INFECTION AND IMMUNITY, Jan. 1987, p. 86–92
0019-9567/87/010086-07$02.00/0

Vol. 55, No. 1

Identification of a New Fimbrial Structure in Enterotoxigenic *Escherichia coli* (ETEC) Serotype O148:H28 Which Adheres to Human Intestinal Mucosa: a Potentially New Human ETEC Colonization Factor

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Three important fimbrial colonization factor antigens (CFAs) designated CFA/I, CFA/II, and E8775 were identified originally in some human enterotoxigenic *Escherichia coli* (ETEC) strains because of their mannose-resistant hemagglutination properties. To identify CFA, in strains lacking mannose-resistant hemagglutination properties we exploited the abillity of human ETEC strains to adhere to human proximal small intestinal mucosa. ETEC strain B7A (O148:H28) was selected for study because it belongs to an epidemiologically important serotype and does not produce a known CFA, and yet it is known to be pathogenic and cause diarrheal disease in human volunteers. Results of an human enterocyte adhesion assay indicated that some bacteria in cultures of B7A produced adhesive factors. To select for such bacteria, cultured human duodenal mucosal biopsy samples were infected with B7A for up to 12 h, after which time a large percentage of the mucosal surface became colonized by bacteria. A new fimbrial structure morphologically distinct from CFA/I, CFA/II, and E8775 fimbriae and consisting of curly fibrils (~3 nm in diameter) was readily identified when bacteria were subcultured from the mucosa and examined by electron microscopy. Identical fimbriae were produced by ETEC strain 1782-77 of the same serotype. Identification of these fimbriae only on bacteria subcultured from human intestinal mucosa strongly suggests that they promote mucosal adhesion of ETEC serotype O148:H28 and thus represent a potentially new human ETEC CFA.

Enterotoxigenic *Escherichia coli* (ETEC) is a major cause of diarrheal disease in infants in less developed countries (29) and in travelers going from developed to less developed countries (23). Adhesion of ETEC to the intestinal mucosa is now recognized as an important, if not essential, early event in colonization and the development of diarrheal disease (13). The ability of ETEC to colonize the gut has been directly correlated with the presence of adhesion fimbriae that allow ETEC to bind to specific receptors on proximal small intestinal enterocytes (13, 26). The specificity of adhesin-receptor interactions often extends, fortuitously, to agglutination of erythrocytes, and this has proved useful in screening ETEC for adherence properties that are of potential significance in intestinal colonization (25). Three important fimbrial colonization factors antigens (CFAs) designated CFA/I, CFA/II, and E8775 were originally identified in some human ETEC strains because of their mannose-resistant hemagglutination (MRHA) properties (9, 12, 30). Heterogeneity within the entities CFA/II (2, 28) and E8775 (31) are now known to exist. Many ETEC isolates, however, do not cause MRHA of erythrocytes from a wide range of species, nor have their CFAs been identified.

To study the adhesive properties of human ETEC strains we developed in vitro adhesion assay systems employing host small intestinal enterocytes (15, 17). When a collection of 40 ETEC isolates was examined in the assay, those strains producing CFA/I and CFA/II showed good brush border adhesion, whereas strains lacking these CFAs were nonadherent (17). Our inability to detect fimbriae produced by the nonadherent strains by electron microscopy led us to conclude that nonadherence is due to the lack of expression of CFAs rather than the absence of brush border receptors. To examine this possibility we selected a single strain for further study. Strain B7A (O148:H28) (8) was selected because it is a well-characterized strain belonging to a serotype that is frequently isolated throughout the world. It does not possess MRHA properties and has never been found to possess CFA/I, CFA/II, or E8775 fimbriae (19); and yet it is known to be pathogenic and cause diarrheal disease when fed to volunteers (20). In this report we show that some bacteria in cultures of B7A produce fimbriae that are distinct from known human ETEC CFA fimbriae, and we also describe a technique in which we employed cultured human intestinal mucosa to select and enrich for such bacteria.

