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Serotypes of Attachment Pili of Enterotoxigenic *Escherichia coli* Isolated from Humans

CARL F. DENEKE,* GRACE M. THORNE, AND SHERWOOD L. GORBACH

Infectious Diseases Service, Department of Medicine, Tufts-New England Medical Center, Boston, Massachusetts 02111

Pili from enterotoxigenic *Escherichia coli* isolated from humans have been partially purified, and antisera have been prepared. These pili were initially attached to erythrocytes and then removed by thermal elution for purification. Three distinct antigenic types of pili have been identified. Antisera against these three pili types reacted with 60 of 106 (56%) enterotoxigenic *E. coli* isolated from humans but not with nontoxigenic, normal human fecal isolates of *E. coli* nor with enterotoxigenic *E. coli* strains isolated from animals. There was no correlation between pili serogroup and any of the following: toxin production (heat labile, heat stable, or both), O antigenic type, geographical source of isolation, or mannose-resistant hemagglutination patterns of various erythrocyte types.

The host specificity of enterotoxigenic *Escherichia coli* (ETEC) strains appears to be conferred by adherence antigens. These surface structures, identified morphologically as pili, are responsible for the adhesion of bacterial cells to the small bowel mucosa of the susceptible host. Pathogenic strains for lambs, piglets, and calves possess the K88 or K99 surface antigens as well as the ability to produce enterotoxins (heat labile [LT], heat stable [ST], or both). *E. coli* strains which have lost the genetic ability to synthesize pili (K88) but retained toxin production could not cause diarrhea in piglets, suggesting that pili production is a vital virulence factor (17). The K88 antigen has multiple antigenic forms, identified as K88ab, K88ac, and K88ad(e) (12). All of these pili proteins have similar subunit molecular weights (25,000) and a similar N-terminal amino acid sequence, but they have slightly different amino acid compositions and cyanogen bromide cleavage fragments (10).

Evans et al. (8) have described a class of pili antigens present on ETEC strains infectious for humans (7), termed the colonization factor antigen (CFA). The two serological groups in this class, CFA/I and CFA/II, have also been characterized in part by their mannose-resistant hemagglutination pattern (MR-HA): CFA/I hemagglutinates human type A and bovine but not guinea pig erythrocytes, whereas CFA/II hemagglutinates only bovine and not guinea pig or human type A erythrocytes (9). The CFA/I antigen has been purified (5, 6) and has a subunit molecular weight of 23,800 (5). Although antiserum has been raised against the purified CFA/I pili, most of the studies reported in the literature use antiserum against whole cells of the parent strain, H10407 (LT^+ ST^+ CFA/I^+), cross-adsorbed with strain H10407P (LT^+ ST^- CFA/I^-) (4). Evans et al. reported that anti-CFA/I sera react with 17.5% of the ETEC strains isolated from humans with diarrhea (9). However, other workers using similar antisera have reported a lower incidence of detection of CFA/I on human ETEC strains (11, 15). The second antigen, CFA/II, has been reported to be confined largely to ETEC strains with O6 and O8 somatic antigen (4).

In a previous publication, we described methods to isolate and characterize the surface attachment pili on ETEC strains, and the isolated pili were shown to attach to human buccal mucosal cells (2). This binding occurred under physiological conditions similar to those found in the bowel (18). These pili were also associated with MR-HA of erythrocytes of various species, including human types A and B, guinea pig, and bovine erythrocytes. This report deals with serological studies of these attachment pili.

MATERIALS AND METHODS

Strains and growth conditions. ETEC strains were collected by investigators throughout the world from patients with diarrhea. Nontoxigenic *E. coli* strains were obtained from the feces of healthy volunteers, a single strain being isolated from each individual.

All strains were maintained on peptone agar (2% Difco proteose peptone, 0.5% NaCl, 1.5% agar) slants or as soft (1%) agar stabs. The strain source and maintenance and enterotoxin testing have been previously described (18). Additionally, 31 strains were kindly provided by I. K. Wachsmuth of the Centers for Disease Control, Atlanta, Ga.

