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# Attachment Pili from Enterotoxigenic *Escherichia coli* Pathogenic for Humans

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Pili from enterotoxigenic *Escherichia coli* pathogenic for humans have been isolated by adsorption to the surface of erythrocytes followed by thermal elution. The pili are composed of two protein subunits with molecular weights of 13,100 and 12,500 as determined by sodium dodecyl sulfate-gel electrophoresis. These pili also bind to human buccal cells under temperature conditions (37°C) which prevent the binding of these pili to the erythrocytes. Analogous temperature effects on binding have previously been observed with whole bacterial cells. This binding can be inhibited by antiserum prepared against the isolated pili.

The adhesion of enterotoxigenic *Escherichia coli* (ETEC) to the mucosal surface of the small bowel and their subsequent resistance to clearance mechanisms such as peristalsis are important steps in the pathophysiology of diarrheal disease. This adhesion is an initial step in the "colonization" of the small bowel (8-10, 12, 14, 19, 25). We would reserve the term colonization for demonstration of mucosal attachment, as well as growth and multiplication of bacteria within the small bowel. Because colonization is a complex event, we have chosen to separate these components, studying each individually rather than approaching the entire process with a complex model.

Previous studies have established that K88 and K99 pili are responsible for the host-specific attachment of ETEC to the small bowel mucosa of pigs and calves, respectively (1, 16, 18, 20, 26, 28, 33, 34). These pili are heat-labile protein surface antigens which project out from the bacterial cell like "fur" (35). Bacteria with these surface pili also hemagglutinate (HA) erythrocytes (RBC) in the presence of the sugar mannose (5, 9, 21, 27, 29). This reaction may be termed mannose-resistant hemagglutination (MR-HA). In contrast, the HA reaction mediated by another kind of pili, termed type 1 pili, is inhibited by mannose (6, 30). Type 1 pili are present on a wide variety of pathogenic and nonpathogenic *E. coli* strains (2-4) and therefore are not thought to be correlated with the ability to cause human diarrheal disease. A further distinction is that the MR-HA reaction occurs at 0°C and is reversed at elevated temperatures, whereas the type 1 pili-mediated HA is not temperature sensitive.

The K88 and K99 pili are also morphologically and chemically different from type 1 pili. K88 pili have a reported diameter of 7 to 8.4 nm (35). K99 pili (17) have a diameter of 8.4 nm and are composed of protein subunits with molecular weights of 22,500 (major) and 29,500 (minor). In contrast, the type 1 pili or common fimbriae (3, 4, 6, 31, 32) have been reported to have a diameter of 7 to 13 nm and are composed of a subunit of 17,500 molecular weight (31, 32). Bacteria can also possess sex pili (37) which function in conjugation. The F and I-like sex pili have a diameter in the range of 4.5 to 10 nm, with the F pili having a subunit molecular weight of 11,800 (3, 4, 24).

Evans and co-workers have recently reported the isolation of pili morphologically similar to K88 and K99 on ETEC isolated from human cases of diarrhea (10). These pili, which they have designated as the CFA/I antigen, have been associated with the attachment of *E. coli* to the intestine of infant rabbits and have been implicated in the production of diarrheal disease in volunteers (9-12). CFA/I pili, have been implicated in MR-HA of human A and bovine RBC. These investigators have also reported a second antigen (CFA/II) which is only associated with O6 and O8 serotypes and is involved in MR-HA of only bovine RBC (7).

We have shown that binding to human buccal mucosal cells of ETEC strains isolated from humans with diarrheal disease correlates with toxigenicity of human strains (36; Deneke, Thorne, and Gorbach, manuscript in preparation). We report here the isolation of pili from toxigenic *E. coli* isolated from human beings with diarrhea. These pili bind both to RBC and

to human buccal mucosal cells. Such surface structures may play a role in adherence of ETEC to the small bowel of humans.