MATERIALS AND METHODS

Bacterial strains. Strains B7A and 1782-77 are heat-labile (LT) and heat-stable (ST) enterotoxin-positive human ETEC isolates belonging to serotype O148:H28. The strains were obtained from M. M. Levine, Center for Vaccine Development, University of Maryland, Baltimore, Md., and K. Wachsmuth, Centers for Disease Control, Atlanta, Ga., respectively. Stock cultures were subcultured into broth or onto solid media (see below) and incubated aerobically for 18 h at 37°C. Strains were routinely examined for fimbriae production by electron microscopy and monitored for MRHA of human and bovine erythrocytes and mannose-sensitive hemagglutination of guinea-pig erythrocytes, as described previously (16).

Enterocyte adhesion. Enterocytes were isolated from human duodenal biopsy samples by an EDTA-chelation method as described previously (17). Cells from four biopsies were suspended in 10 ml of HEPES (*N*-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid)-buffered modified

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TABLE 1. Adhesion of ETEC to isolated human duodenal enterocytes

Strain and growth medium[a]	Incubation time (h)	Enterocyte adhesion (mean no. of bacteria/brush border)[b]
B7A		
CFA agar	0.5	0.01
CFA agar	3	2.8
M/H broth	3	2.7
Nutrient broth	3	3.3
1782-77		
CFA agar	0.5	0
CFA agar	3	2.0
M/H broth	3	1.8
H10407, CFA agar[c]	0.5	2.3
M424C1, CFA agar	0.5	2.3

[a] M/H, Mueller-Hinton.
[b] Mean of at least two assays.
[c] Data for H10407 (CFA/I) and M424C1 (CFA/II) were taken from reference 17 and are included for comparison.

Eagle medium containing 0.5% D-mannose. For each adhesion assay 1 ml of the enterocyte suspension was added to 2 ml of a washed suspension of bacteria (10^7 to 10^9/ml) in a bottle (Bijoux), and the contents were incubated for up to 3 h at 37°C on a rotary mixer. Nonadherent bacteria were removed by repeated washing, and adhesion was quantitatively assessed as described previously (15) by counting the bacteria that adhered to the brush border of 50 cells selected at random.

Adhesion to cultured intestinal mucosa. Human duodenal mucosal biopsy samples were maintained in culture as described previously (18). Biopsy samples were oriented with villi facing up and were placed in petri dishes (3 cm in diameter) on sterile sponge supports saturated with culture medium (NCTC 135–Dulbecco modified Eagle medium [1:1] plus 10% newborn calf serum). The level of the medium was adjusted so that a thin layer covered the villous surface of the biopsy sample. Petri dishes were placed in a sealed container gassed with 95% O_2–5% CO_2 and maintained at 37°C on a rocking table. For adhesion studies 25 μl of a bacterial suspension was placed onto the mucosal surface of the biopsy sample, and culturing was continued for up to 12 h. To maintain pH and prevent overgrowth of bacteria the culture medium was replaced with fresh medium every 2 to 3 h. At the end of the incubation period biopsy samples were thoroughly washed with several changes of fresh medium prior to fixation for microscopy.

Tissue processing. Biopsy samples to be examined by light microscopy were fixed in Formalin and embedded in paraffin, and sections were stained either with hematoxylin-eosin or Giemsa. Tissue for electron microscopy was fixed in 3% glutaraldehyde, postfixed in 1% osmium tetroxide, and embedded in Epon. For ruthenium red staining, ruthenium red was added to both fixative and wash solutions at a final concentration of 0.075%. Sections were stained with aqueous uranyl and lead salts and examined in an electron microscope (EM301; Philips). For scanning electron microscopy fixed tissue samples were dehydrated through graded acetone solutions and dried to the critical point. Mounted tissue was coated with gold and examined in a scanning electron microscope (S4; Cambridge). For negative staining of bacteria, 10 μl of a washed bacterial suspension was mixed with equal volumes of bacitracin (150 μg/ml) and