MR-HA. Bacteria were grown for either 4 h or

overnight on peptone agar. A small amount was removed and mixed with phosphate-buffered saline (PBS) (8.9 g of NaCl, 0.34 g of NaH_2PO_4, and 1.07 g of Na_2HPO_4 per liter; pH 7.2) containing 1% mannose to form a milky suspension, and a drop of washed erythrocytes was added. The mixture was incubated on ice and scored for MR-HA reaction.

Pili preparation. The attachment pili were prepared by a refinement of the thermal elution procedure previously described (2). Bacteria were grown overnight on peptone agar on 8 to 10 large (150-mm diameter) petri dishes and suspended in PBS, pH 7.2. After being washed once, the bacterial pellet was resuspended in 30 to 40 ml of PBS containing 0.5% mannose, and pili were removed from the cell surface with a Sorvall Omnimixer (DuPont Instruments, Newtown, Conn.), using 2 min of blending at half the maximum speed. Bacteria and cellular debris were removed by centrifugation (27,000 × *g* for 15 min), leaving pili in the supernatant. The supernatant was mixed with washed erythrocytes of various species, depending on the MR-HA pattern of the particular ETEC strain. For strain 334 pili preparations, guinea pig, bovine, or human type A erythrocytes were used. Bovine erythrocytes were used for strain M9800-5, and human type A erythrocytes were used for strain D542 pili preparations. The mixture was cooled on ice to allow attachment of those pili responsible for MR-HA. The binding of type 1 or common pili is inhibited in the presence of mannose (14, 16). After 15 min on ice, the erythrocytes were washed three times with cold PBS containing 5% mannose to remove unbound bacterial components. Throughout this wash sequence, the centrifuge and washing buffers were maintained at 4°C. The attached pili were eluted from the erythrocytes by raising the incubation temperature; i.e., the washed erythrocyte pellet was suspended in PBS and incubated at 37°C for 10 min. The erythrocytes were then removed by centrifugation (500 × *g*) for 10 min. The eluted pili preparations were filter sterilized (0.2-μm Nuclepore filter) and then used for immunization of rabbits. The pili preparations were stored at 4°C with NaN_3.

Immunization. Adult New Zealand white rabbits were immunized at monthly intervals by injection of 1 ml of pili preparation into the marginal ear vein. After two to four injections, test bleeds were positive for bacterial agglutination of the homologous strain at 1:1,000 or greater dilutions; 50-ml blood samples were then collected from the ear artery 7 to 10 days after the last immunization.

Titration. Whole-bacterium agglutination titers were determined in round-bottom microtiter trays, using a 200-μl final volume. Antisera and preimmunization (control) sera were diluted from 1:8 to 1:8,192 in PBS containing 10 mM sodium azide using 100-μl volumes. The final row of wells contained only PBS containing 10 mM sodium azide without antiserum. Bacterial strains were grown overnight on peptone agar, suspended in PBS containing 10 mM sodium azide, and diluted to approximately 10^8 colony-forming units per ml. This bacterial suspension (100 μl) was added to each well containing antisera or buffer. Microtiter trays were incubated for 24 h and read. The endpoint was considered to be the last well which was different from the control well containing buffer. A strain was considered to have reacted specifically with the antisera when the titer was ≥512 and was at least two dilutions above the nonspecific (control) reaction. In a few cases, such as strain 193-4, the bacteria self-aggregated to such an extent that the agglutination titer could not be determined for these strains.

Pili cross-linking. The pili described here are composed of low-molecular-weight subunits (12,500 and 13,100) which are antigenically monovalent and are not precipitating antigens. Therefore, we chemically cross-linked these pili by using the bifunctional reagent *N,N'-p*-phenylenedimaleimide (PDM) (Aldrich Chemical Co., Milwaukee, Wis.), an analog of *N*-ethylmaleimide. PDM (10 mM in acetone) was added in excess to pili preparations maintained at 37°C. After 15 min of incubation, sufficient cysteine (100 mM in distilled water) was added to destroy the PDM. These preparations were then used directly in immunodiffusion studies.

