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Purification, Morphology, and Genetics of a New Fimbrial Putative Colonization Factor of Enterotoxigenic *Escherichia coli* O159:H4

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The ability to colonize the small intestine is essential for the pathogenesis of diarrhea caused by enterotoxigenic *Escherichia coli* (ETEC). Colonization is mediated by fimbriae (pili), of which there are several antigenically distinct types, including colonization factor antigen I, colonization factor antigen II (CS1, CS2, and CS3), and PCF8775 (CS4, CS5, and CS6). These fimbriae are associated with certain ETEC O serogroups. Serogroup O159 has had no known colonization factor. We found a distinct plasmid-encoded fimbria composed of 19-kilodalton protein subunits associated with ETEC serotype O159:H4. Rabbit antibody against this purified fimbria reacted with a single 19-kilodalton protein band as seen by Western immunoblot of sheared-cell preparations. The rabbit antibody, treated with colloidal-gold-labeled goat anti-rabbit immunoglobulin G, bound specifically to fimbriae when cells were examined with an electron microscope. Of 10 available ETEC O159:H4 strains from Europe, Bangladesh, and Kenya, 6 expressed this type of fimbria; its true prevalence among ETEC strains is unknown. This putative colonization factor of O159:H4 joins other ETEC fimbriae as potentially useful immunogens against human diarrhea.

In developing countries, enterotoxigenic *Escherichia coli* (ETEC) is a leading cause of diarrhea and dehydration in children younger than 2 years of age (10, 28); ETEC is the most common cause of traveler's diarrhea (9, 20) and is occasionally responsible for outbreaks of food- and waterborne disease (27, 30). Investigations of the pathogenesis of ETEC-induced diarrhea have identified factors essential for virulence which might be manipulated to produce a vaccine. These virulence factors include fimbrial colonization factors, which allow the organism to adhere to the epithelium of the proximal small intestine, and heat-stable and heat-labile enterotoxins (ST and LT). The ability of these toxin-producing *E. coli* to adhere to intestinal epithelium and resist the clearing action of peristalsis is essential for virulence. In studies with volunteers, diarrhea did not occur after ingestion of an organism that produced LT but which had lost a plasmid encoding a fimbrial colonization factor and ST; the plasmid-containing fimbriate parent strain did cause diarrhea (6).

The ability of some ETEC strains to cause mannose-resistant hemagglutination led to the identification of several colonization factors of human ETEC. These include colonization factor antigen I (CFA/I), CFA/II complex (which consists of three antigenically distinct fimbriae designated CS1, CS2, and CS3), PCF8775 (which also has three distinct fimbriae designated CS4, CS5, and CS6), and perhaps a fimbria of strain 260-1 (5, 7, 12, 16, 31, 32). These colonization factor antigens are associated with the following O serogroups: O25, O63, O78, and O128 (CFA/I); O6, O8, O80, and O85 (CFA/II); and O25, O27, O115, O148, and O167 (PCF8775). All of these colonization factors are fimbriae, the genes for which are plasmid encoded. The genes encoding ST (and sometimes LT) are found on the same plasmid as the genes encoding either CFA/I or CS1, CS2, and CS3. The same is usually, but not invariably, true of genes encoding CS4, CS5, and CS6.

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When large collections of ETEC strains have been screened for the presence of the known colonization factors, the prevalence of these factors has varied widely (11, 17, 19, 25, 31, 33). When strains that had been isolated from patients with diarrhea, but which lacked CFA/I or CFA/II, were fed to volunteers, the volunteers nevertheless became ill with diarrhea (15). These observations suggested that other, antigenically distinct, colonization factors may be present on these strains and on other ETEC strains lacking known colonization factors. Indeed, ETEC strains of serogroup O159 are commonly associated with diarrhea in many geographic areas, but they do not cause mannose-resistant hemagglutination and lack a known colonization factor. In this report we describe an antigenically distinct fimbrial colonization factor associated with the common ETEC serotype O159:H4.

MATERIALS AND METHODS

Bacterial strain. ETEC 350C1 serotype O159:H4 was chosen for study (17). This serotype has been commonly associated with diarrhea, and strain 350C1 does not agglutinate with antisera against CFA/I, CFA/II, or PCF8775. 350C1 is a nonhemagglutinating strain which lacks type 1 pili when grown on solid agar and produces both ST and LT (17).