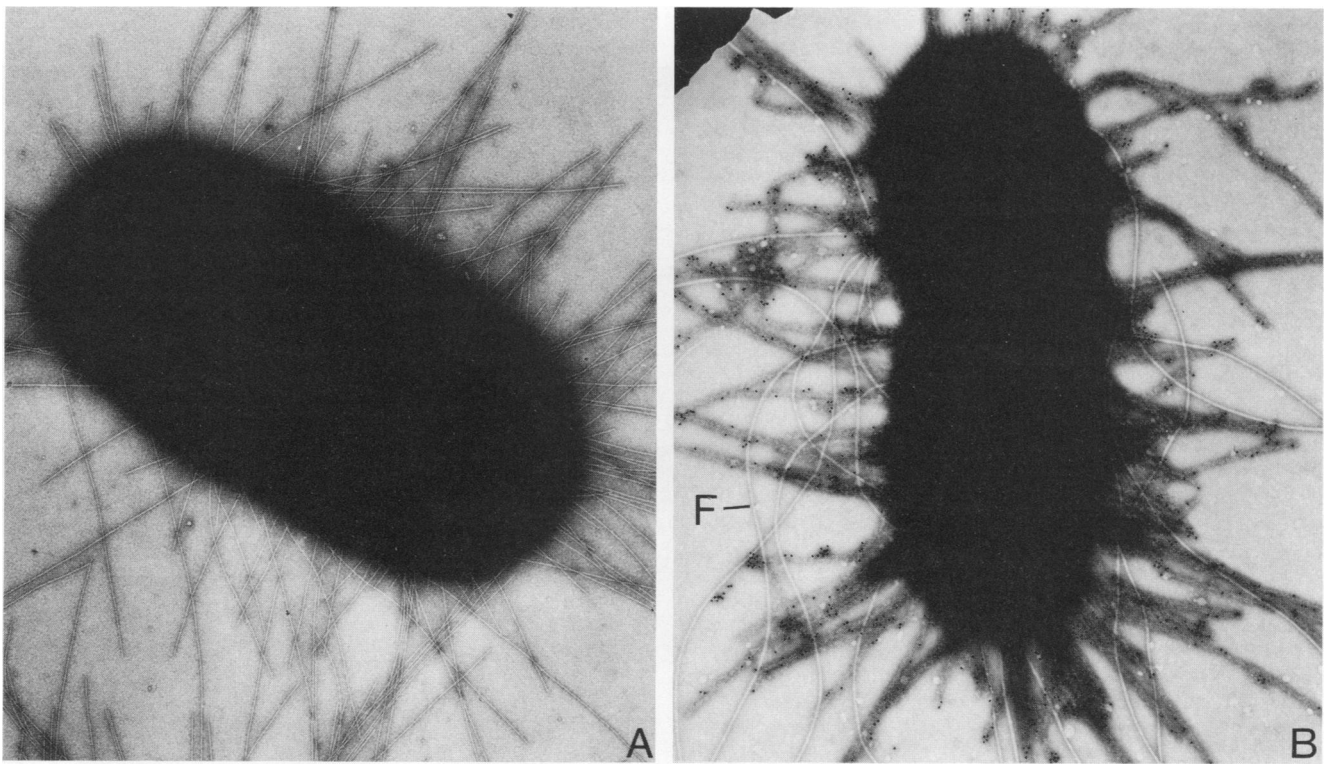

FIG. 1. Production by B7A and 1782-77 of long, rigid, rodlike fimbriae (~7 nm in diameter) (a) that were labeled specifically with anti-type 1 fimbrial antibody (B). F, Flagella. Magnification: A, ×40,000; B, ×35,000.

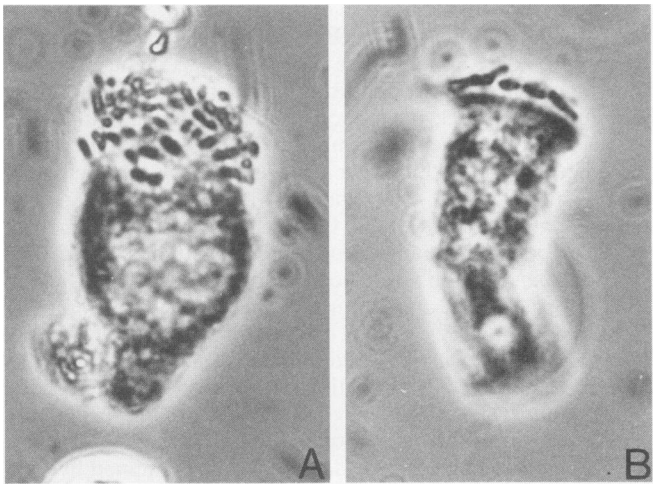

FIG. 2. Phase-contrast micrographs illustrating specific brush border adhesion of ETEC strains B7A (A) and 1782-77 (B). Magnification: ×1,000.

