEp-2 cells (Fig. 1B). Acquisition of plasmids pMAR8 and pMAR15 by nonadherent *E. coli* K-12 strains P678-54 and HB101, respectively, was accompanied by acquisition of localized HEp-2 adhesion (Fig. 1C and D). In Giemsa-stained preparations bacteria appeared to adhere as a monolayer to the HEp-2 cell surface (Fig. 1A to C), but scanning electron microscopy revealed that bacteria were also piled up on each other (Fig. 1D).

By electron microscopy, strain E2348 was seen to adhere to HEp-2 cells in a manner that closely resembled EPEC adhesion to intestinal mucosa. Bacteria were intimately attached to cuplike projections of the apical cell surface (Fig. 2 to 4), with just 10 nm separating bacterial and HEp-2 cell membranes (Fig. 3). In regions of attachment cell surface microvilli were absent and the associated cytoskeletal elements were disrupted. Instead of the normal cell surface cytoskeletal organization, concentrations of short filaments were present beneath attached bacteria. The filaments,


MV
A
MV
B
C

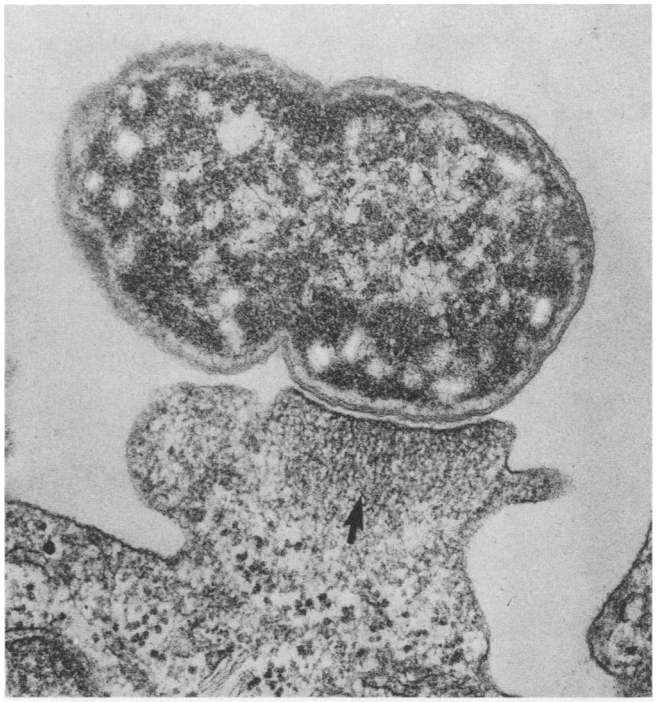


FIG. 3. Electron micrograph showing a typical cell surface projection with an adherent E2348 bacterium. Approximately 10 nm separated bacterial and HEp-2 cell membranes. Concentrations of short filaments are present beneath the attached bacterium (arrows). Magnification: ×65,000.

which appeared to be anchored to and radiate from the plasma membrane, were of a dimension similar to those of the core filaments of cell surface microvilli (Fig. 3). Destruction of microvilli was localized to sites of bacterial attachment, but many intact microvilli were still apparent between attached bacteria. This was most noticeable with rounded cells, the surface of which was covered with large numbers of microvilli (Fig. 2A).

EPEC adhesion to HEp-2 cells was also demonstrated in freeze-fracture replicas (Fig. 4). The fracture plane shown in Fig. 4 passed through the HEp-2 cell plasma membrane at the site of a microcolony of adherent E2348 bacteria. Intimate attachment of bacteria was revealed at the points at which HEp-2 and bacterial membranes were cross-fractured; oblique fractures through both membranes revealed the large surface area of bacteria-cell contact. Other bacteria not visualized were so tightly attached that they left an impression of their location on the HEp-2 cell membrane fracture face (Fig. 4). Typical fractured cell surface microvilli were seen between adherent bacteria but were absent from sites of bacteria attachment. The inset to Fig. 4 shows the freeze-fracture appearance of one of the cytoplasmic projections on which bacteria appeared to sit. Most of the attached bacterium was removed during the fracturing process, although small fragments of fractured cell wall and cell membrane remained (inset, Fig. 4). The cuplike appearance of the cytoplasmic projection conforming to the shape of the bacterium was readily apparent.

Endocytosis of some bacteria appeared to have occurred after a 3-h incubation with HEp-2 cells (Fig. 2B and C); after a 6-h incubation large numbers of endocytosed bacteria were seen inside some cells.