Purification of fimbriae. A suspension of strain 350C1 was streaked onto aluminum pans (32 by 42 by 2 cm) containing 1 liter of CFA agar (1% Casamino Acids, 0.15% yeast extract, 0.005% $MgSO_4$, 0.0005% $MnCl_2$, 2% agar; pH 7.4) (5) and incubated overnight at 37°C. The bacterial cells were harvested, suspended in 20 ml of phosphate-buffered saline (PBS), pH 7.2, and sheared for 90 s on ice in a Sorvall Omnimixer at setting 5. Cells and particulate debris were pelleted by centrifugation at 8,000 × *g* for 30 min; outer membrane vesicles were removed by centrifugation at 39,000 × *g* for 30 min. The supernatant was collected and centrifuged at 180,000 × *g* for 1.5 h in a Beckman L8-80 centrifuge. The fimbria-containing pellet was suspended in 0.1 M Tris–0.2 mM EDTA with a magnetic stirrer at 4°C.

This suspension was applied to a self-generating isopycnic cesium chloride gradient with 1.5% (wt/vol) sodium *N*-lauroyl sarcosine to a density of 1.3 g/cm^3 by ultracentrifugation at 117,000 × *g* for 44 h. Opaque bands visible under strong illumination were collected by tube puncture and dialyzed overnight in PBS at 4°C. The presence of fimbriae was monitored by electron microscopic examination and examination of fimbrial subunits by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (16).

SDS-PAGE. SDS-PAGE gels were prepared by the method of Laemmli (13) with the modifications of Lugtenberg et al. (18) and Shapiro et al. (29). Stacking and separating acrylamide gels of 5% (pH 6.9) and 12.5% (pH 8.7), respectively, were prepared in a water-cooled minigel apparatus (93 by 102 by 1.5 mm; Hoefer Scientific Instruments, Inc., San Francisco, Calif.). Electrophoresis buffer was the Tris-glycine preparation of Ornstein (24) and Davis (3) with 0.1% SDS. Samples were boiled for 5 min with equal volumes of 62.5 mM Tris base, 2% SDS, 10% glycerol, 0.05% bromphenol blue, and 5% 2-mercaptoethanol, and approximately 1 μg per lane was loaded with a syringe into the wells. End wells contained prestained molecular weight markers (Bethesda Research Laboratories, Inc., Gaithersburg, Md.) for estimation of size and electrophoresis time. Electrophoresis was conducted at 25 mA until the samples stacked and then at 35 mA until the markers were well resolved. Gels were stained with Coomassie brilliant blue R-250.

Anti-fimbria antiserum. An adult male rabbit weighing 2 kg was inoculated subcutaneously between the scapulae and into the rear footpads with purified fimbriae in Freund complete adjuvant. At week 8, a booster injection of fimbriae with Freund incomplete adjuvant was given subcutaneously. The rabbit was bled 2 weeks later. Specific antiserum was prepared by multiple absorptions with nonfimbriate cells grown at 18°C (7).

Bacterial agglutination. Slide agglutination of bacterial cells was performed by mixing a drop (0.05 ml) of antiserum with organisms on a toothpick at room temperature.

Immunoblotting. Crude sheared-cell preparations and purified fimbriae were electrophoresed in duplicate on SDS-PAGE gels; one of these was stained with Coomassie brilliant blue, and the other was electroblotted onto nitrocellulose. Western blots were developed as described by Towbin et al. (35) with the modifications of Batteiger et al. (1). Absorbed rabbit antiserum was diluted in 0.5% Tween 20 in PBS and incubated with the nitrocellulose filters overnight at 4°C. Filters were washed three times for 10 min each in diluent and agitated in 1:1,000 goat anti-rabbit immunoglobulin G (IgG) conjugated to horseradish peroxidase (Cooper Biomedical, Inc., West Chester, Pa.) for 2 h at room temperature. Filters were again thoroughly washed and developed with hydrogen peroxide substrate and 4-chloronaphthol chromagen. Dot immunoblots of whole-cell preparations were performed by suspending cells from an overnight culture in PBS and dotting the suspension on nitrocellulose filters. The filters were incubated with absorbed rabbit antiserum, washed, reacted with goat anti-rabbit IgG, and developed as for the Western blot.

Electron microscopy. Bacterial cells were grown at 37°C on CFA agar, harvested, and examined directly with a Siemens Elmiskop IA transmission electron microscope operated at 80 kV with negative staining by 2% phosphotungstic acid (16).

Immunolabeling with colloidal-gold-labeled goat anti-rabbit antiserum was performed (16). A drop of a washed suspension of strain 350C1 was placed on carbon-coated grids and allowed to partially dry under a light bulb for 5 min. Excess liquid was removed, and each grid was placed face down on a drop of antiserum for 15 min. After being washed in 3 successive drops of distilled water, the grids were each placed on a drop of 10-nm-gold-labeled goat anti-rabbit serum (Janssen Pharmaceutical, Piscataway, N.J.) for 15 min (8). Each grid was washed in 3 drops of distilled water. The grids were then stained with 2% phosphotungstic acid. Grids incubated without the primary antibody or with anti-CFA/I antibody were used as controls.