ammonium molybdate (2%, pH 7.0). A total of 10 μl was applied to carbon-coated grids for 30 s, and the excess liquid was removed with filter paper. Immunogold labeling of type 1 fimbriae was carried out as follows. A total of 10 μl of a washed suspension of bacteria was applied to carbon-coated grids for 2 to 3 min. Excess liquid was removed, and the grid was immediately placed face down onto a drop of a suitable dilution of antiserum for 15 min. Antiserum to purified type 1 fimbria was a generous gift of M. M. Levine, Center for Vaccine Development, and was prepared as described previously (16). After a thorough washing, grids were placed on drops of goat anti-rabbit immunoglobulin adsorbed onto gold particles (10 nm in diameter; Janssen Pharmaceuticals, Wantage, United Kingdom) for 15 min. After a further thorough washing, grids were negatively stained with 1% ammonium molybdate. All incubation and wash solutions contained 1% bovine serum albumin and 1% Tween 20 to reduce nonspecific labeling. Grids incubated without the primary antibody or with heterologous antibody were used as controls.

RESULTS

Adhesion to isolated enterocytes. We have previously demonstrated good brush border adhesion of strains producing CFA/I and CFA/II fimbriae following the incubation of enterocytes with bacteria for 30 min at 37°C (15, 17). Under these assay conditions strains B7A and 1782-77 were nonadherent (17) (Table 1). Levels of brush border adhesion comparable to those observed for CFA/I- and CFA/II-positive ETEC were obtained, however, when the incubation time was increased from 30 min to 3 h, irrespective of whether bacteria were grown on solid media or in broth (Table 1). Bacterial cultures used in adhesion assays were routinely examined by electron microscopy for production of fimbriae. Rigid, rodlike fimbriae (~ 7 nm in diameter) were produced by many bacteria in agar cultures of strain 1782-77 and in static broth cultures of both strains (Fig. 1A). On the basis of mannose-sensitive hemagglutination of human and guinea pig erythrocytes (7) and immunoelectron microscopy (Fig. 1B), these were identified as type 1 fimbriae. Other fimbriae were not detected. Type 1 fimbriae promoted adhesion of bacteria to enterocyte basolateral surfaces, but in the presence of D-mannose adhesion of both strains was specifically localized to the enterocyte brush border (Fig. 2). Because type 1 fimbriae do not function to