Adherent E2348 cells were intimately attached to the HEp-2 cell surface; other bacteria within a microcolony that were piled on each other and not bound to the HEp-2 cell surface were separated from each other by a clear (up to 0.5-μm wide) electron-translucent zone (Fig. 2). Ruthenium red staining revealed numerous rigid, rodlike fimbrial structures (~7 nm in diameter) in this clear zone that appeared to connect bacteria to each other (Fig. 5).

HEp-2-adherent P678-54(pMAR8) and HB101(pMAR15) did not show intimate attachment, nor was there loss of cell surface microvilli or disruption of the associated cell cytoskeleton (Fig. 6A to C). Bacteria were generally seen to be separated from the cell surface and from each other by a distinct electron-translucent zone, although in this case fimbrial structures connecting bacteria to the cell surface or to each other were not revealed in ruthenium red-stained preparations (Fig. 6B). Rigid, rodlike fimbriae (~7 nm in diameter), however, were observed at the surface of a few P678-54(pMAR8) bacteria when bacteria were subcultured from HEp-2 cells onto solid media and examined by negative stain electron microscopy (Fig. 6D).

DISCUSSION

Following the initial observation of Cravioto et al. (4) that most strains of EPEC causing outbreaks of diarrhea adhere to cultured HEp-2 cells, whereas non-EPEC strains do so rarely, adhesion to cultured HeLa and HEp-2 cells has been thought to be useful in screening EPEC strains of human origin for adhesive properties of potential significance in intestinal colonization (1, 8, 14, 15, 18). This conclusion is directly supported by the results of this study, in which it has been shown that EPEC adheres to HEp-2 cells in a manner that closely parallels EPEC adhesion to intestinal mucosa. Intimate attachment of bacteria to the cell surface in tissue culture, with localized disruption of the cell cytoskeleton and loss of cell surface microvilli, is identical to the intestinal histopathological lesion seen in natural EPEC infections in infants (17, 21, 22) and experimental EPEC infections in animals (13, 16, 20).

Mucosal adhesion of some bacteria, e.g., enterotoxigenic *E. coli*, appears to be a simple receptor-ligand interaction between bacterial adhesins and specific mucosal receptors (5). This type of bacterial adhesion occurs without any apparent mucosal damage (6). Mucosal adhesion of EPEC is clearly more complex. Using genetic techniques to transfer the 60-MDa plasmid from EPEC strain E2348 to nonadherent *E. coli* K-12 strains, we have been able to define a distinct plasmid-encoded stage of the adhesion process. Plasmid-encoded factors promote HEp-2 cell adhesion, but this is of a nonintimate type; they do not appear to be responsible for subsequent localized disruption of the cell cytoskeleton, loss of microvilli, and intimate attachment of

FIG. 2. Electron micrographs of HEp-2 cell monlayers infected with E2348. Bacteria are intimately attached to cuplike projections of the apical cell surface (arrows). Bacteria within a microcolony not attached to the HEp-2 cell surface are separated from other bacteria by a clear electron-translucent zone; endocytosis of some bacteria appeared to have occurred (stars, panels B and C). MV, microvilli. Magnification: A and B, ×30,000; C, ×22,000.