Immunodiffusion. Immunodiffusion was performed in 1% HGT(P) agarose (Marine Colloids, Rockville, Maine) using barbital buffer (pH 8.6). The barbital buffer used contained 3.28 g of sodium barbital, 0.152 g of barbituric acid, and 0.5 g of sodium azide per liter. Immunodiffusion plates were incubated at 25 to 30°C for 4 days, pressed, washed twice in 0.9% saline containing barbital buffer (pH 8.6), stained briefly with Coomassie brilliant blue R (0.1 g of stain in 100 ml of ethanol-acetic acid-water, 45:10:45), and then destained in ethanol-acetic acid-water (2:1:17).

Immune electron microscopy. Bacteria were grown for 4 h on peptone agar, suspended in PBS, and mixed with either immune serum or normal rabbit serum, both diluted 1:10. These mixtures were incubated for 2 h, applied to carbon-coated grids (E. F. Fullam, Schenectady, N.Y.), and stained with 1% phosphotungstic acid.

RESULTS

Serological characterization of isolated pili. Based on preliminary studies, the attachment pili of strains 334, M9800-5, and D542 were selected as the prototypes of the serological groups. High specificity for bacterial agglutination was noted when these organisms were reacted with their respective pili antisera (Table 1). Only limited cross-reactivity between these organisms and the heterologous antisera was detected. Ouchterlony immunodiffusion analysis

TABLE 1. *Reciprocal titers of antipili sera with type strains*

Strain	Titer of: Antisera against pili of strain: 334	M9800-5	D542	Control serum
334	8,192	128	128	256
M9800-5	128	8,192	256	256
D542	256	64	4,096	64

of PDM-linked pili from these three strains with respective pili antisera confirmed the high specificity and the low level of cross-reactivity (Fig. 1). Precipitation lines of identity were seen between homologous pairs of antigen and antisera, whereas lines of nonidentity were detected between heterologous pairs.

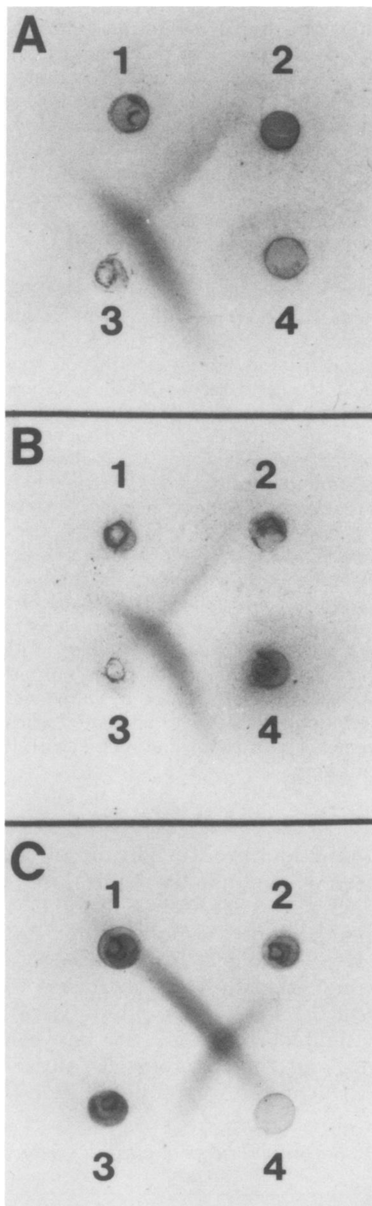

FIG. 1. *Immunodiffusion patterns of pili serogroups 1, 2, and 3. Isolated pili preparations were cross-linked with PDM. (A) Well 1, anti-334 pili; well 2, anti-M9800-5 pili; well 3, M9800-5 pili; well 4, 334 pili. (B) Well 1, anti-D542 pili; well 2, anti-M9800-5 pili; well 3, M9800-5 pili; well 4, D542 pili. (C) Well 1, anti-334 pili; well 2, anti-D542 pili; well 3, D542 pili; well 4, 334 pili.*