## MATERIALS AND METHODS

**Bacterial strains.** *E. coli* strains used in this study were isolated from a variety of human sources including infants, travelers, and indigenous populations. The sources of these strains are detailed in a previous paper (36). The prototype strain for this work was *E. coli* 334 (O15:H11), originally isolated by one of us (S.L.G.) from a case of acute diarrhea in Calcutta, India. Nontoxigenic control strains included those from a healthy person (CD-1), a well-described strain (H10405), and a plasmid-free derivative of 334, termed 334LL. The individual strains are detailed in Table 1.

**Isolation of pili.** Bacteria were grown overnight at 37°C on peptone salt agar, composed of 2% peptone (Difco Laboratories, Detroit, Mich.), 0.5% NaCl, and 1.5% agar. The cells were harvested in phosphate-buffered saline, pH 7.2 (PBS). The bacterial cells were washed once and resuspended in PBS containing 0.5% mannose (mPBS). Pili were removed from the washed bacteria by blending for 3 min with short (about 30-s) bursts; the blender was cooled in ice to minimize thermal denaturation. Intact bacteria and large debris were removed by centrifugation at 27,000 × *g* for 10 min, leaving the pili in the supernatant. This mPBS supernatant, containing pili, was mixed with freshly drawn, washed guinea pig RBC and cooled on ice to allow attachment of the pili to the RBC. In the presence of mannose, type 1 pili could be removed with mPBS in the cold by low-speed centrifugation. Only those bacterial components which bound to RBC in the presence of mannose and sedimented at low speed were retained.

Attachment pili were eluted from RBC under conditions which reversed the HA reaction, i.e., resuspension in PBS and incubation for 15 min at 37°C. The RBC were removed by centrifugation at 500 × *g* for 10 min with a Dynascan clinical centrifuge at room temperature. This washing-elution procedure was repeated with temperatures of 37, 42, and 50°C. Finally, RBC membrane fragments and debris were removed by ultracentrifugation at 45,000 rpm for 2 h, leaving partially purified pili in the supernatant.

**Electron microscopy.** Bacteria were prepared for negative staining by growing them for either 6 h or overnight. Bacteria were resuspended in saline and applied for 2 min to carbon-coated grids (E. F. Fullam, Schenectady, N.Y.) which had been pretreated for 1 min with a 0.1% solution of bovine serum albumin. The grids were washed with deionized filtered water before staining with 2% (wt/vol) phosphotungstic acid. Pili preparations were similarly treated with a 3-min absorption time and either 2 or 4% uranyl acetate stain. The preparations were examined in a Jelco JEM-100B electron microscope.

**SDS-PAGE.** Molecular weight determinations of the pili subunits were determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) by the method of Laemmli (23) with the following modifications. For the separation gel, the acrylamide concentration was increased to 16.8% with 0.45% bisacrylamide; for the stacking gel, the acrylamide concentration was 5.8% with 0.15% bisacrylamide. The separating gel was allowed to polymerize overnight. The molecular weight standards were myoglobin (molecular weight 17,200), lactalbumin (molecular weight 18,400), and lysozyme (molecular weight 14,300), all obtained from Sigma Chemical Co. (St. Louis, Mo.).

Samples were dissolved in 2% SDS, 5% mercaptoethanol, and 10% glycerol (pH 8.8) and were heated in a steam bath for 20 min to ensure complete denaturation. The samples were electrophoresed for 4 to 6 h with a current of 50 mA. After electrophoresis, gels were stained overnight in a solution of 225 ml of ethanol, 45 ml of glacial acetic acid, and 225 ml of water containing 0.5 g of Coomassie brilliant blue R (Sigma). The destaining solution was ethanol-acetic acid-water (2:3:35). Staining and destaining were done in a shaking water bath at 50°C.