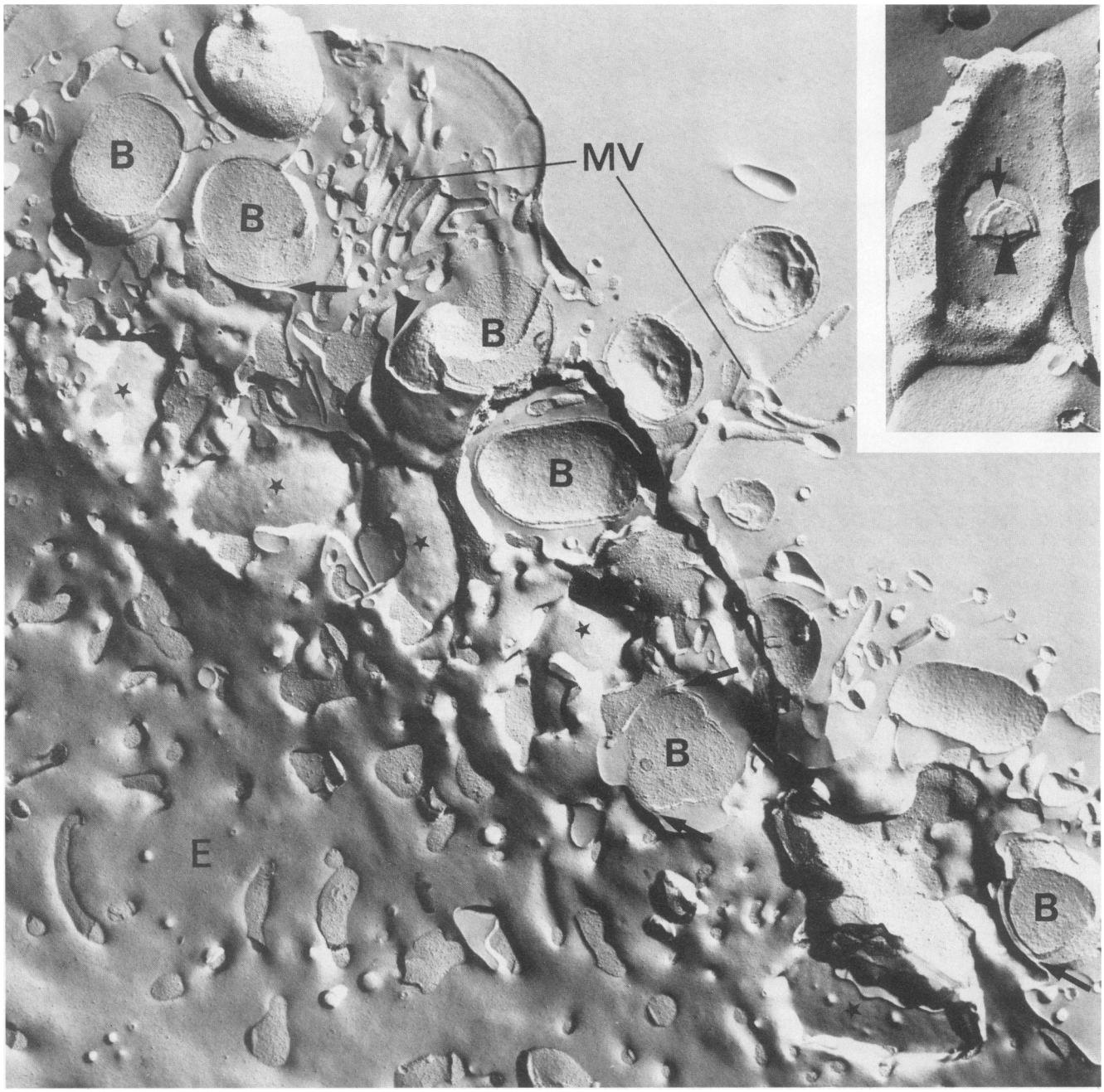


FIG. 4. Freeze-fracture electron micrograph showing the exoplasmic (E) face of a fractured HEp-2 cell and numerous adherent fractured E2348 bacteria (B) readily identified by their double membrane profile. Intimate attachment is seen where cross (arrows) or oblique (arrows) fractures through both HEp-2 and bacterial cell membranes occurs. MV, Microvilli. Magnification: ×24,000; inset, ×35,000.