Number of reactive strains. Of the 106 ETEC strains examined, 60 (56%) reacted with one or more of the antisera developed against the three pili types. The remaining 46 ETEC strains were nonreactive in these three antisera (Table 2). One strain, H10407, reacted with a high titer (1:4,096) with antisera against the pili of both strains 334 and D542. Five strains reacted with a titer of 1:1,024 or greater with antisera against the pili of strains 334 and M9800-5. These six strains are listed in Table 2 as 1, 3 and 2, 3 reactive strains, respectively. The antisera were also nonreactive with the 16 strains of nontoxigenic *E. coli* isolated from healthy adults, the rabbit pathogenic strain RDEC-1 (1), and the porcine pathogenic strain P307 ($K88^+$).

Geographic distribution. The geographic sources of these strains and their reactivity with the pili antisera is given in Table 3. Whereas clustering of one pili serotype within a given outbreak was noted (e.g., all three strains from the Crater Lake outbreak were reactive with pili serogroup 2), there did not seem to be an association between pili serogroup and locale.

Enterotoxins. The toxin profiles of these 106 strains are recorded in Table 4. Again, there seemed to be no pattern of toxin production and pili serogroup.

Serotype. The distribution of O antigens within each of the pili serogroups is given in Table 5. No absolute correlation between pili serogroups and O antigenic groups was observed, although there was some tendency for group 1 (334-like) strains to be O15 (57%) and for group 2 (M9800-5-like) to be O6 (32%). However, no pili group included only a single somatic antigenic type, and each somatic serotype occurred in at least two pili reactive groups.

MR-HA. The MR-HA patterns are shown in Table 6. Of the 106 strains, 38 (36%) were reactive with at least one species of the erythrocytes tested. The MR-HA reaction detected fewer ETEC strains than did the pili antisera, which reacted with 60 (56%) of the strains. The only discernible pattern is that strains which showed MR-HA with human A erythrocytes also reacted with human B erythrocytes. Of those strains which did react with erythrocytes, there was no pattern of pili serogroups.

Immune electron microscopy. Electron micrographs of strain D542 treated with immune or normal rabbit serum are shown in Fig. 2 and 3, respectively. The immune serum reacted with the pili on the intact organisms and formed

TABLE 2. *Reactivity of ETEC strains of human origin with antipili sera*[a]

Serogroup	No. of reactive strains (%)
1	12 (11.3)
2	34 (32.1)
3	8 (7.5)
1, 3	1 (0.9)
2, 3	5 (4.7)
Negative (nonreactive)	46 (43.4)

[a] ETEC strains obtained from throughout the world were titrated with antisera prepared against MR-HA pili of strain 334 (serogroup 1), strain M9800-5 (serogroup 2), and strain D542 (serogroup 3).

TABLE 3. *Geographic source of ETEC strains by pili serotype*

Pili serotype	Source (no. of isolates)
1	India (2), Dacca (2), Pakistan (1), United States (5), unknown (2)
2	U.S. Army (2), foreign (2), United States (6), Kenya (5), Dacca (4), Sweden (2), Ethiopia (2), Honduras (2), Vietnam (1), Mexico (2), unknown (6)
3	Dacca (3), Mexico (2), Honduras (2), Ethiopia (1)
1, 3	India (1)
2, 3	Texas (2), Dacca (3)
Negative	Pakistan (1), United States (3), Kenya (5), Vietnam (2), Morocco (2), Mexico (6), Ethiopia (4), Honduras (11), cruise ships (2), unknown (10)

TABLE 4. *Enterotoxin profile of E. coli strains by pili serogroup*

Pili serogroup	No. of strains producing toxin			
	LT and ST	LT	ST	ND[a]
1	6	3	3	0
2	20	4	8	2
3	4	1	3	0
1, 3	1	0	0	0
2, 3	3	0	2	0
Negative	11	16	11	8

[a] ND, Toxin profile either not reported or determined by nonstandard methods.

clumps (Fig. 2). The normal serum had no effect (Fig. 3).

