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Characterization of Fimbriae Produced by Enteropathogenic *Escherichia coli*

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Enteropathogenic *Escherichia coli* (EPEC) express rope-like bundles of filaments, termed bundle-forming pili (BFP) (J. A. Girón, A. S. Y. Ho, and G. K. Schoolnik, Science 254:710–713, 1991). Expression of BFP is associated with localized adherence to HEp-2 cells and the presence of the EPEC adherence factor plasmid. In this study, we describe the identification of rod-like fimbriae and fibrillae expressed simultaneously on the bacterial surface of three prototype EPEC strains. Upon fimbrial extraction from EPEC B171 (O111:NM), three fimbrial subunits with masses of 16.5, 15.5, and 14.7 kDa were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Their N-terminal amino acid sequence showed homology with F9 and $F7_2$ fimbriae of uropathogenic *E. coli* and F1845 of diffuse-adhering *E. coli*, respectively. The mixture of fimbrial subunits (called FB171) exhibited mannose-resistant agglutination of human erythrocytes only, and this activity was not inhibited by α-D-Gal(1-4)-β-Gal disaccharide or any other described receptor analogs for P, S, F, M, G, and Dr hemagglutinins of uropathogenic *E. coli*, which suggests a different receptor specificity. Hemagglutination was inhibited by extracellular matrix glycoproteins, i.e., collagen type IV, laminin, and fibronectin, and to a lesser extent by gangliosides, fetuin, and asialofetuin. Scanning electron microscopic studies performed on clusters of bacteria adhering to HEp-2 cells revealed the presence of structures resembling BFP and rod-like fimbriae linking bacteria to bacteria and bacteria to the eukaryotic cell membrane. We suggest a role of these surface appendages in the interaction of EPEC with eukaryotic cells as well as in the overall pathogenesis of intestinal disease caused by EPEC.

Enteropathogenic *Escherichia coli* (EPEC) is an important cause of infantile diarrhea in developing countries (27). The term EPEC is commonly used to describe classical serotypes of *E. coli* that have been epidemiologically implicated in diarrheal disease and which commonly do not elaborate heat-labile or heat-stable enterotoxins (27). Although several authors have reported the ability of EPEC strains to invade tissue culture cells (1, 8, 39), EPEC does not cause the invasive dysentery characteristic of *Shigella* spp. or enteroinvasive *E. coli* (27).

The mechanisms by which EPEC causes diarrhea are not yet elucidated, but adhesion to the intestinal mucosa and effacement and destruction of the epithelial microvilli are important features of their pathogenicity. Cravioto et al. (6) reported that 80% of the EPEC strains previously isolated from diarrheal outbreaks showed mannose-resistant adherence to HEp-2 cells. Commonly, EPEC strains adhere to restricted areas of the HEp-2 or HeLa cells in tight clusters or microcolonies in a pattern termed localized adherence (LA) (43). The LA property is associated with the presence of a 50- to 60-MDa plasmid (2) referred to as the EPEC adherence factor (EAF) plasmid (34), but the nature of the actual adhesin mediating LA remains uncertain. Jerse et al. (16) identified in strain E2348/69 a 94-kDa membrane protein as the product of the *eae* (*E. coli* attaching and effacing) chromosomal locus, necessary for the production of the attaching-effacing activity on human intestinal tissue culture cells.

In contrast to enterotoxigenic *E. coli* or uropathogenic *E. coli* (UPEC) in which a repertoire of colonization factors have been characterized in detail (21), the nature of potential virulence factors associated with EPEC adherence to intestinal mucosal surfaces and tissue culture cells remains poorly elucidated. Type I (9, 40) fimbriae, type I-like fimbriae, and a fibrillar hemagglutinin (18, 22, 36, 52, 55) have been reported in some EPEC strains. Scotland et al. (44) reported that the adhesion to HEp-2 cells of outbreak EPEC strains is not dependent on the presence of fimbriae. Yakubu et al. (55) described an ~3-nm-wide fibrillar mannose-resistant hemagglutinin in two EPEC strains (O111:H12) among several strains studied. However, no detailed characterization of these appendages or the type I-like fimbriae observed on EPEC has been reported nor has their correlation with LA or pathogenesis been demonstrated. On the other hand, outer membrane proteins were also suggested to mediate LA, and a 32-kDa component of the outer membrane was described as the LA factor in EPEC (42). However, Chart et al. (5) demonstrated that this 32-kDa protein corresponds to OmpF and that it may not be involved in the LA of EPEC to HEp-2 cells.

Recently, Girón et al. (14) described inducible bundle-forming pili (BFP) expressed by EPEC. The BFP forms rope-like structures, and the pilin subunit isolated from strain B171 (O111:NM) had a mass of 19.5 kDa. Its N-terminal amino acid sequence showed considerable homology with the toxin-coregulated pilus of *Vibrio cholerae* and the family of type IV or methyl-phenylalanine pilins (14, 48). Antibodies raised against the purified BFP recognized common epitopes on similar morphologic fimbrial structures elaborated by different EPEC serotypes. Production of BFP was associated with the presence of the EAF plasmid and moreover with the LA phenotype (14).

This article describes the identification and characterization of two additional morphologic types of fimbriae expressed by EPEC B171 (O111:NM) and demonstrates a relationship between their structural subunits and other well-characterized

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adhesins described in *E. coli* strains from extraintestinal infections. The fimbriae described here together with BFP may contribute to the overall pathogenesis of intestinal disease caused by EPEC.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Prototype EPEC strains B171, E2348/69, and 5368-64 belong to classic EPEC serotypes, namely, O111:NM, O127:H6, and O128:H1, respectively (14, 22). These EPEC strains show LA on tissue culture cells, harbor a ca. 60-MDa plasmid that hybridizes with the 1-kb EAF DNA probe, and express BFP (13, 14). To investigate the expression of fimbriae, strains were grown in Luria broth with aeration and on colonization factor antigen (CFA) agar (9) for 18 h at 37°C or for 48 h at 18 or 22°C and examined by electron microscopy. *E. coli* K-12 strain CAB 70 (donated by Craig A. Bloch) was used as a reference strain for type I fimbriae production. C1845 is a diffuse-adhering *E. coli* (DAEC) strain that expresses a fimbrial adhesin termed F1845 (3).

Isolation and purification of fimbriae from EPEC B171 (FB171). EPEC B171 was grown overnight at 37°C on CFA agar to inhibit expression of type I fimbriae (9). The cells were harvested in 50 mM Tris buffer (pH 8.0), and the fimbriae were detached from the bacterial cells by shearing two times at setting 4 for 10 min in an Omnimixer (Dupont Sorvall, Newton, Conn.). The bacteria were removed by centrifugation at 23,500 × *g* for 30 min, and fimbriae present in the supernatant were precipitated overnight with 50% ammonium sulfate at 4°C. The resulting pellet was dissolved overnight in Tris buffer at 4°C, and contaminating outer membranes were removed by centrifugation at 20,800 × *g* for 45 min. A second cycle of ammonium sulfate precipitation rendered a fairly pure fimbrial preparation (designated FB171) free of most outer membranes as judged by electron microscopy and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (25).

Production of antiserum to FB171. One 11-week-old New Zealand White rabbit was immunized subcutaneously with 100 μg of FB171 in complete Freund adjuvant. Three weeks later, the animal was boosted weekly with the same dose of fimbrial protein in incomplete Freund adjuvant. Blood was collected at each immunization. Lipopolysaccharide (LPS) antibodies present in the FB171 antiserum were removed by repeated adsorptions with latex beads (Particle Technology, Indianapolis, Ind.) coated with O111 LPS extracted from strain B171 as described previously (39). The absorbed antiserum (designated absorbed FB171 antiserum) did not agglutinate fresh LPS-coated beads or strain B171 grown in Luria broth. The presence of FB171 antibodies was monitored by slide agglutination of bacterial cells grown on CFA agar, immunoelectron microscopy, and Western blots (immunoblots).