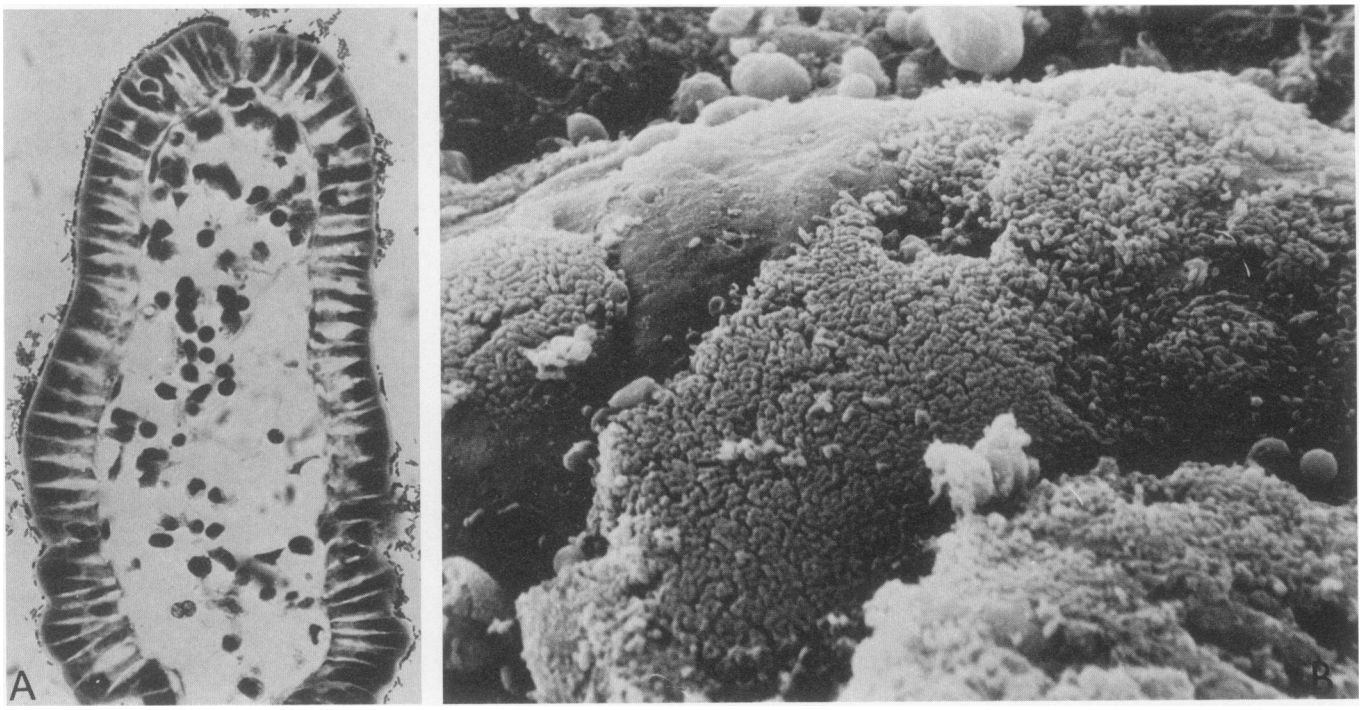

FIG. 3. Cultured human duodenal mucosa infected with B7A for 10 h. Histologic (A) and scanning electron microscopic (B) examination revealed extensive bacterial colonization of the mucosal surface. Magnification: A, ×350; B, ×1,000.