**Buccal cell binding by pili.** Buccal cells were collected from 10 to 15 volunteers by scraping the buccal epithelium with wooden tongue depressors. The buccal cells were then washed three times with mPBS. Isolated pili preparations were mixed with washed buccal cells, incubated for 5 min, and washed four times with mPBS with a Beckman microfuge. Each wash supernatant and final precipitate were dissolved in an equal volume of the SDS solubilization reagent, heated for 20 min in a steam bath, and analyzed by SDS-PAGE.

**Antisera.** Antisera were prepared in rabbits with purified pili preparations. By the method of Stirm et al. (35), pili were injected into the marginal ear vein of adult New Zealand white rabbits twice weekly for 3 weeks. After a 1-week resting period, the animals were bled. Immunoglobulin G was isolated from the whole serum by sequential sodium sulfate precipitation, first with 18% and then 14% (both wt/vol). The precipitate was dissolved in 15 mM phosphate buffer (pH 8.0) and was passed through diethylaminoethyl-Sephadex. Monovalent $F_{ab}$ fragment was prepared by papain cleavage. The remaining immunoglobulin G was removed from the $F_{ab}$ by gel chromatography with Sephadex G-100.

## RESULTS

**Electron microscopy.** Short, needle-like pili (5 to 10 by 300 nm) were present on the surface of a number of different ETEC strains (Fig. 1A to F). These include 334, 193-4, TX-1, D481, and D563. An electron micrograph of the attachment pili of ETEC strain 193-4 isolated by the method described above is shown in Fig. 2. Pili from strain 334, obtained in the same manner, are shown in Fig. 3. The pili preparations appeared to be homogeneous, without the presence of the larger structures which were visible on the intact cells. The isolated pili had the same diameter as the small pili on the intact bacterial cells, supporting the impression that the smaller needle-like structures are the attachment pili. It is these