Electron microscopy and immunogold labelling. Ultrastructural studies of bacteria and of the different fractions obtained during the isolation and purification of FB171 were done by negative staining with 1% phosphotungstic acid as described earlier (14). For immunogold labelling studies, goat anti-rabbit immunoglobulin G conjugated with 10-nm-diameter gold particles was used to detect type I fimbriae antibodies or FB171 antibodies (Jansen Pharmaceutical, Piscataway, N.J.). Type I fimbria antiserum was kindly provided by C. A. Bloch.

PAGE and Western blots. The purity of the fimbrial preparation and the apparent mass of the fimbrial subunit were estimated by SDS-PAGE in a 16 to 20% gradient gel by the method of Laemmli (25). The fimbrial proteins were solubilized in sample buffer and boiled for 5 min. After electrophoresis, the separated proteins were visualized by staining with Coomassie blue and silver. Proteins were electrophoretically transferred to nitrocellulose membranes (for immunoblots) or onto polyvinylidene difluoride membranes (Millipore Corp., Bedford, Mass.; for N-terminal amino acid sequencing of fimbrial subunits) and stained with amido black to visualize the transferred proteins. For the immunoblots, FB171 was detected by absorbed FB171 antiserum (1:1,000) and then by goat anti-rabbit immunoglobulin G conjugated to alkaline phosphatase (Sigma Chemical Co., St. Louis, Mo.). The substrate was a mixture of nitroblue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate (BCIP; Sigma). The protein concentration was estimated by the method of Bradford (4). In addition, FB171 was tested in Western blots with F1845 antiserum kindly donated by S. L. Moseley. F1845 antiserum recognizes the F1845 fimbriae expressed by DAEC C1845 (3).

Amino-terminal amino acid sequencing. Purified FB171 and the FB171 protein subunits electroblotted onto a polyvinylidene difluoride membrane (Millipore) were subjected to N-terminal sequencing by using automated Edman degradation (890M; Beckman Instruments, Inc., Palo Alto, Calif.) in the presence of Polybrene (46). Sequences obtained were compared with published sequences of *E. coli* fimbrial proteins (21, 41).

HA and inhibition of HA of FB171. Hemagglutination (HA) of human A, B, AB, and O blood group erythrocytes by FB171 was tested in the absence and presence of 1% D-mannose. Other animal erythrocytes tested for HA were chicken, ox, sheep, horse, calf, and turkey. Serial twofold dilutions of 50 μl of FB171 were done in phosphate-buffered saline (PBS) containing 1% bovine serum albumin and 1% mannose in 96-well microtiter plates (Nunc, Roskilde, Denmark). To each well was subsequently added 50 μl of a 0.3% suspension of the different erythrocytes to be tested, and the HA titer was determined after 4 h of incubation at 22°C. HAs were recorded when a pellet of erythrocytes was observed in the well containing only PBS plus erythrocytes. The highest dilution of FB171 showing HA was considered 1 HA unit. Neuraminidase-treated human erythrocytes were assayed for HA with FB171 and prepared with 1 U of *V. cholerae* neuraminidase (Sigma) as described earlier (32).

For HA inhibition tests, serial twofold dilutions of the putative inhibitor (50 μl per well) were done as described above, and an equal volume of 4 HA units of FB171 was added to each well and allowed to stand at room temperature for 20 min. An aliquot of 50 μl of an 0.3% suspension of human erythrocytes was then added. The concentration of the substance that inhibited the HA activity of 4 HA units of FB171 was recorded as the MIC. When two or three substances were tested in combination, the amount of each substance used in the mixture was the same as the amount used when the substance was tested alone. The level of inhibition was recorded on a scale of 0 to 10, in which 0 means no inhibition at the lowest dilution of a substance and 10 means the highest titer of inhibition of the strongest inhibitor.

Different compounds known to be part of receptor structures for different bacterial fimbriae were tested as putative inhibitors of the mannose-resistant HA (MRHA) shown by FB171. Galactose α-(1-4)-galactose, glycophorin A, *N*-acetylglucosamine, *N*-acetylgalactosamine, *N*-acetylneuraminic acid, *N*-acetylneuraminlactose, GM_1, fucose, fetuin, asialofetuin, α-acid glycoprotein (orosomucoid), gelatin, collagens type I, II, and V, and chloramphenicol were purchased from Sigma. Trihexosylceramide and globoside were purchased from Matreya, Inc. (Pleasant Gap, Pa.). Fibronectin, collagen type IV,

and laminin were obtained from Collaborative Research, Inc. (Bedford, Mass.). A tetrapeptide (RGDS) was available from this laboratory. The 40-kDa fragment of fibronectin was purchased from Calbiochem Corp. (San Diego, Calif.). Anti-type I fimbriae and anti-FB171 were also tested for inhibition of MRHA properties of FB171. Antifibronectin antibodies (Sigma) were used in studies of the inhibition of adherence of strain B171 to HEp-2 cells and in studies of its effect on the HA inhibitory activity of fibronectin.

Effect of different antisera on adherence of EPEC B171. The LA assay onto HEp-2 cells was performed as described by Cravioto et al. (6). Briefly, 10 μl of an overnight bacterial culture was incubated with a monolayer of HEp-2 cells in the presence of 1% D-mannose in Dulbecco minimal essential medium (GIBCO) for 3 h at 37°C. The cells were washed six times with PBS, fixed with methanol, and stained with Giemsa. The LA pattern was visualized by light microscopy.

To study the role of FB171 on the initial adherence of EPEC to HEp-2 cells, bacteria were incubated with HEp-2 cells for 60 min. For adherence inhibition experiments, 10 μl of an overnight bacterial culture was added to individual wells in triplicate on microtiter plates and incubated for 20 min at room temperature with 50 or 5 μl of the following antisera: normal rabbit serum, antifibronectin antiserum, rabbit anti-O111 LPS (Difco Laboratories, Detroit, Mich.), absorbed anti-FB171, and a combination of adsorbed anti-FB171 and anti-BFP antiserum (1:1). The mixture of bacteria plus antiserum was then added to HEp-2 cell monolayers which had been preincubated with 300 μl of Dulbecco minimal essential medium containing the same dilution of the respective antibodies. The cells were allowed to incubate for 60 min at 37°C and then washed, fixed, and visualized as indicated above. The percentage of bacterial attachment was calculated by counting the number of cells with attached bacteria in a total number of cells in a field.

Scanning electron microscopy of EPEC–HEp-2 cell interaction. The HEp-2 adherence assay was performed as described before, and after washing off unbound bacteria, the HEp-2 cells with bound bacteria were fixed with 2% glutaraldehyde in 0.1 M PBS and the coverslips were prepared for scanning electron microscopy as described earlier (47).

RESULTS

Ultrastructural studies of EPEC. Negative staining of strain B171 grown in Luria broth at 37°C with aeration revealed the expression of two different morphologic types of fimbriae, namely, ~7-nm-wide rod-like fimbriae and 2- to 3-nm-wide fibrillar structures (Fig. 1A). These two types of appendages were also detected in EPEC E2348/69 and 5368-64 (not shown). The fibrillar structures were not always expressed under the growth conditions described above, and attempts to optimize their expression under different growth conditions were unsuccessful. In contrast, rod-like fimbriae appeared to be expressed in liquid or solid media and at different incubation temperatures (18, 25, or 37°C). B171 also expresses an inducible BFP, which is a distinct morphologic fimbrial type found on EPEC strains (12, 13) (Fig. 1B and C). Expression of BFP is favored in tissue culture media and Trypticase soy agar plates supplemented with sheep blood (14).

Analysis of EPEC FB171 fimbriae by SDS-PAGE. Upon purification of FB171 from EPEC B171 grown on CFA agar at 37°C, electrophoretic separation of FB171 subunits by SDS-PAGE revealed a prominent band with an apparent mass of 16 kDa and a less prominent band of 14.7 kDa (Fig. 2A). FB171 appeared to be largely free of contaminating outer membrane proteins, as shown by SDS-PAGE. Negative staining of this preparation revealed the presence of rod-like fimbriae. It is possible that the fibrillar structures seen in Fig. 1A are not apparent in Fig. 2B because of the extraction procedure or negative staining technique employed. A more sensitive staining procedure with silver nitrate revealed the presence of additional bands (~8, 12, 31, and 60 to ~90 kDa) which might represent minor fimbrial subunits or contaminants present in lesser amounts (Fig. 3A). In addition, it was shown that the 16-kDa band seen in Fig. 2A was composed of two bands that comigrated closely together. These bands had apparent masses of 16.5 and 15.5 kDa (Fig. 3A).