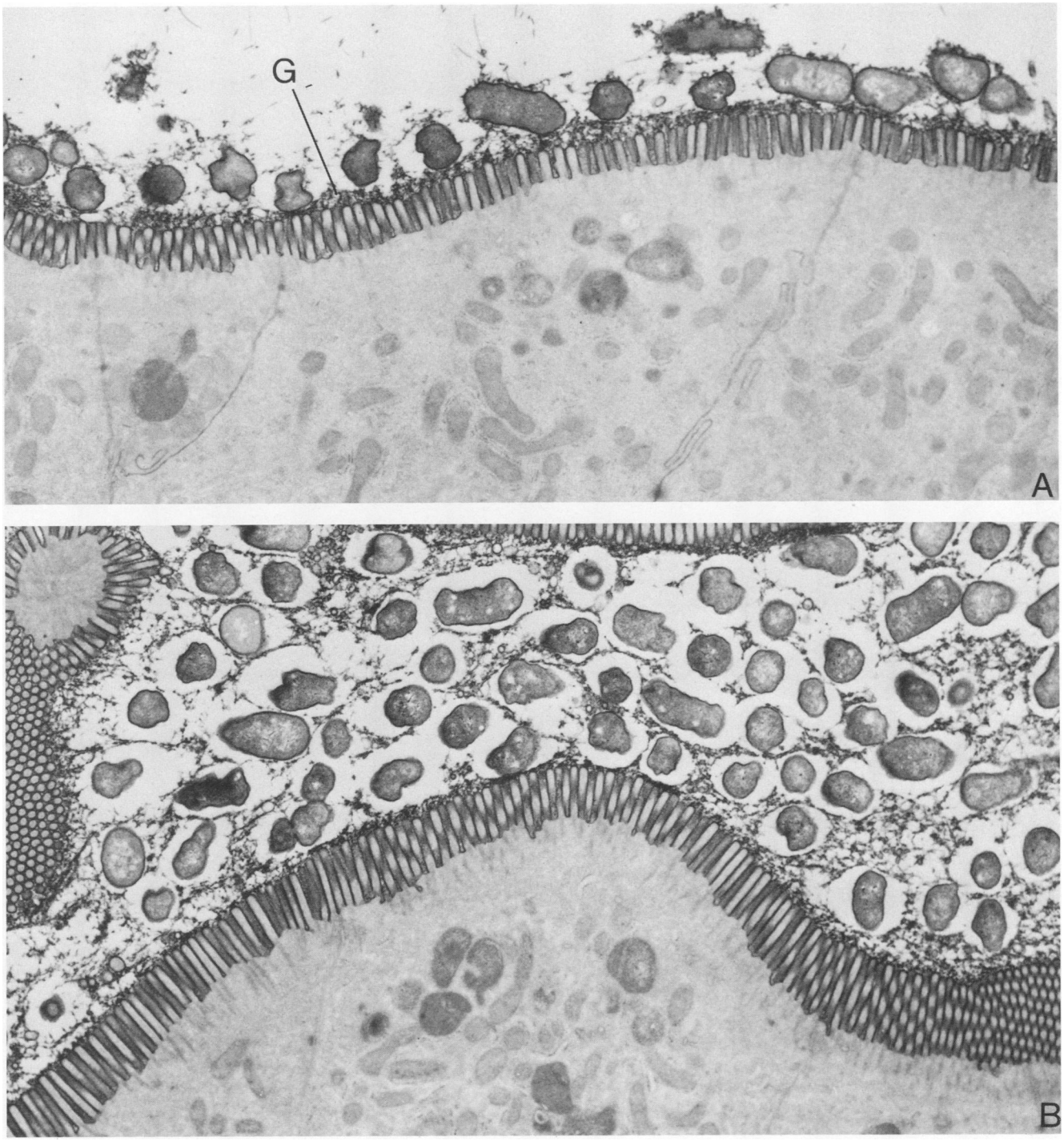

FIG. 4. Electron micrographs of cultured intestinal mucosa colonized by B7A. In ruthenium red-stained preparations bacteria are seen adhering to the enterocyte glycocalyx (G) (A). Note the distinct electron-translucent halo which surrounds adherent bacteria (B). Magnification: ×12,000.

promote brush border attachment of ETEC (15, 17), other adhesins must be produced by strains B7A and 1782-77. The long incubation time required to observe good brush border adhesion of B7A and 1782-77 and our inability to detect adhesion fimbriae by electron microscopy suggested that only a small number of bacteria in our cultures produced the adhesin. A technique employing cultured human small intestinal mucosa was therefore developed to select for bacteria in cultures of B7A and 1782-77 that were producing CFAs.

Adhesion to cultured human intestinal mucosa. Intestinal mucosal biopsy samples maintained in culture for up to 12 h routinely showed good morphological preservation of the mucosa by both light and electron microscopy. To assess the ability of strain B7A to adhere to cultured human duodenal