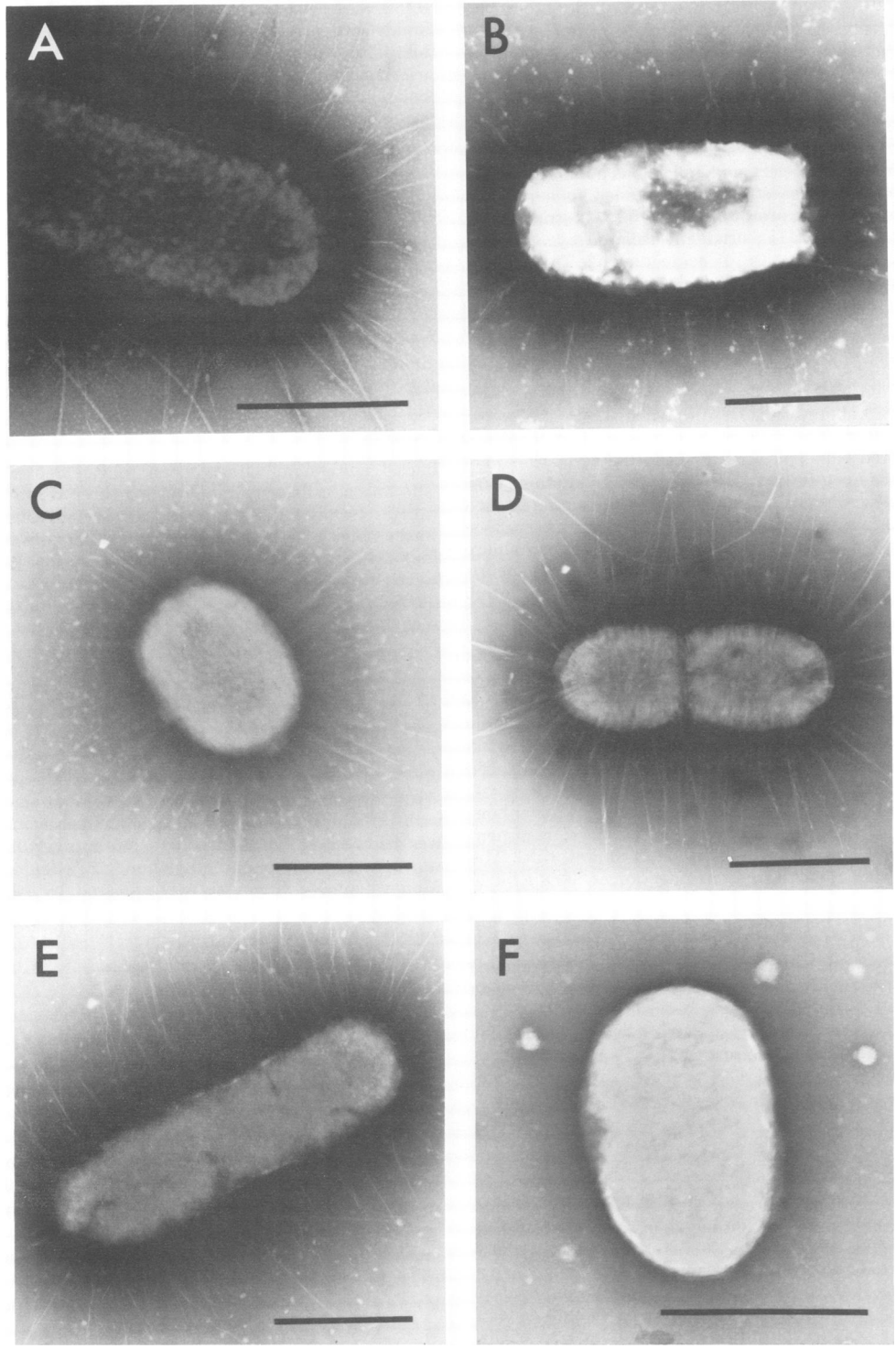


FIG. 1. *Five strains of ETEC and one non-enterotoxigenic strain negatively stained with 2% phosphotungstic acid. (A) 334; (B) 193-4; (C) D563; (D) D481; (E) TX-1; (F) 334LL (negative control). Attachment pili are small, needle-like structures (4 to 6 nm). Bar = 1.0 μm.*