Absorbed FB171 antiserum detected the 16.5-, 15.5-, and 14.7-kDa subunits in Western blots (Fig. 3B). Native epitopes exposed on the surface of the pilus filaments were detected by using FB171 antibodies and immunogold labelling of strain B171 (Fig. 4) as well as strains E2348/69 and 5368-64 (not shown). In addition, CFA agar-grown EPEC strains were agglutinated by absorbed FB171 antiserum. F1845 antibodies recognized a 14.7- and a 31-kDa polypeptide (Fig. 3B). Most likely, this 31-kDa polypeptide is a dimer of FB171-14. Moreover, absorbed FB171 antiserum cross-reacted with F1845 expressed on the surface of strain C1845 (3), which confirms the presence of common antigenic determinants between FB171 and F1845 (data not shown).

Type I fimbriae were not likely present in the FB171 preparation since FB171 was resolved by SDS-PAGE under conventional denaturation conditions without HCl treatment. Such treatment is needed to depolymerize type I fimbriae of *E. coli* K-12 and many wild-type *E. coli* strains to monomer subunits that can enter the polyacrylamide gel (31). Heat and acid treatment of FB171 destroyed the fimbrial subunits (not shown). Type I fimbria antiserum was used to determine any serological relation between type I fimbriae and rod-like fimbriae in strain B171. This antiserum recognized type I fimbriae produced by *E. coli* CAB 70 (Fig. 5A) but did not recognize the rod-like fimbriae in strain B171 (Fig. 5B).

Amino acid analysis of FB171. N-terminal sequencing analysis was performed on FB171 and on FB171 blotted onto an Immobilon polyvinylidene difluoride membrane. Three sequences which correlated with the sequences obtained from the individual 16.5-, 15.5-, and 14.7-kDa protein bands electroblotted onto the Immobilon polyvinylidene difluoride membrane were obtained from pure FB171. The sequences shown in Fig. 6 correspond to the sequences of the 16.5- and 15.5-kDa subunits, respectively. The third sequence, which was present in a low percentage ratio in the mixture, corresponds to the minor protein band of 14.7 kDa (Fig. 7).

When the sequences obtained were compared with other N-terminal amino acid sequences from colonization factors reported in enterotoxigenic *E. coli* and UPEC, it was found that the sequence of the 16.5-kDa subunit (designated FB171-16) showed 100% identity with the first 19 residues of the structural 21-kDa subunit of F9 fimbriae (a P fimbria) of UPEC (Fig. 6) (7, 46). We do not discard the possibility that differences in the amino acid sequence in the C-terminal region of these two fimbrial subunits which may account for other biochemical or biological differences between these two fimbriae may exist. Relative homologies are also observed between F9, FB171-16, FB171-15, and type I fimbriae. Eleven residues were common between $F7_2$, FB171-15 (the 15.5-kDa subunit of FB171), FB171-16, and F9. The sequence of the FB171-15 subunit showed 81% homology with the structural subunit of $F7_2$ fimbriae (another P fimbria) of UPEC which has a mass of 17 kDa (Fig. 6) (51). The first three amino acids of $F7_2$ fimbriae are not present in FB171-15. Considerable ho-

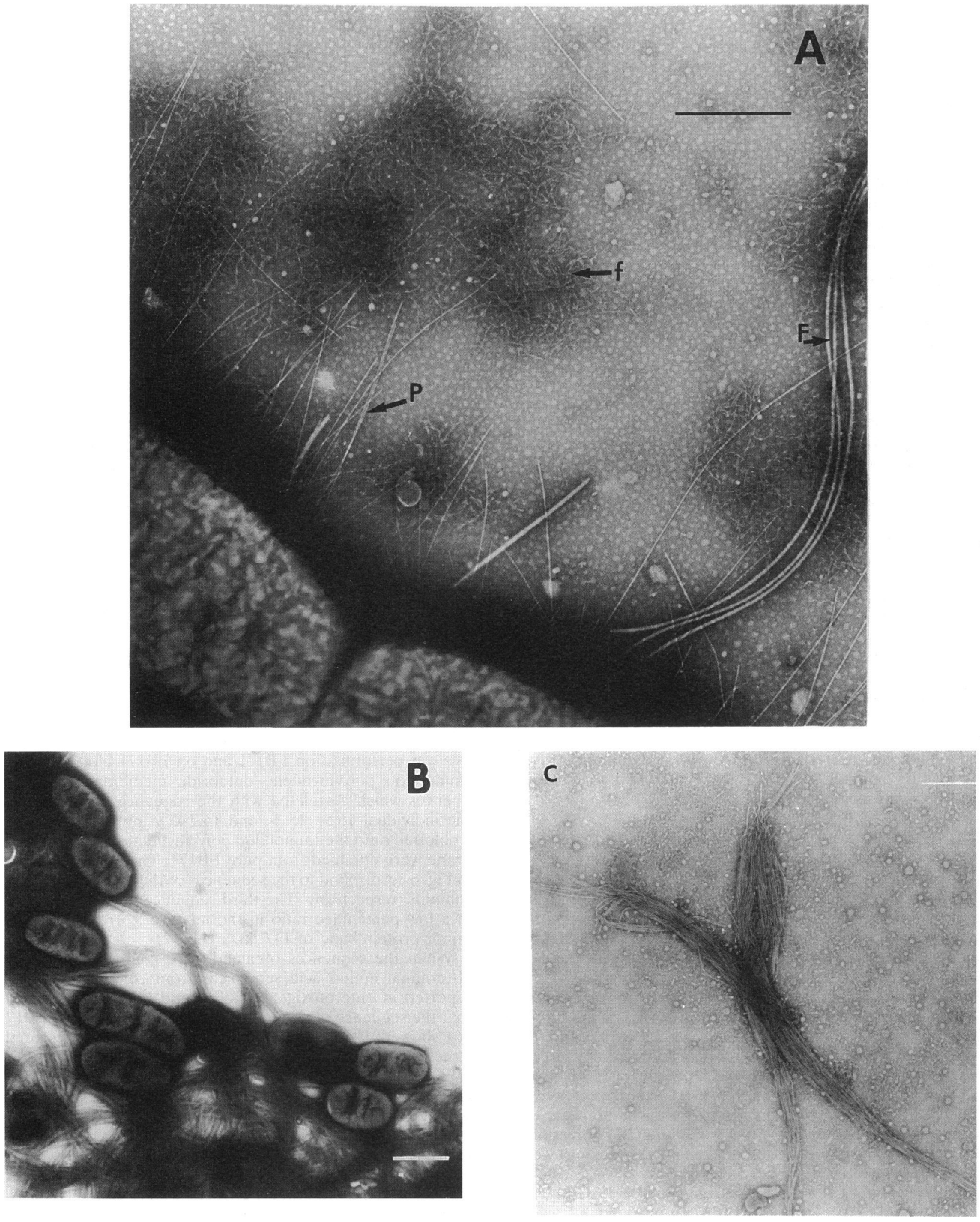

FIG. 1. Ultrastructural studies. (A) Transmission electron micrographs of EPEC B171 (O111:NM) grown in Luria broth at 37°C with aeration. Three types of appendages are shown: pilus-like structures (7 nm wide) protruding from the cell surface (P); fibrillae (~2 to 3 nm wide; f); and flagella (F). Bar, 0.5 μm. Similar structures are also expressed on CFA agar. (B) EPEC grown on tryptic soy agar supplemented with 5% defibrinated sheep blood expressing BFP. Bar, 0.56 μm. (C) High magnification of BFP. Bar, 0.4 μm.