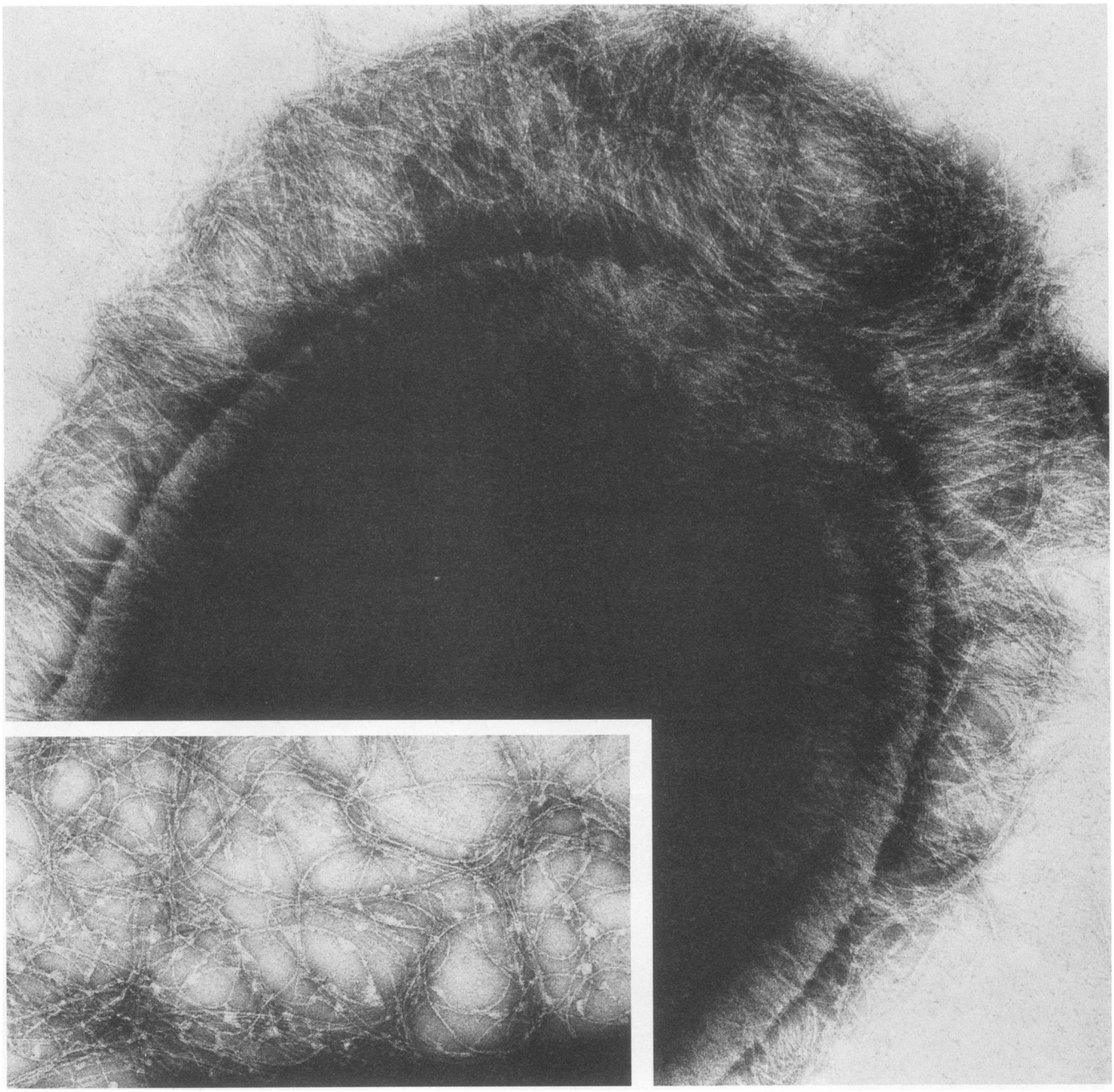

FIG. 5. Electron micrographs of B7A cultured from intestinal mucosa. Curly fibrillar fimbriae (~3 nm in diameter) were produced by many bacteria. Magnification: ×150,000; inset, ×120,000.

mucosa, biopsy samples were examined histologically and by scanning electron microscopy at various time intervals following infection. Mucosally adherent bacteria were detected after 2 to 3 h. The numbers of adherent bacteria increased with time, and after 12 h extensive colonization of the mucosal surface was observed (Fig. 3).

Transmission electron microscopy of colonized mucosa revealed a single layer of adherent bacteria, although the bacterial surface structures mediating attachment were not visualized in this type of preparation (Fig. 4A). Nevertheless, a distinct electron-translucent zone that separated bacteria from the mucosal surface was indicative of fimbrially mediated attachment. This electron-translucent halo surrounding adherent bacteria was most noticeable in those areas of ruthenium red-stained preparations in which there was a large amount of ruthenium red-positive mucus material, i.e., in invaginations of the mucosa (Fig. 4B).

Bacterial fimbriae are most clearly visualized by negative staining. We therefore subcultured bacteria from colonized mucosa on to CFA agar (12) and examined bacteria by negative staining. A surface coat (thickness, 0.3 μm) consisting of a dense mass of curly fibrils (~3 nm in diameter) was seen at the surface of many bacteria (Fig. 5); much longer and less densely arranged fibrils were seen at the surface of some bacteria (Fig. 5, inset). Identical fibrils were produced by both B7A and 1782-77. Bacteria cultured from

intestinal mucosa were MRHA negative for human and bovine erythrocytes.