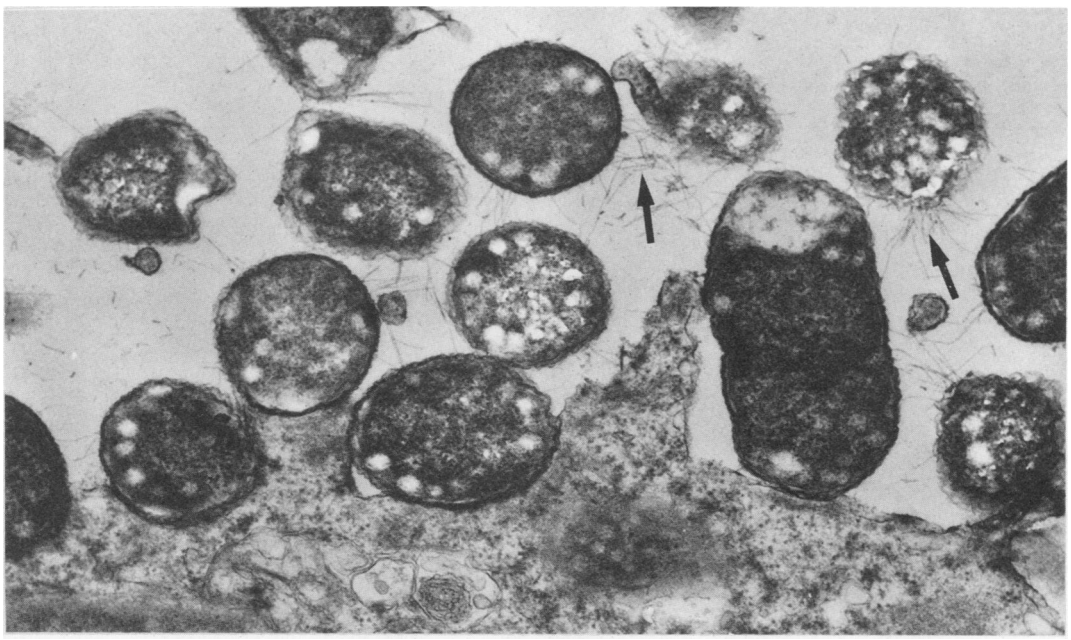


FIG. 5. Electron micrographs of a ruthenium red-stained preparation showing HEp-2 adherent E2348. Long, rigid fimbriae (arrows; ~7 nm in diameter) appear to connect bacteria to each other within the microcolony. Magnification: ×30,000.

bacteria. These events presumably require chromosomally encoded factors. It is possible that the cell-damaging property of E2348 and other EPEC strains is plasmid encoded, but this possibility was excluded in this case by the insertion or the deletion of transposons. This is unlikely, however, because in an accompanpying paper (7) we show that E2348 cured of pMAR2 can cause the characteristic EPEC intestinal histopathological lesion i.e., it can cause disruption of the enterocyte cytoskeleton and the loss of brush border microvilli.

The molecular nature of the adhesin encoded on pMAR2 has yet to be established. The ultrastructural appearance of HEp-2 adhesion by plasmid-containing *E. coli* K-12 strains is suggestive of fimbrially mediated adhesion, and fimbriated bacteria were detected in cultures of P678-54(pMAR8) by negative staining. P678-54 itself does not produce fimbriae, and so it is likely that these structures are encoded by the EPEC plasmid. Morphologically similar fimbriae were detected on adherent E2348 bacteria by ruthenium red staining. These could be type 1 fimbriae, which are known to be produced by this strain (11), although aggregation of bacteria mediated by type 1 fimbriae would not be expected to occur in the presence of the concentrations of D-mannose used in our adhesion assay. The different patterns of ruthenium red staining suggest that fimbriae produced by E2348 and by P678-54(pMAR8) are different, although the presence or absence of acidic polysaccharides with which ruthenium red is thought to bind (3) could explain the positive staining of fimbriae in one case and not the other.

We have presented preliminary evidence that the EPEC adhesion factor may be fimbrial in nature. Scotland et al. (19) concluded that adhesion of EPEC to HEp-2 cells is not dependent on the presence of fimbriae. There are, however, a number of reasons why it might be difficult to detect adhesion fimbriae in such a study, given that EPEC adhesion involves a fimbrial adhesin. Scaletsky et al. (18) showed that LA could be detected at the end of a 30-min infection period and that small microcolonies of bacteria simply increased in size during the subsequent 3-h multiplication period, with the number of colonies remaining the same during the entire experiment. The conclusion of Scaletsky et al. (18) was that receptors related to LA were not distributed over the whole surface of HeLa (or HEp-2) cells. An alternative explanation might be that receptors are in fact present over the entire cell surface, but that only a few adherent microcolonies are seen because only a small fraction of bacteria in EPEC cultures produce the adhesin. If this is the case, and it is our experience with some well-characterized adhesins that they are often only produced by a small fraction of bacteria in a culture (G. T. Hinson, S. Knutton, M. K. L. Lam-Po-Tang, A. S. McNeish, and P. H. Williams, Infect. Immun., submitted for publication), then it may be difficult to detect fimbriae produced by a small number of bacteria by negative staining. The culturing of bacteria directly from HEp-2 cell monolayers serves to enrich for bacteria that produce adhesins, but detection by electron microscopy would again depend that they be expressed under the in vitro growth conditions used.

The fimbriae produced by E2348 and detected by ruthenium red staining appear to promote the adhesion of bacteria to each other. The difference between LA and DA, therefore, could be that LA adhesins promote adhesion of bacteria to each other as well as to the cell surface, whereas DA adhesins promote only cell adhesion.