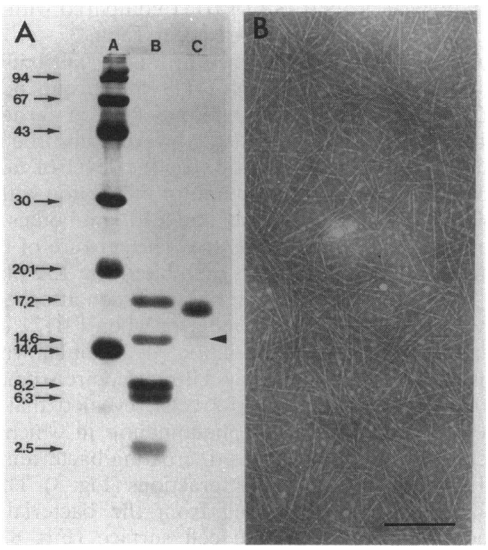

FIG. 2. Electrophoresis of purified EPEC B171 fimbriae (FB171). (A) SDS-PAGE (with a 16 to 20% gradient polyacrylamide gel) analysis of FB171 after denaturation in gel sample buffer and boiling for 10 min. Lanes: A and B, molecular weight markers; C, final FB171 preparation. The arrowhead indicates a 14.7-kDa subunit. (B) Purified fimbriae were negatively stained with sodium phosphotungstic acid and observed by electron microscopy. Only rod-like fimbriae are visible in this micrograph by our negative staining procedure. Bar, 0.3 μm.

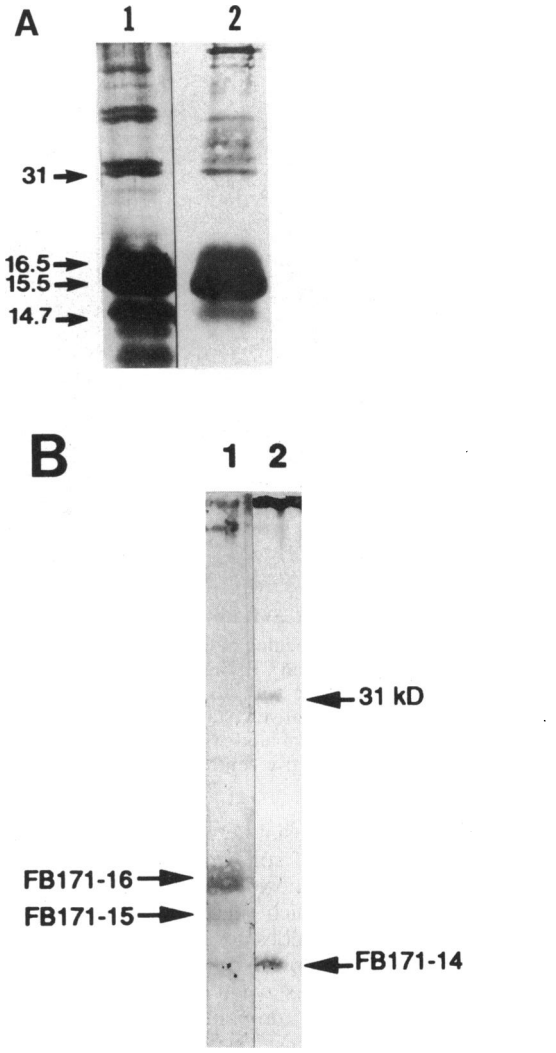

FIG. 3. (A) SDS-PAGE analysis of purified FB171. Lanes: 1, silver staining of purified FB171 shown in Fig. 2A; 2, biotinylated FB171 (described in Materials and Methods). (B) Western immunoblots. Lanes: 1, immunoblot of FB171 by using absorbed FB171 antiserum; 2, immunoblot of FB171 by using antiserum raised against F1845 fimbrial adhesin (3). Electrophoretic conditions were the same as those described in the legend to Fig. 2A.

mologies are also observed with $F7_1$ and PapA fimbriae of UPEC and to a lesser extent with type I fimbriae. All of these fimbriae appear to share certain sequence homologies in their structural subunits.

The N-terminal sequence of the 14.75-kDa subunit (designated FB171-14) showed 100% homology with the N-terminal sequence of the F1845 fimbriae found in DAEC C1845 isolated from a child with protracted diarrhea (Fig. 7) (3). The fibrillar morphology of F1845 is similar to the fibrillar structures observed in EPEC B171 (Fig. 1A). F1845 antiserum also recognized the FB171-14 subunit in Western blots (Fig. 3). FB171 antibodies recognized F1845 on strain C1845 as determined by immunogold labelling (not shown).

MRHA of FB171 and inhibition by receptor analogs. FB171 did not agglutinate sheep, chicken, horse, calf, or guinea pig erythrocytes. FB171 showed MRHA at 22°C of human erythrocytes, regardless of the ABO blood antigen. However, differences in the titers of HA were observed among the donors studied. The minimal concentration of FB171 to show HA was 2 to 6 ng/ml. Higher titers of HA were obtained when sialic acid residues were enzymatically removed from the erythrocytes with neuraminidase. Direct dose-dependent binding of FB171 to erythrocytes was shown by using iodinated FB171 (data not shown). FB171 antibodies inhibited MRHA of FB171, while type I fimbriae antibodies had no effect.

Given the N-terminal sequence homologies between P, F1845, and FB171, we attempted to study whether receptor specificities were also shared among their adhesins. Table 1 shows that known specificities for P, S, M, G, and Dr hemagglutinins found in UPEC did not inhibit MRHA by FB171, even at concentrations higher than those used by other workers to inhibit MRHA of UPEC fimbriae. Since FB171 is composed of a mixture of different fimbriae, we also tested different combinations of substances in the HA assay. As shown in Table 1, none of these substance alone or in combination showed any effect on MRHA exhibited by FB171.

Compounds that inhibited MRHA of FB171 are shown in Table 2. Collagen type IV and its breakdown product gelatin, followed by fibronectin and laminin, showed the highest inhibitory activity. Less potent inhibitors were collagens type I, II, V, and mixed gangliosides, and the weakest inhibitors were fetuin and asialofetuin. An RGDS tetrapeptide and a 40-kDa fragment of the N-terminal region of the fibronectin molecule did not show any inhibitory activity (Table 1), suggesting that the binding site for FB171 on the fibronectin molecule is different from the collagen and cell binding domains. To learn more about the interaction of FB171 and matrix glycoproteins, we used a combination of substances in the MRHA assay (Table 2). The combination of asialofetuin and collagen resulted in approximately 50% reduction of the inhibition exhibited by collagen type IV alone, which suggests that the binding site on

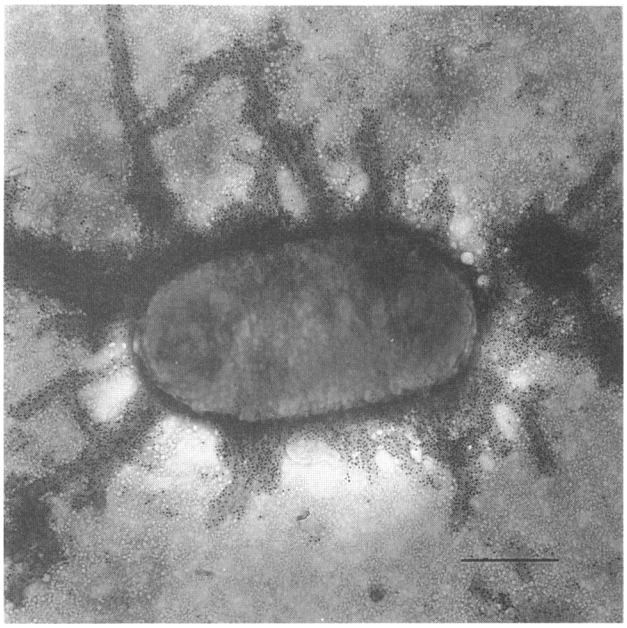

FIG. 4. Immunogold labelling of EPEC B171 with absorbed FB171 antiserum. Bacteria grown on CFA agar were incubated with absorbed FB171 antiserum and then with 10-nm colloidal gold-labelled goat anti-rabbit serum and negatively stained with 1% sodium phosphotungstic acid. Bacteria incubated with PBS instead of the primary antiserum served as a negative control. Bar, 0.5 μm.