DISCUSSION

Antisera raised against isolated pili from three ETEC strains were found to react with 56% of the ETEC isolated from humans, but not with control *E. coli* strains or animal pathogens. In previous studies, we have shown that strains with this type of pili bind to human buccal cells and that the specific antisera developed against the pili prevent this binding. Nontoxigenic *E. coli* did not adhere to buccal cells, nor did they agglutinate with pili antisera. Some strains, such as 214-4 and B7A (3), demonstrated to cause human disease, do not react with our three pili antisera. Thus, our three pili serogroups do not encompass all of the attachment mechanisms of ETEC strains. These nonreactive ETEC may possess other mannose-resistant pili types, mannose-sensitive pili, or non-pili attachment components. Some strains which fail to react with the three pili antisera undergo the MR-HA re-

TABLE 5. *Somatic O antigens of 106 ETEC strains by pili serogroup*

Pili serogroup	O antigens (no. of strains)
1	O15 (8), O78 (1), O128 (2), ND[a] (1)
2	O6 (11), O8 (1), O20 (1), O25 (1), O27 (3), O78 (1), O148 (2), ND (14)
3	O6 (1), O78 (1), O128 (1), ND (5)
1, 3	O78 (1)
2, 3	O78 (2), ND (3)
Negative	O6 (1), O15 (1), O25 (4), O27 (2), O78 (5), O128 (3), O148 (2), ND (28)

[a] ND, O antigen not determined.

TABLE 6. *MR-HA of 106 ETEC strains by pili serotype*

Pili serotype	MR-HA with erythrocytes[a]			No. of strains
	Human A and B	Bovine	Guinea pig	
1	+	+	+	4[b]
	+	−	+	2
	−	−	−	6
2	−	+	−	4[b]
	+	−	−	2
	−	−	−	23
	−	−	+	3
	+	+	−	1
3	+	+	−	5[b]
	+	−	−	2
	−	−	−	1
1, 3	+	+	−	1
2, 3	+	+	−	4
	−	−	+	1
Negative	−	−	−	40
	+	+	−	2
	+	−	+	1
	+	−	−	3

[a] MR-HA was determined at 0°C using 1% mannose and washed erythrocytes.
[b] Includes prototype strain.