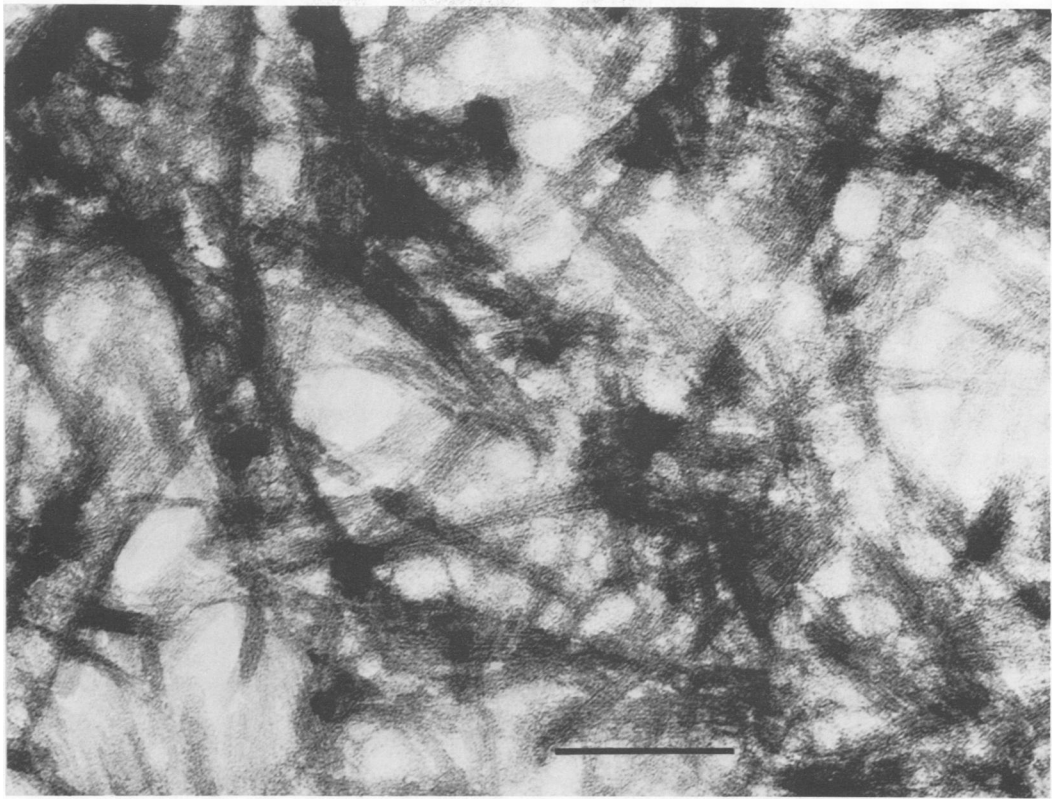

FIG. 2. *Pili preparation of ETEC strain 193-4 stained with 4% uranyl acetate. Bar = 1.0 μm.*

purified attachment pili which we have further characterized.

The purified pili of *E. coli* 334 were found to possess two polypeptide chains by SDS-PAGE (Fig. 4A). These doublet bands were present in each of the supernatant fractions of the purification procedures and in each of the cold mPBS washes. After the first supernatant, which contained material not bound to RBC, there were no protein bands other than the pili doublet. Pili with identical molecular weights were also found on the other *E. coli* strains (Table 1).

The presence of pili polypeptides throughout the cold mPBS washes suggests that the pili existed in an equilibrium between unattached and free states, even in the cold. This equilibrium was probably not seen in the macroscopic scale of HA because we were observing the overall reaction of a large number of pili on each bacterium and multiple attachment sites on each RBC. When the incubation temperature was raised, this equilibrium was apparently shifted toward the unbound state. Thus, both reversal of the HA reaction and desorption of isolated pili were observed when the temperature was raised to 37°C.

Pili prepared with the above isolation and purification procedure bind to isolated human buccal cells (Fig. 4). Buccal cells without bacterial products did not demonstrate a band at the characteristic position of the pili doublet in the SDS-PAGE system (Fig. 4B). Moreover, no pili bands appeared in subsequent washes; this suggests that those pili which were bound existed in a relatively tight association and were not removed by washing, in contrast to the RBC binding reaction.

Finally, when the buccal cells were dissolved in SDS, pili were still present (Fig. 4H). Thus, although not all of the pili isolated by our RBC adsorption method were reactive, at least some of them attached rather tightly to human buccal cells. This implies that some of the pili involved in HA and buccal cell binding are identical and that the two subunit polypeptide chains bind to both types of eucaryotic cells.

The effect of the $F_{ab}$ fragment of anti-pili immunoglobulin G antibody on buccal binding by whole bacterial cells is shown in Table 2. The addition of the $F_{ab}$ fragment inhibits the binding of intact organisms to human buccal cells. The monovalent $F_{ab}$ fragment was used because in-