collagen for FB171 and asialofetuin are the same or are closely related. The mixtures of collagen type IV and fibronectin or gelatin and fibronectin showed a similar effect as collagen type IV or gelatin alone, which suggests that the binding site on collagen for fibronectin does not bind FB171. Asialofetuin and fibronectin combined showed no effect on the inhibition exhibited by these substances when used alone, suggesting that FB171 binds two distinct domains on these molecules that do not interact among themselves. Some workers (20, 56) have reported that specific gangliosides or related sialic acid-containing glycoconjugates on the cell surface may act as the receptors for fibronectin. We observed that the fibronectin inhibitory activity was not affected by mixed gangliosides or vice versa, which suggests that the fimbriae recognize a binding site on these molecules different from the one involved in fibronectin-ganglioside interaction. We studied the effect of antifibronectin serum on the inhibitory activity of fibronectin. Inhibition by fibronectin was not inhibited by antifibronectin serum. In fact, the combination of fibronectin and antifibronectin antibodies exhibited a higher degree of inhibition of HA. When asialofetuin, collagen, and fibronectin were used in combination, a 20% reduction of the inhibitory activity exhibited by collagen was observed. This reduction could be attributed to the interaction of collagen and asialofetuin since, as discussed above, fibronectin does not affect the inhibition observed with collagen. These results support the hypothesis that the inhibitory activity displayed by the substances in Table 2 is most likely due to the direct interaction between binding domains on these molecules and FB171 and not to steric hindrance which might block FB171 binding sites on the erythrocytes. Moreover, fibronectin and collagen alone do not agglutinate human erythrocytes, suggesting that inhibition by these substances is not due to blocking of binding sites on erythrocytes but rather to blocking of adhesins on FB171 (data not shown).

The biological properties of FB171 compared with those of F9, $F7_2$, and F1845 are summarized in Table 3.

Inhibition of HEp-2 adherence by FB171 antibodies. Neither normal rabbit serum, anti-O111 antiserum, nor antifibronectin antiserum had any inhibitory effect on the adherence of strain B171 to HEp-2 cells. However, absorbed FB171 antiserum showed 75% reduction of the level of adherence exhibited by this strain after 60 min of incubation with HEp-2 cells. We have shown previously that BFP antibodies reduced the level of LA by strain B171 (14). The mixture of anti-BFP and anti-FB171 antibodies (1:1) inhibited the LA phenotype ~100%. Only a few single bacteria were seen attached to less than 5% of the HEp-2 cell monolayer when FB171 and BFP antibodies were used in combination (data not shown).

Scanning electron microscopy. Ultrastructure studies of the interaction of EPEC and HEp-2 cells revealed that the LA phenotype is a multifactorial phenomenon in which cellular and bacterial factors are involved, creating bacterium–HEp-2 cell and bacterium-bacterium interactions (Fig. 8). Thin fibers resembling fimbriae protruding from the bacterial surface appeared to interact with the cell surface (Fig. 8A). This fimbria-mediated interaction has been referred to as initial attachment or stage one of infection (21). In some instances, the bacterium is in close association with the cell membrane, causing the characteristic pedestal formation or stage two of infection (Fig. 8B) proposed to be mediated by outer membrane proteins (21). High magnification analysis of the LA cluster shows fimbriae interacting with the cell membrane, cellular microvilli, and other bacterial cells (Fig. 8C and D). Rope-like structures resembling BFP, most likely promoting the tight clustering of dividing bacteria or facilitating the anchorage of clustered bacteria present in the supernatant, are also visible (Fig. 8C and D).

DISCUSSION

We report here that EPEC expresses, in addition to BFP, a variety of fimbrial structures with different morphological, biochemical, and biological properties. Two filamentous surface structures (rod-like fimbriae and fibrillae) were identified by electron microscopy in three prototypic EPEC strains. Three structural subunits were obtained after extraction and purification of fimbriae (FB171) from EPEC B171. The rod-like fimbriae present in FB171 shared no biochemical, serological, or functional properties with type I fimbriae. This conclusion is based on the following data: differences between the N-terminal amino acid sequence of type I fimbriae and FB171, MRHA activity with human erythrocytes (not inhibited by anti-type I antiserum), expression on CFA agar (which is commonly used to inhibit expression of type I fimbriae) (9), resolution in SDS-polyacrylamide gels under normal denaturation conditions without HCl treatment (31), and the lack of reactivity with type I fimbria antiserum. Nevertheless, we do not rule out the fact that EPEC strains express type I fimbriae under different growth conditions (9). B171 does express type I fimbriae, but its expression is inhibited upon growth on CFA agar (data not shown).

N-terminal sequence analysis of the three subunits contained in FB171 revealed homologies with F9 and $F7_2$ fimbriae of UPEC and F1845 of DAEC (3, 37, 41, 49). However, differences in mass are observed among the related subunits, which may account for the differences in receptor specificities (Table 3). F9 agglutinates human, sheep, and ox but not guinea pig erythrocytes, and $F7_2$ shows MRHA of sheep erythrocytes (7, 41, 46, 51). FB171 only agglutinated human erythrocytes irrespective of their A, B, O, and Rh phenotypes, which