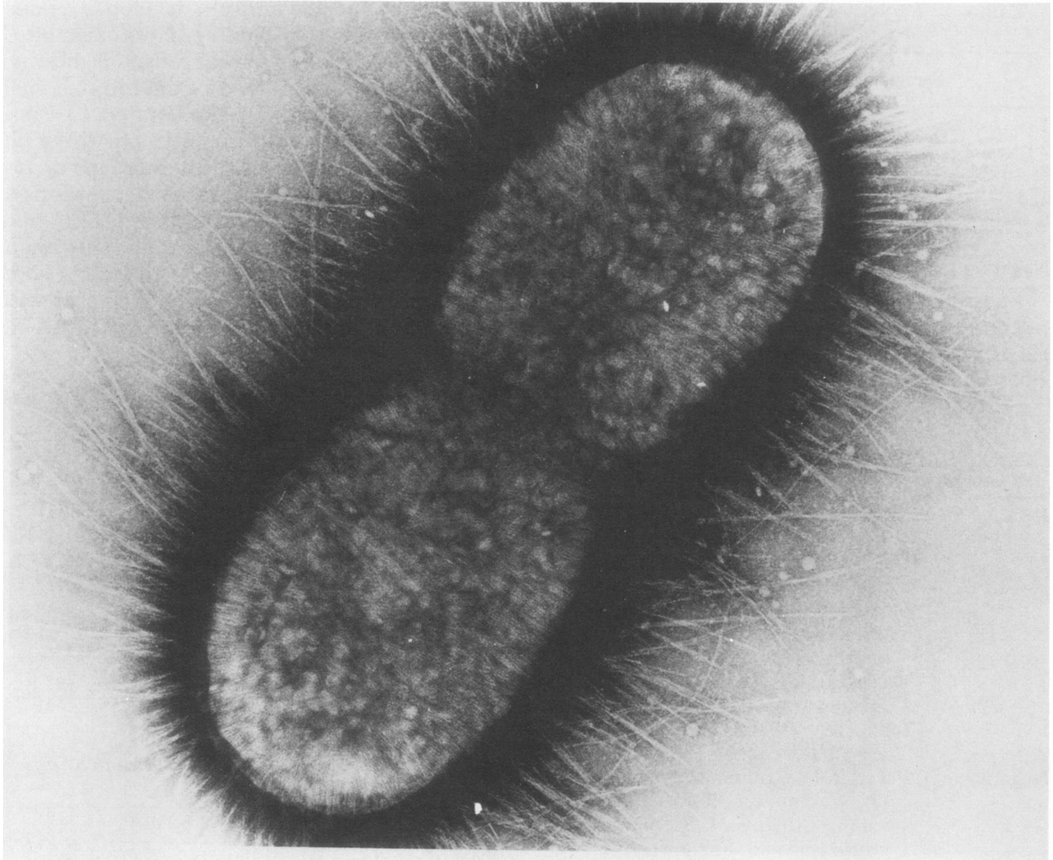

FIG. 2. *ETEC strain D542 treated with antipili serogroup 3 sera diluted 1:10 and then negative stained. Note that pili have clumped.*

action (CDC 70-5206, H410C1, and 2016-10). These strains most likely possess as yet unclassified pili, perhaps with the same basic structure as that reported here.

Thus, ETEC strains found to react with the three pili antisera are piliated when examined by electron microscopy, and they are able to bind to human buccal cells. This adherence is inhibited by the pili specific antisera. Laboratory-derived strains, such as 334LL, which are pili negative by electron microscopy and which do not react with these antisera, do not bind to human buccal cells. Also, the isolated pili shown here to react with the antisera have been radiolabeled, and these labeled pili bind to buccal cells. All these data support the conclusion that these pili represent an attachment mechanism for some ETEC strains. At this time, there is no information on the target receptors or the thermodynamic binding properties of pili within the three serogroups.

The ability to identify ETEC strains by CFA antisera or by MR-HA patterns has varied widely. Evans et al. (6) have detected CFA/I or CFA/II on 65 of 179 strains (36.3%), finding roughly equal numbers of each antigen. Ørskov and Ørskov (15) reported that 16 of 77 ETEC strains (20.8%) reacted with CFA/I antisera. Gross et al. (11) found that only 6 of 89 (6.7%) human ETEC strains reacted with their CFA/I antisera, and 9 of the strains (10.1%) showed MR-HA of human erythrocytes. In our study of 106 human ETEC, 11 strains showed the CFA/I MR-HA pattern, i.e., hemagglutinated human and bovine but not guinea pig erythrocytes, and 4 strains displayed the CFA/II pattern; i.e., they hemagglutinated only bovine and not human or guinea pig erythrocytes (Table 6). Thus, the ability to detect CFA/I or CFA/II on ETEC of human origin by antisera or MR-HA patterns varies from 10 to 36% among different investigators.

Our three pili antisera reacted with ETEC that produced both LT and ST as well as with