DISCUSSION

ETEC serotype O148:H28 has been repeatedly isolated throughout the world as LT and ST strains and yet the CFA of this serotype has not been identified (19). Strains could have lost plasmids coding for known CFAs, although the ability of B7A to colonize the gut and cause disease in human volunteers (20) indicated that B7A produces a CFA distinct from CFA/I, CFA/II, and E8775. B7A and 1782-77 produce type 1 fimbriae, but in this study and elsewhere (15, 17) we have shown that type 1 fimbriae do not promote adhesion of ETEC to human small intestinal enterocyte brush borders. In this study we have shown that B7A and 1782-77 adhere specifically to human small intestinal enterocyte brush borders and produce, in addition to type 1 fimbriae, fimbriae consisting of curly fibrils (~3 nm in diameter). The identification of this new fimbrial structure only on bacteria subcultured from human intestinal mucosa strongly suggests that these structures promote mucosal attachment of strains B7A and 1782-77. Curly fibrils (~3 nm in diameter) are morphologically different from CFA/I (6, 10), CFA/II (21), E8775 (30), and other putative human ETEC adhesion fimbriae (3, 5, 6, 14) and thus represent a potentially new human ETEC CFA. Studies are currently in progress to prepare an antiserum to the fimbrial antigen so that a more definitive assessment of their role as a potential CFA can be made.

Curly fibrillar fimbriae have been reported previously in two O8:H9 human ETEC isolates (27), but these are probably different than the O148 fimbriae. Their reported diameter was greater than that of the O148 fimbriae described here, and cultures of these strains were MRHA positive for bovine erythrocytes, whereas B7A and 1782-77 were MRHA negative. One possibility that must be considered is that the O148 fimbriae described and the CS6 antigen of E8775-positive ETEC are the same antigen. Three surface antigens designated coli surface antigens 4, 5, and 6 (CS4, CS5, and CS6, respectively) were recently identified within the entity E8775 now designated PCF8775; CS4 and CS5 were shown to be fimbriae but CS6 was not identified (31). Long curly fibrils similar to those produced by B7A and 1782-77 were found in some PCF8775-positive strains, but these were not thought to be CS6 because their presence did not correlate with that of CS6, nor did they react antigenically as did CS6 when it was tested by immunoelectron microscopy (31). In view of the recent identification of CS6 in two O148 ETEC isolates (22), however, the conclusion that the long curly fibrils described by Thomas et al. (31) are not CS6 should probably now be reevaluated.

Long, rigid, rodlike fimbriae (~7 nm in diameter) similar to CFA/I (16), the CS1 and CS2 fimbriae of CFA/II-positive ETEC (21), and the CS4 and CS5 fimbriae of PCF8775-positive ETEC (31) are relatively easy to visualize by electron microscopy. It is becoming clear, however, that other human ETEC adhesion fimbriae can have a much finer fibrillar structure similar to the K88, K99, and F41 fimbriae of animal ETEC (13). Some of these fimbriae can be more difficult to visualize by electron microscopy. The 2- to 3-nm-diameter fibrillar CS3 fimbriae of CFA/II-positive ETEC, for example, initially eluded detection and were thus thought to be nonfimbrial (24, 28). The findings of this study suggest that other ETEC CFAs classified as nonfimbrial (4) may yet turn out to have a fimbrial structure. Fimbriae like those described in this study, which are relatively easy to visualize by electron microscopy, may have previously eluded detection because insufficient bacteria in cultures actually produced fimbriae. It is well known that ETEC exhibits fimbrial phase variation, the random reversible on-off switching of fimbriae production (1), and that expression of adhesion fimbriae is profoundly affected by the culture conditions. CFA fimbriae, for example, are preferentially expressed when bacteria are grown on minimal solid media (11), whereas type 1 fimbriae production is favored in static broth cultures (7). Results of this study indicate that some bacteria in both broth and agar cultures produce adhesins, and we were able to enrich cultures sufficiently to identify a new type of fimbria by electron microscopy. Further enrichment and cloning of fimbriate bacteria will allow these structures to be purified and characterized further.

Known adhesion fimbriae are found in approximately 65 to 75% of LT^+ and ST^+ strains, in 20 to 25% of LT^- and ST^+ strains, and rarely in LT^+ and ST^- strains (19). The feasibility of immunoprophylaxis of ETEC diarrhea in humans by means of purified fimbrial vaccines will depend on the identification of CFAs common to most human ETEC pathogens. The procedures described in this report are one approach to the identification of adhesion fimbriae in the many ETEC isolates that currently lack known CFAs.

ACKNOWLEDGMENTS

We thank R. Cockel for providing intestinal tissue and K. Wachsmuth and M. M. Levine for bacterial strains.

This work was supported by Medical Research Council project grant G8517290SB to S.K.

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