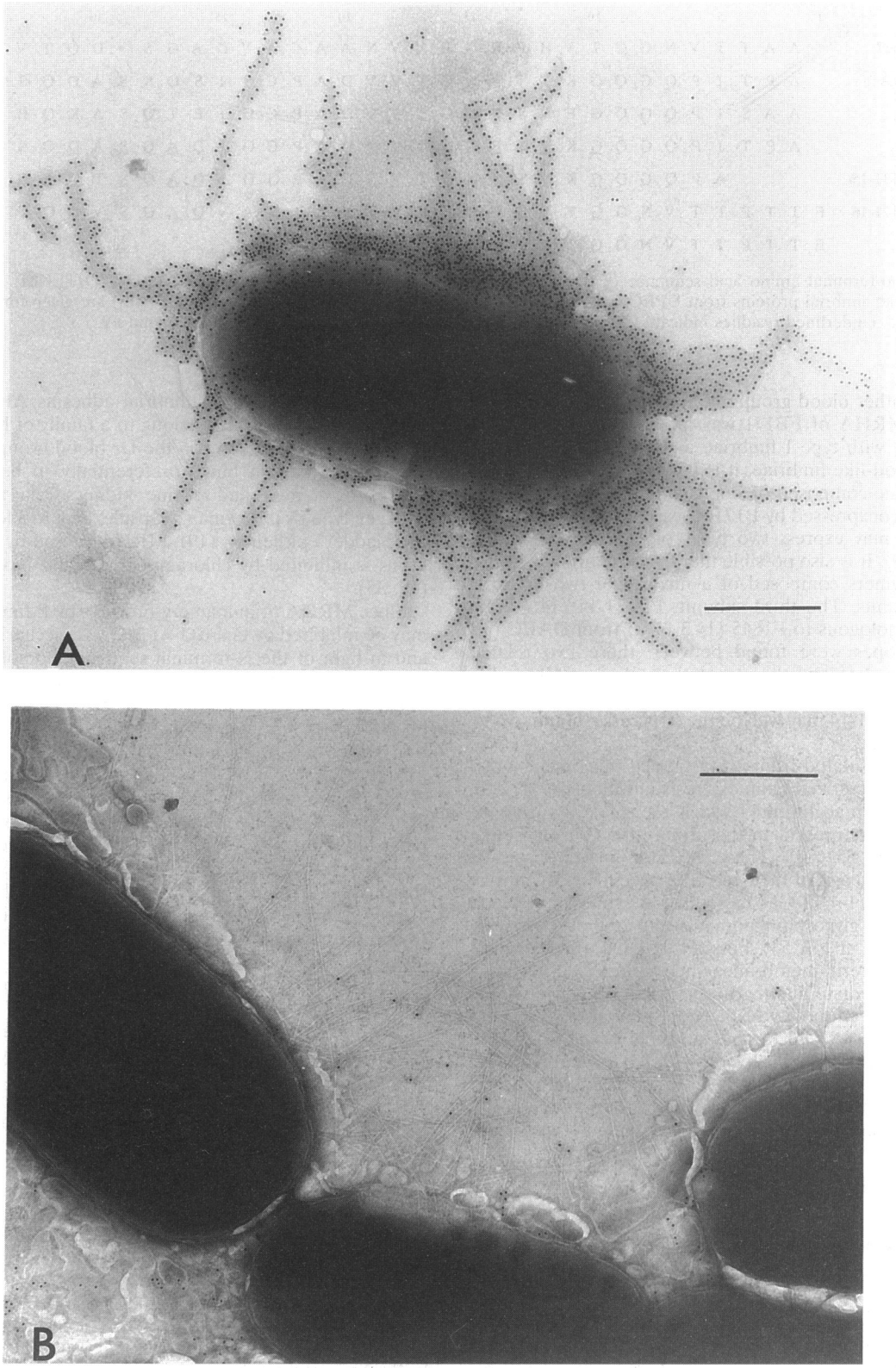

FIG. 5. Detection of type I fimbriae by immunogold labelling. (A) *E. coli* CAB 70 (type I fimbria positive) labelled by the immunogold technique with specific antiserum against type I fimbriae. The long, rigid, 7-nm structures are clearly labelled by this technique. Bar, 0.5 μm. (B) EPEC B171 labelled by the immunogold technique with specific antiserum against type I fimbriae. Rod-like structures produced by strain B171 are not recognized by the type I antiserum. No fibrillae are seen in this micrograph. Bar, 0.5 μm. Cells incubated with PBS instead of the primary antiserum served as a negative control in the experiments illustrated in panels A and B.

	1					5					10					15					20					25					30				
Type I	A	A	T	T	V	N	G	G	T	V	H	F	K	G	E	V	V	N	A	A	C	A	V	D	A	G	S	V	D	Q	T	V	Q
Pap A	A	P	T	I	P	Q	G	Q	G	K	V	T	F	N	G	T	V	V	D	A	P	C	S	I	S	Q	K	S	A	D	Q	S	I
$F7_1$	A	A	S	I	P	Q	G	Q	G	E	V	S	F	K	G	T	V	V	D	A	P	C	G	I	E	T	Q	S	A	K	Q	E	I
$F7_2$	A	P	T	I	P	Q	G	Q	G	K	V	T	F	N	G	T	V	V	D	A	P	C	G	I	D	A	Q	S	A	D	Q	S	I
FB171-15				A	P	Q	G	Q	G	K	V	T	F	N	G	T	V	V	D	A	P	G	G	I	D	A	Q	S	T	D	X	X	I
FB171-16	E	T	T	P	T	T	V	N	G	G	T	V	H	F	K	G	E	V	V	N	A	A	S	A	V	D	A	G	S	V	D	Q	T	V
F9	E	T	T	P	T	T	V	N	G	G	T	V	H	F	K	G	E	V	V															

FIG. 6. Amino-terminal amino acid sequence of the 16.5- and 15.5-kDa subunits of FB171 of EPEC B171 (O111:NM). The N-terminal sequences of other fimbrial proteins from UPEC (i.e., type I [20], PapA [20], $F7_1$ [41], $F7_2$ [49], and F9 [37, 46]) are given for comparison. X, unknown residue. Underlined residues indicate identical residues found between $F7_2$, FB171-15, FB171-16, and F9.

suggests that other blood group antigens could be involved in the binding. MRHA of FB171 was inhibited by FB171 antibodies but not with type I fimbriae antibodies. Since F9 and $F7_2$ are both rod-like fimbriae, it is possible that two types of rod-like fimbriae composed of single subunits of FB171-16 or FB171-15 are coexpressed by B171. It is well known that some UPEC strains may express two types of P fimbriae simultaneously (21, 29). It is also possible that these fimbrial filaments are heteropolymers composed of a mixture of two or three structural subunits. The third subunit, FB171-14 (14.7 kDa) was 100% homologous to F1845 (14.3 kDa) from DAEC (3). Common epitopes were found between these two related subunits. These data and the morphological resemblance between the fibrillar structures visible in Fig. 1A and F1845 suggest that FB171-14 may well be the structural subunit of the fibrillar structures.

UPEC strains isolated from urinary tract infections express several well-characterized mannose-resistant fimbriae (P, F, S, G, M, and Dr hemagglutinins) which recognize a variety of chemical structures present in the urinary tract or on animal erythrocytes (21, 35, 50). These MRHA fimbriae can be subdivided on the basis of their receptor specificity. P fimbriae bind the α-D-Gal(1-4)-β-Gal moiety of the human P blood group antigen and glycosphingolipids of the uroepithelium (18, 26). Westerlund et al. (53, 54) showed that P fimbriae also have affinity for basolateral membranes and immobilized fibronectin. F adhesins mediate binding to the galactose-*N*-acetyl-α(1-3)-galactose-*N*-acetyl moiety of the Forssman antigen present on sheep erythrocytes and the human renal pelvis (21, 22). S fimbriae recognize sialyl galactosides (24, 32), G fimbriae bind to terminal *N*-acetylglucosamine residues (38), and M adhesins adhere to glycophorin A on the M blood group antigen (49). Another important heterogenous group of mannose-resistant

	1					5					10					15				
AFA-I	N	F	T	S	S	G	T	N	G	K	V	D	L	T	I	T	E-			
950 Dr	G	F	T	P	S	G	T	T	G	T	T	K	L	T	V	T	E-			
F1845	T	F	Q	A	S	G	T	T	G	I	T	T	L	T	V	T	E-			
FB171-14	T	F	Q	A	S	G	T	T	G	I	T	T	L	T	V	T	E-			

FIG. 7. Amino-terminal amino acid sequence of the 14.7-kDa subunit of FB171 EPEC B171 (O111:NM). The N-terminal sequences of other afimbrial and fimbrial proteins from UPEC (i.e., AFA-I [19, 35], Dr hemagglutinin from *E. coli* 950 [O75:K5:H^-] [19], and F1845 [3]) are given for comparison. Underlined residues indicate discordant residues between FB171-14 and the sequences given.

hemagglutinins includes afimbrial adhesins AFA-I, AFA-III, F1845, and O75X, which belong to a family of hemagglutinins recognizing the receptor on the Dr blood group antigen (19). The O75X adhesin binds preferentially to basement membranes of human and canine kidneys (via recognition of collagen type IV), Bowman's capsule, and, to a lesser extent, to the bladder epithelium (19). MRHA caused by Dr hemagglutinins is inhibited by chloramphenicol and modified tyrosine (19, 35).

Since MRHA of human erythrocytes by P-fimbriated UPEC may be inhibited by Gal-α(1-4)-β-Gal disaccharide (17, 23, 26) and in light of the N-terminal sequence homologies between FB171 and P fimbriae, we explored the possibility that FB171 might recognize Gal-α(1-4)-β-Gal residues. MRHA produced by FB171 was not inhibited by the Gal-α(1-4)-β-Gal disaccharide or by any other minimum receptor analogs known to inhibit MRHA of S, M, F, G, and Dr hemagglutinins (Table 1). These data suggest that although FB171 represents a mixture of two biochemically related P fimbrial subunits and one Dr hemagglutinin, it shows different receptor specificities. Since FB171 is composed of different fimbrial subunits, it may not be reasonable to expect a single inhibitor to show an effect. Therefore, some of the substances were tested in combination to determine a change in the level of inhibition. No effect was observed in any combination tested (Table 1).

Sialic acid residues on the erythrocyte membrane appeared not to be involved in the interaction with FB171, and instead, higher titers of HA were obtained after sialic acid residues were enzymatically removed from the erythrocyte membrane. There are at least two explanations for this result. First, other surface receptors may be exposed after neuraminidase treatment (32) or, alternatively, the net negative charge of the cell is consequently reduced, favoring the interaction of FB171 and the erythrocytes. In addition, asialofetuin and fetuin showed equal inhibitory effects, suggesting that sialyl galactosides are not recognized by FB171. Moreover, sialyl-containing compounds like orosomucoid, *N*-acetylneuraminlactose and *N*-acetyllactosamine did not affect MRHA by FB171.