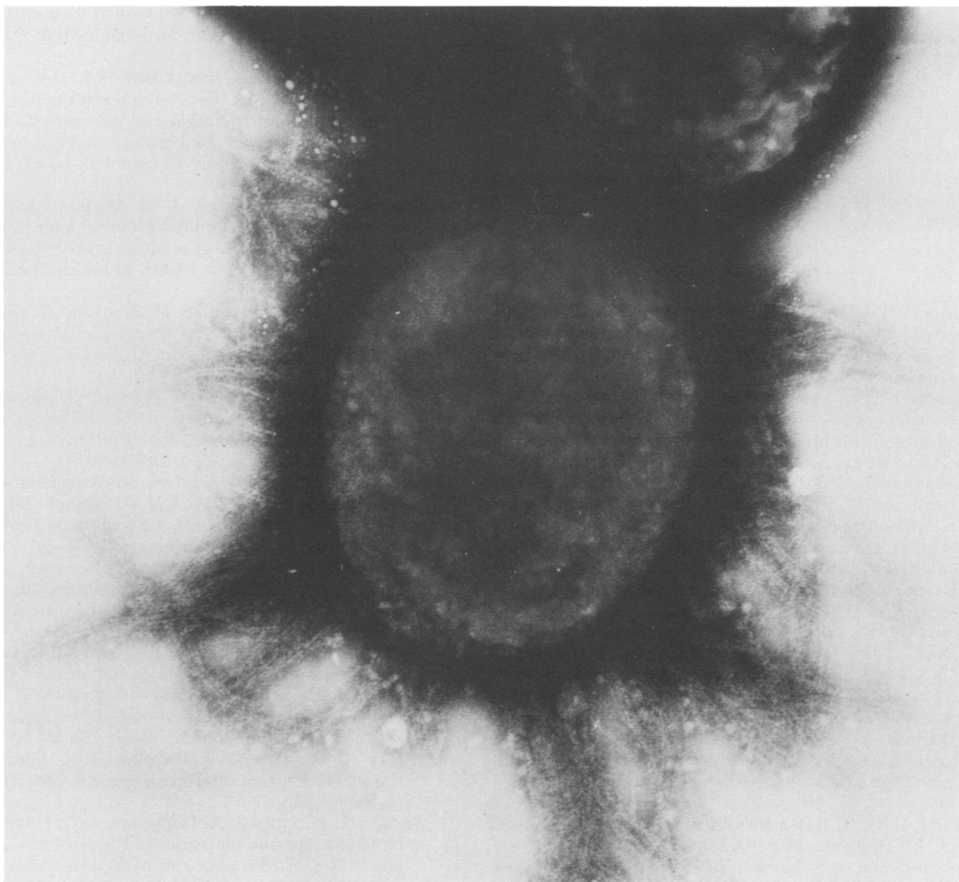

FIG. 3. *ETEC strain D542 treated with normal rabbit sera diluted 1:10 and then negatively stained. Pili appear similar to those of preparations which have not been treated with sera.*

strains producing only LT or only ST (Table 4). Strains of all three toxin profiles have been shown to cause human disease (3, 13). The relative detection percentages were 75.6, 33.3, and 59.2%. In contrast, the CFA system as reported by Evans et al. (9) reacts primarily with ETEC strains that produce both LT and ST, detecting 91.1% of these strains, but detecting only 17.3% of LT-only strains and 7.0% of ST-only strains.

The molecular weight of the protein subunits of the pili described here are similar for each of the three serogroups. We have found that these pili have two subunits with molecular weights of 13,100 and 12,500 (2). In contrast, CFA/I has a single subunit with a molecular weight of 23,800 (5). As stated above, these pili demonstrate different MR-HA patterns from those of CFA/I and CFA/II. Therefore, CFA/I and CFA/II are different from the attachment pili that we have characterized. These isolated pili are homogeneous by sodium dodecyl sulfate gel electrophoresis, giving two lines without additional bands. The lack of additional bands suggests that if additional protein contamination is present, the contamination is at a low level. In addition, the final pili preparations are negative for lipopolysaccharide by *Limulus* amebocyte assay. The absence of major contaminants is also shown by the single immunoprecipitation line by immunodiffusion.

Attachment to the intestinal epithelium is considered a prerequisite stage in the diarrheal disease process. Those ETEC strains pathogenic for animals possess K88 and K99 pili that are responsible for adhesion. The various serogroups of pili reported here may be analogous structures involved with human disease, suggesting that serological identification of attachment pili might be useful in the diagnosis of human diarrheal disease. Ultimately, these antigens may be employed as a vaccine to prevent diarrheal disease caused by ETEC in humans.

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