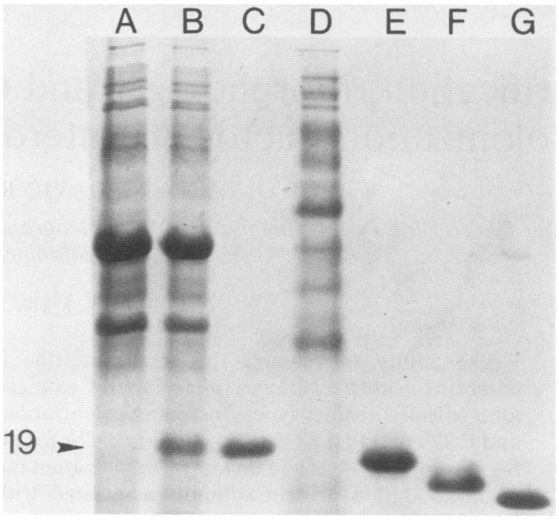

FIG. 1. SDS-PAGE of crude and purified fimbrial preparations. Lanes: A, proteins of a sheared-cell preparation of *E. coli* O159:H4 strain 350C1 grown on CFA agar at 18°C under conditions which inhibited expression of fimbriae; B, proteins of a sheared-cell preparation of the same organism grown at 37°C under conditions which encouraged expression of fimbriae (→, 19-kDa band representing fimbrial subunits); C, purified O159:H4 fimbrial subunits; D, proteins of a sheared-cell preparation of the O159:H4 serotype strain cured of the plasmid that encodes fimbriae (no 19-kDa band is visible); E, F, and G, purified subunits of other ETEC fimbriae (CS1, CS3, and CFA/I, respectively).

Characterization of plasmid DNA. Plasmid DNA was extracted by the Birnboim alkaline extraction technique (2) and examined by electrophoresis on a 0.4% agarose gel. Plasmids were cured by multiple passages on tryptic soy agar at 37°C and by incompatibility matings. Matings were performed by mixing suspensions of each parent and incubating the mixture overnight on a Nuclepore filter on an L agar plate (22). Transconjugates were recovered from the filter by plating on appropriate selective media.

The 27-megadalton (MDa) plasmid was marked with drug resistance by using the ampicillin resistance transposon Tn*801* and transformed into HB101. Cells were plated on L agar containing 200 μg of ampicillin per ml to select for ampicillin-resistant transformants. Transformants were examined for fimbriae with an electron microscope.

DNA hybridization. Colony blots were performed under stringent conditions as described by Moseley et al. (22). The LT-B DNA probe was prepared from pWD615, and the ST DNA probe was prepared from pSLM004 by nick translation with [α-^{32}P]dATP (New England Nuclear Corp., Boston, Mass.) (22).

ELISA for ST and LT. The direct culture method GM1 ganglioside enzyme-linked immunosorbent assay (ELISA)