Recently, the interaction of matrix basement membrane glycoproteins with *E. coli*, P fimbriae, and O75X hemagglutinins has been reported (12, 53, 54). The interaction between P fimbriae and fibronectin does not involve the PapG subunit [pilus tip protein responsible for binding to Gal-α(1-4)-β-Gal residues] of the fimbriae (28) or the RGDS cell binding domain of fibronectin (54). The active regions for binding of P fimbriae are located at the N- and C-terminal fragments of fibronectin (54). The 40-kDa amino-terminal region of fibronectin binds to some bacterial pathogens (15) and contains

TABLE 1. Receptor specificities that were recognized by fimbrial hemagglutinins of UPEC and that did not show any inhibitory effect on MRHA exhibited by FB171 of EPEC B171

Compound(s)	Fimbria(e)	Concn used (mg/ml)[a]	MIC reported (mg/ml)[b]	Reference(s)
Gal-Gal disaccharide	Pap	8.3	0.068	18, 26
N-Acetylglucosamine	G	1.3	2.2	38, 49
N-Acetylgalactosamine	G	166.6	11	18, 49
N-Acetylneuraminic acid	S	1.3	1.0	24, 32
N-Acetylneuraminlactose	S	1.3	0.19	24
GM_1		33.3	0.2	26
Globoside	Pap	1.66	0.2	18, 26
Glucocerebroside		0.66	0.2	18, 26
Trihexosylceramide	Pap	0.33	0.2	18, 46
Fucose		33.3	2.5	46
D-Mannose	Type I	3.33	0.29	41
Chloramphenicol	Dr	2.0	0.00065	35
Orosomucoid		1.66	1.0	32
Glycophorin A	M,S	0.66	0.24	50
Asialoglycophorin		0.166	0.11	49
RGDS		1.66		37
40-kDa Fibronectin		0.01		37
Chloramphenicol + gangliosides		—[c,d]		
Chloramphenicol + Gal-Gal		—		
Gal-Gal + gangliosides		—		
Gal-Gal + fucose		—		
Gal-Gal + glucocerebroside		—		
Gal-Gal + globoside		—		

[a] Highest concentration of inhibitor tested in the MRHA assay.
[b] The MIC is that reported by other authors as inhibitory of MRHA of fimbriae found in UPEC. MRHA of FB171 and inhibition assays are described in Materials and Methods.
[c] —, substances were mixed 1:1 at the same concentrations as those when used alone.
[d] Weak inhibition due to gangliosides (see Table 2).

TABLE 2. MICs of compounds that inhibited MRHA of human erythrocytes by FB171

Inhibitor	MIC	Level of inhibition[a]
Collagen type IV	0.2 ng/ml	8
Gelatin	0.1 ng/ml	10
Fibronectin	2.1 ng/ml	3
Laminin	35 ng/ml	6
Collagen type I	2.5 μg/ml	3
Collagen type III	1.2 μg/ml	3
Collagen type V	2.5 μg/ml	3
Mixed gangliosides	0.13 μg/ml	2
Fetuin	0.2 mg/ml	2
Asialofetuin	0.1 mg/ml	2
Collagen type IV + fibronectin	—[b]	8
Gelatin + fibronectin	—	10
Collagen type IV + asialofetuin	—	4
Asialofetuin + fibronectin	—	3
Asialofetuin + collagen type IV + fibronectin	—	6
Gangliosides + fibronectin	—	2
Fibronectin + antifibronectin serum	—	10

[a] Range, 0 to 10. 0, no inhibition at the highest concentration of a substance; 10, highest titer of inhibition of the strongest inhibitor.
[b] Substances were mixed 1:1 at the same concentrations used when used alone.

a binding domain for collagen, gelatin, and gangliosides (30, 37). MRHA by FB171 was inhibited by fibronectin but not by the 40-kDa domain of the N-terminal region of fibronectin or the RGDS tetrapeptide, which suggests that other regions of the fibronectin molecule are involved in the interaction with FB171. UPEC strains carrying F8, F9, F10, F12, and F13 were reported by Speziale et al. (45) to bind laminin but not soluble fibronectin. P fimbriae of serotypes $F7_1$, $F7_2$, F8, F11, F13, and F14 were shown to bind immobilized fibronectin but not soluble fibronectin (54). Thus, differences in the reactivity of the different serovariants of P fimbriae with fibronectin may depend on conformational differences between the soluble and the insoluble forms of fibronectin (15, 54).

In addition to fibronectin, high inhibitory activities were also observed with collagen type IV, laminin, and gelatin and to a lesser extent with collagen types I, II, and V, mixed gangliosides, and fetuin. The effect observed with these inhibitors is less likely to be due to steric hindrance since not all molecules of comparable mass show the same effect. Sialic residues

TABLE 3. Comparison of properties of FB171 of EPEC and P fimbriae of UPEC

Strain	Fimbriae	Mass (kDa)	Agglutination of erythrocytes
UPEC	F9	22	Sheep, human, ox
EPEC B171	FB171-16	16.5	Human
UPEC	$F7_2$	16	Sheep, ox
EPEC B171	FB171-15	15.5	Human
DAEC	F1845	14.3	Human
EPEC B171	FB171-14	14.7	Human