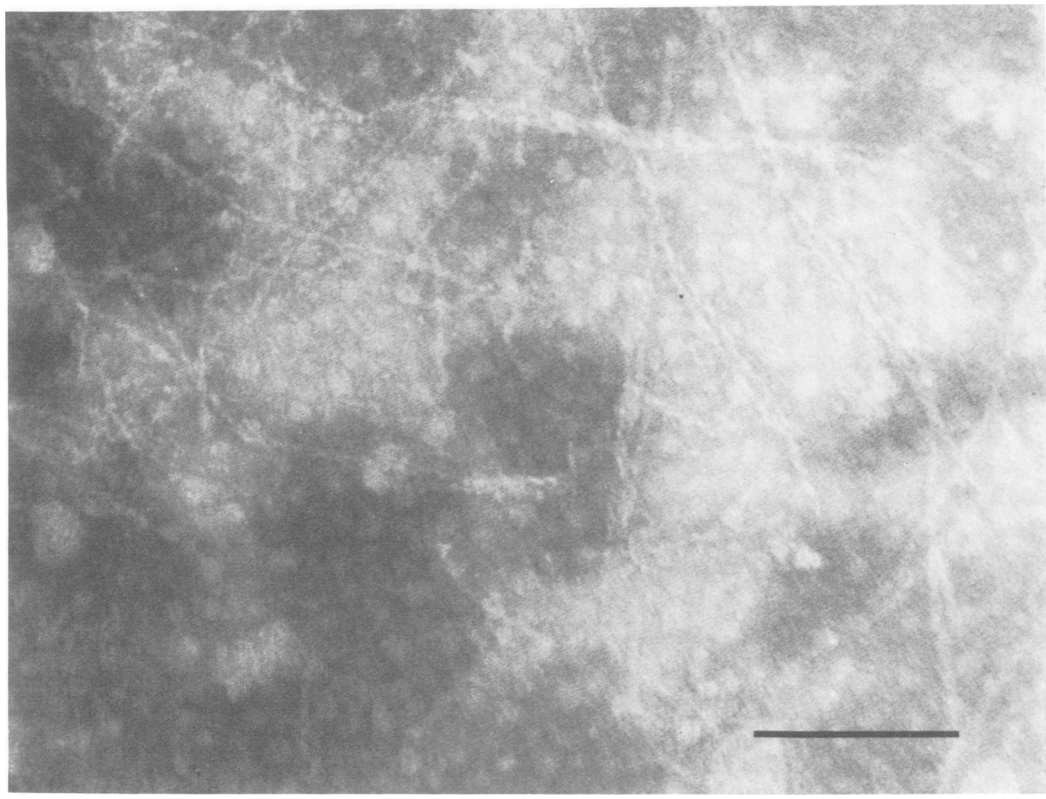

FIG. 3. *Pili preparation of ETEC strain 334 negatively stained with 2% uranyl acetate. Bar = 1.0 μm.*

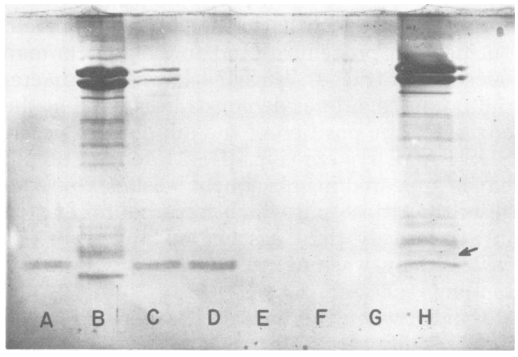


FIG. 4. *Isolated pili attach to buccal cells. Pili, isolated by attachment to RBC, also attach to buccal cells as the characteristic pili bands are present in the buccal cell preparation after washing. SDS-PAGE. (A) Purified pili alone; (B) buccal cells alone; (C) initial mixture of buccal cells and pili; (D) supernatant from first wash; (E) supernatant from second wash; (F) supernatant from third wash; (G) supernatant from fourth wash; (H) final buccal cell precipitate, arrow points to pili bands.*

tact anti-pili immunoglobulin G agglutinates the whole bacteria, interfering with the filter assay. We have further shown that the isolated pili are precipitated by this antibody.

## DISCUSSION

The pili of ETEC which appear to be responsible for the attachment to human mucosal cells are biochemically different from those associated with attachment in animals, i.e., K88 and K99 pili. The pili reported here had a diameter of 5 to 10 nm compared with a diameter of 8 to 13 nm for K88 and 7 to 8 nm for K99. The polypeptide chain subunits had a lower molecular weight, being 12,500 and 13,100 for the human attachment pili and 22,500 for K99. The molecular weights that we report are closer to that observed for the F sex pilus, which was 11,800. Brinton (3) has reported type 2 pili in the same size range as our preparation, but they were not chemically characterized.