The EPEC adhesion factor responsible for LA has been detected in the serogroups most frequently implicated in EPEC outbreaks worldwide, including O55, O111, O119, O127, and O128. The EPEC adhesion factor was not found in the less common serogroups O44, O86, and O114; but in one O44:H18 EPEC strain an adhesin distinct from the EPEC adhesion factor and conferring DA was shown to be encoded on a 65-MDa plasmid (15). Most O44, O86, and O114 serogroup EPEC strains are HEp-2 nonadherent (15). Several different plasmid-encoded adhesins therefore appear to be involved in adhesion of EPEC. Because these adhesins appear to be required for full expression of EPEC pathoge-

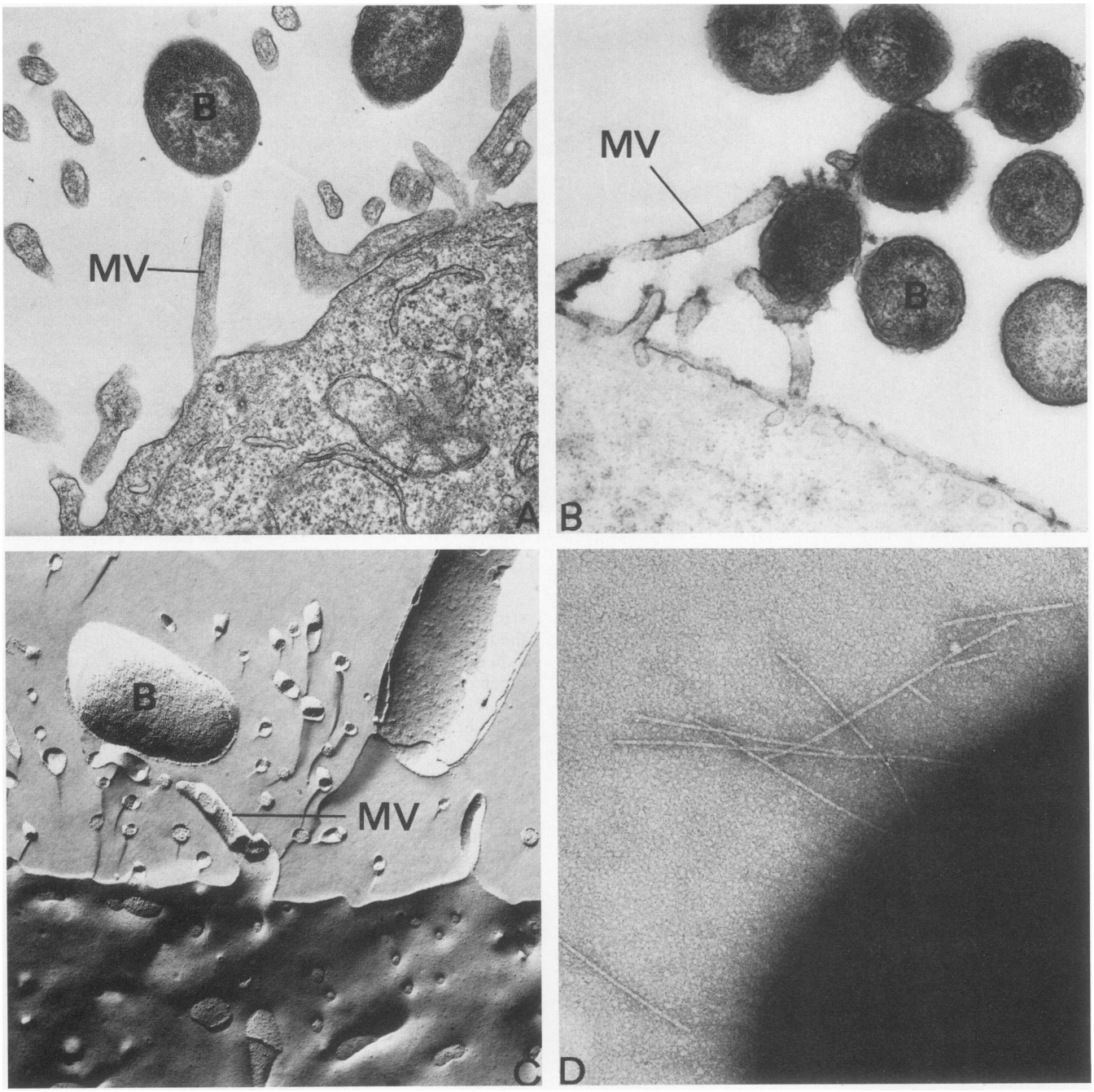


FIG. 6. Transmission (A and B) and freeze-fracture (C) electron micrographs showing HEp-2-adherent HB101(pMAR15) (A) and P678-54(pMAR8) (B and C). Bacteria (B) do not show intimate attachment nor is there any disruption of cell surface architecture. Fimbriae are not seen in ruthenium red-stained preparations (B), although long, rigid fimbriae (~7 nm in diameter) are seen at the surface of some P678-54(pMAR8) bacteria by negative staining (D). MV, Microvilli. Magnification: A, ×28,000; B, ×32,000; C, ×23,000; D, ×120,000.

nicity (10), it is important that they be identified and characterized. This is the aim of studies currently in progress.

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