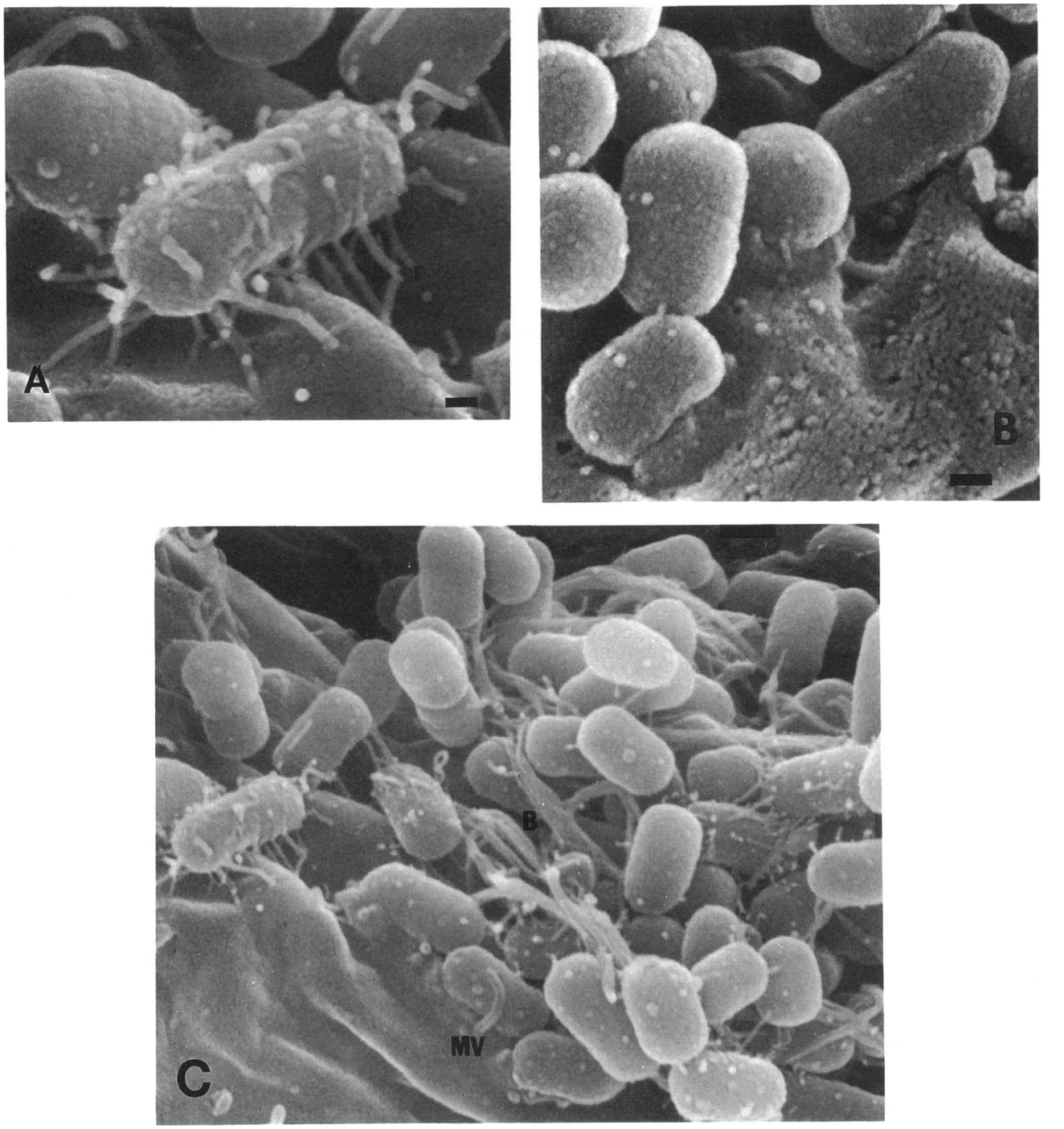

FIG. 8. Scanning electron microscopy of EPEC showing LA on HEp-2 cells. (A) High magnification of a bacterium adhering to the HEp-2 cell surface via fimbria-like (F) structures. This micrograph illustrates the first stage of EPEC adherence, referred to as initial attachment. Bar, 0.14 μm. (B) Cup-like or pedestal formation of the cell membrane induced by EPEC. This process has been referred to as the second stage of EPEC infection. Bar, 0.25 μm. (C) Multiple interactions between cellular and bacterial surface structures, for example, cell membrane engulfing bacteria; microvilli (MV) promoting close attachment, rope-like structures resembling BFP (labelled B) interlinking bacteria. Bar, 0.56 μm. (D) Presence of structures resembling BFP (labelled B) clearly demonstrated within the LA cluster. Arrowheads indicate putative BFP structures. Microvilli can be readily differentiated from BFP because microvilli have a tubular structure as opposed to BFP which resemble braids of hair or wicks. Fimbriae (F) also appear to tether individual bacteria to other bacteria. Bar, 0.56 μm.

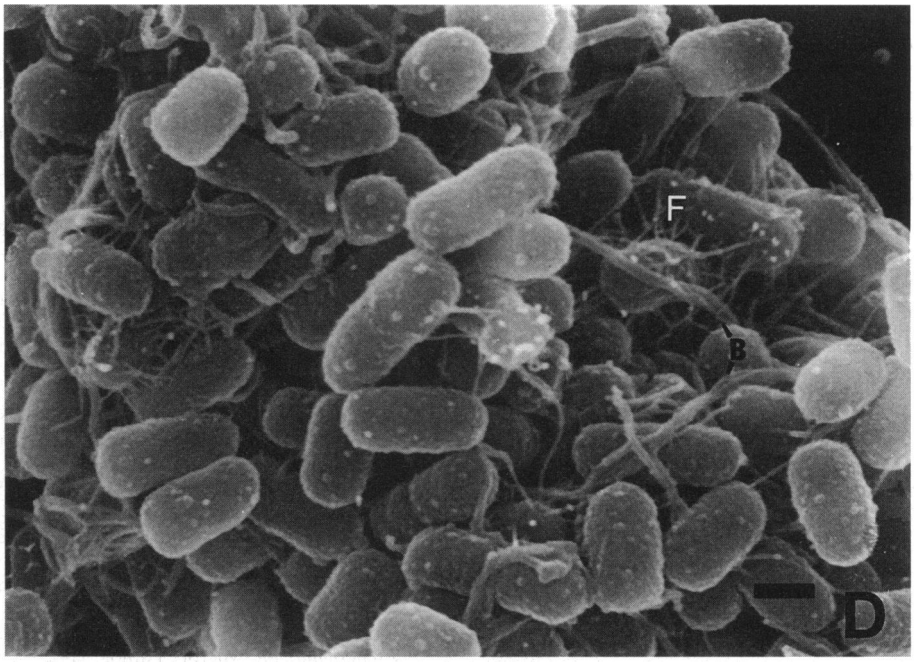

FIG. 8—*Continued.*

present on gangliosides (56) are most likely not involved in the interaction between FB171 and gangliosides. Fibronectin and collagen did not agglutinate human erythrocytes, which suggests that the inhibitory activity of these molecules is not through blocking of FB171 receptors on the erythrocytes. Most likely, the inhibitory activity is due to direct binding of FB171 to the inhibitors. The studies with mixtures of inhibitors suggest that FB171 recognizes binding sites on matrix glycoproteins (Table 2) through one or more adhesins. Whether the properties of HA, the interaction with the matrix glycoproteins, or the adherence to HEp-2 cells or to the intestinal mucosa resides in one or more adhesins of FB171 or similar appendages in EPEC needs to be further studied in detail. These kinds of studies might be helpful in mapping binding domains on matrix glycoproteins for bacterial adhesins and in learning more about the interaction among these molecules. Our data do not answer the question of whether the subunits found in FB171 form different filamentous structures or heteropolymeric filaments. Thus, the genetic dissociation of the adhesins present in FB171 will aid in answering this question and in determining which is the adhesin(s) or sequence responsible for the binding specificity to matrix glycoproteins as well as in helping characterize their contribution to virulence.

The significance of the interaction between matrix glycoproteins and EPEC fimbriae in the pathogenesis of EPEC remains unclear. Histopathology of EPEC infection shows close adherence to and pedestal formation on epithelial cells, accompanied by an increase in intracellular calcium, which leads to disruption of the brush border cell integrity in the small bowel, tissue damage, and inflammation (16, 22, 33). Exposure of subepithelial connective tissue proteins would facilitate adhesion to basolateral membranes via recognition of matrix glycoproteins. This type of interaction could be useful for EPEC at later stages of infection, allowing persistence in intestinal tissues after disruption of apical membranes and invasion of the gut epithelium (1, 8, 33, 39). Our data highlight the versatility of these organisms to persist in different environments via recognition of a repertoire of receptors mediated by fimbrial adhesins.

The scanning microscopic study of the interaction of EPEC with cultured eukaryotic cells and the inhibition of initial adherence by anti-FB171 antiserum support the model proposed by Knutton et al. (22) which suggests that fimbrial structures are important for initial attachment of EPEC. Francis et al. (11) demonstrated that while the EAF plasmid was necessary and sufficient for efficient association of EPEC to HEp-2 cells, both the EAF plasmid and a chromosomally encoded factor (*eae*) were required for the ability to efficiently efface, cause formation of attaching and effacing lesions, and enter these cells. These workers proved that the EAF plasmid-mediated initial adherence of EPEC to HEp-2 cells can be substituted in EAF-negative, EAF-positive strains by an afimbrial adhesin or type I fimbriae of UPEC, supporting a role of fimbriae in the association of EPEC with eukaryotic cells. In this study, structures resembling fimbriae are seen interacting with the surface of the eukaryotic cell, microvilli, and other bacteria (Fig. 8). Whether these fimbria-like structures correspond to FB171 remains to be elucidated. FB171 antibodies were shown to recognize the native fimbriae by immunogold, to inhibit HA, and to inhibit by 75% the initial attachment of EPEC B171 to HEp-2 cells in a modified 60-min adherence assay. Collectively, these data suggest a role of FB171 in the interaction with HEp-2 cells. Previously, we have shown that BFP antibodies reduced LA of EPEC to HEp-2 cells (12). The combination of FB171 and BFP antibodies totally inhibited the LA phenotype. BFP are seen by scanning electron microscopy to connect bacteria to bacteria and appear to facilitate the formation of tight clusters or infectious units. Similar functions have been attributed to structures resembling BFP expressed by different pathogens like *Neisseria gonorrhoeae*, *Pseudomonas aeruginosa*, *Moraxella bovis*, *Bacteroides nodosus*, and *V. cholerae* (47, 48).

The scanning microscopic studies suggest that the LA phe-

nomenon is a multifactorial process in which bacterial and eukaryotic cell factors are coparticipating. The formation of typical LA clusters or microcolonies may take place by replication of attached bacteria and/or recruitment of proximate bacteria or clusters of bacteria (infectious units) present in the supernatant in the vicinity of the cell surface, linked together via BFP. Bacterial surface hydrophobicity conferred by LPS and outer membrane proteins may also contribute to bacterium-bacterium interactions. It is possible that environmental signals are controlling expression of adherence determinants in EPEC. The multifaceted nature of the host-parasite interaction indicates that more than one virulence determinant is typically involved in pathogenesis (10). Future studies of the unique aspects of EPEC adherence will enable a better understanding of the interactions between adherent bacteria and eukaryotic cells.

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