The attachment pili discussed here differ in HA pattern from CFA/I and CFA/II. For example, we were unable to show a direct correlation between mannose-resistant HA of human types A or B, guinea pig, and bovine RBC with either the presence of the attachment pili described here or buccal binding as discussed previously (36). This is unlike the case of CFA/I and CFA/II which mediate MR-HA human A and bovine RBC or only bovine RBC, respectively.

TABLE 1. *Detection of K-like pili on strains of ETEC isolated from humans*

| Strains | Serotype | Entero-toxins | Buccal cell adherence | MRHA[a] | | | Pili detected by | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | Human | Guinea pig | Bovine | SDS-PAGE[b] | Electron microscopy | |
| | | | | | | | | 5 to 10 nm | 13 to 20 nm[c] |
| ETEC | | | | | | | | | |
| 334 | O15:H11 | LT/ST | + | + | + | + | + | + | + |
| 193-4 | NT[d] | LT/ST | + | + | + | + | + | + | + |
| TX-1 | O78:K80:H12 | ST | + | + | − | + | + | + | + |
| D542 | NT | LT/ST | + | + | − | + | + | + | + |
| D481 | NT | LT/ST | + | + | − | + | + | + | + |
| D563 | NT | LT/ST | + | + | − | + | + | + | + |
| Control nontoxigenic | | | | | | | | | |
| 334LL | O15:H11 | − | − | − | − | − | ? | − | − |
| CD-1 | NT | − | − | − | − | − | ? | − | − |
| K12,K88 | NT | − | − | − | + | − | + | + | − |

[a] Performed at 0°C.
[b] Proteins of 12,500 and 13,100 molecular weight detected by SDS-PAGE after RBC attachment and elution procedure.
[c] These larger structures (13 to 20 nm) morphologically resemble flagella.
[d] NT, Not typable.

TABLE 2. *Inhibition of buccal binding by anti-334 pili $F_{ab}$*[a]

| Bacterial strain | Type of antiserum | Amt of antisera (μl) | Mean no. of bacteria per buccal cell |
|---|---|---|---|
| 334 | | | 100 |
| 334 | $F_{ab}$ | 200 | 60 |
| 334 | $F_{ab}$ | 400 | 10 |
| 334 | Normal rabbit serum (heat inactivated) | 200 | 110 |

[a] Bacteria alone were pretreated for 15 min with either $F_{ab}$ or heat-inactivated normal rabbit serum before the addition of buccal cells.

Although we found that the same pili appeared to be binding to RBC and buccal cells, the receptors on these cell types are unlikely to be the same since pili remain bound to buccal cells at 37°C, whereas they are eluted from RBC under the same conditions. It seems likely that a cross-reacting receptor structure is involved.

Our studies show that these pili are found on ETEC strains isolated from humans, they are physically and chemically distinct from previously reported pili types, and the same isolated pili attach to human mucosal cells. Further evidence which suggests that these pili are involved in mucosal cell attachment is that the $F_{ab}$ fragment from anti-pili immunoglobulin G blocks the attachment of whole bacteria to buccal cells. Other workers have also shown inhibition of bacterial binding by antibodies (13, 15, 22). Because of potential changes in surface conformation, charge, and other steric factors, the inhibition of buccal cell binding by anti-pili $F_{ab}$ does not preclude additional attachment mechanisms.

Previously (36) we have reported that the ability to adhere to human buccal mucosal cells occurs to a greater extent and more frequently in human ETEC strains than in *E. coli* strains isolated from healthy people or from animals. Many, but not all, of the ETEC strains exhibit MR-HA in the cold, but we have not found, as have Evans et al. (9), a direct correlation between HA of RBC from a particular animal species and the presence of these attachment pili. Attachment of human ETEC strains to buccal mucosal cells appears to be mediated via these attachment pili, which are not found on control strains, and it is possible that these pili might also function in the attachment of ETEC to human intestinal mucosa, the initial event in the pathophysiology of acute diarrheal disease.

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