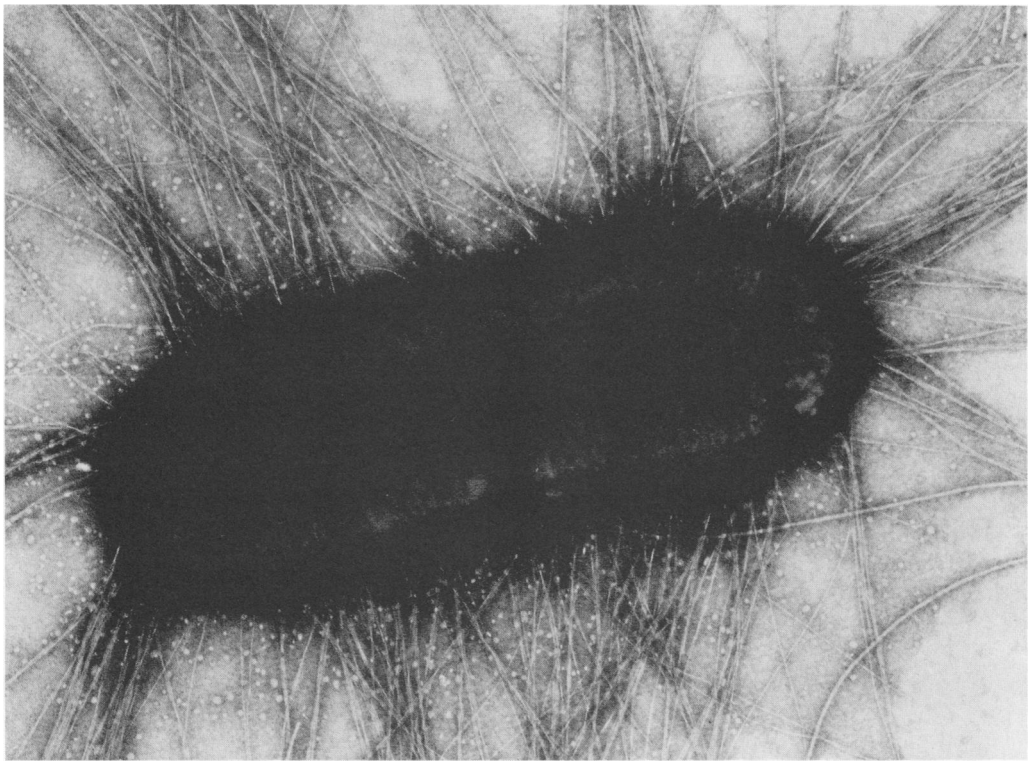

FIG. 2. ETEC O159:H4 strain 350C1 negatively stained with 2% phosphotungstic acid. Filamentous fimbriae 6 to 8 nm in diameter are visible. Magnification, ×31,500.

for LT (26) and monoclonal antibody ELISA for ST (34) were performed with ETEC 350C1 and its plasmid-cured derivatives.

RESULTS

Purification of fimbriae. Colonization factor antigens are characteristically expressed when organisms are grown at 37°C but not when they are grown at 18°C (15). Crude sheared-cell preparations of O159:H4 cells grown at 37°C on CFA agar were subjected to SDS-PAGE and compared with sheared-cell preparations of O159:H4 cells grown at 18°C. The protein banding patterns were identical except for a 19-kDa band present in the 37°C cell preparation (Fig. 1, lane B) that was absent from the 18°C cell preparation (Fig. 1, lane A). Differential centrifugation and isopycnic CsCl gradient banding yielded purified fimbriae. On SDS-PAGE, this preparation produced a single polypeptide subunit band of 19 kDa (Fig. 1, lane C).

Electron microscopy. Examination of strain 350C1 grown at 37°C on CFA agar revealed rigid filamentous fimbriae 6 to 8 nm in diameter (Fig. 2). Cells grown at 18°C on CFA agar lacked fimbriae. Figure 3 shows purified fimbriae which are morphologically identical to those seen on the cell surface.

Figure 4 shows strain 350C1 treated with absorbed rabbit serum against fimbriae and reacted with gold-labeled goat anti-rabbit IgG. Only fimbriae, not flagella or background, are labeled with gold, indicating the specificity of the absorbed rabbit serum for this bacterial organelle.

Immunologic studies. Preimmune, immune, and absorbed immune rabbit serum all failed to agglutinate whole 350C1 cells grown on CFA agar at 37°C.

Figure 5 is an immunoblot of the crude and purified fimbrial preparations shown in Fig. 1. Preimmune serum did not react with any of the proteins in these preparations. Absorbed immune rabbit serum reacted with a single 19-kDa band in the sheared-cell preparation of strain 350C1 (Fig. 5, lane B) and with purified fimbriae of strain 350C1 (Fig. 5, lane C). This specific antiserum against 350C1 fimbriae did not react with the subunits of CS1, CS3, CFA/I (Fig. 5, lanes E through G), or PCF8775 (data not shown). Conversely, antisera against CFA/I, CS1, and CS3 did not react by immunoblot with the 19-kDa antigen.

Plasmid studies. The plasmid content of strain 350C1 included two plasmids of nearly identical size (27 and 28 MDa) (Fig. 6, lane B). The derivative that was cured of the 28-MDa plasmid (Fig. 6, lane C) produced both ST and LT and expressed fimbriae as seen by electron microscopic examination. The derivative cured of the 27-MDa plasmid (Fig. 6, lane A), however, was nontoxigenic as seen by colony blots with ST and LT gene probes and by ELISA for ST and LT and lacked fimbriae, indicating that the genes for ST and LT and for the fimbria are coded on the 27-MDa plasmid. SDS-PAGE of a sheared-cell preparation of this derivative lacked the 19-kDa fimbrial subunit (Fig. 1, lane D). HB101, transformed with the 27-MDa plasmid that had been marked with Tn*801*, expressed fimbriae that were visualized in the electron microscope and contained the 19-kDa subunit when examined by SDS-PAGE of a sheared-cell preparation. Fimbriae were not expressed when the transformant was grown at 18°C.

Prevalence of fimbriae among other ETEC strains. ETEC strains from the Center for Vaccine Development collection were screened by dot immunoblot of whole cells and Western blot of sheared-cell preparations. Of 10 available ETEC O159:H4 strains from Europe, Bangladesh, and Kenya (including several strains kindly provided by Frits Ørskov, World Health Organization *Escherichia* Collaborating

FIG. 3. Purified ETEC O159:H4 fimbriae negatively stained with 2% phosphotungstic acid. Magnification, ×43,875.

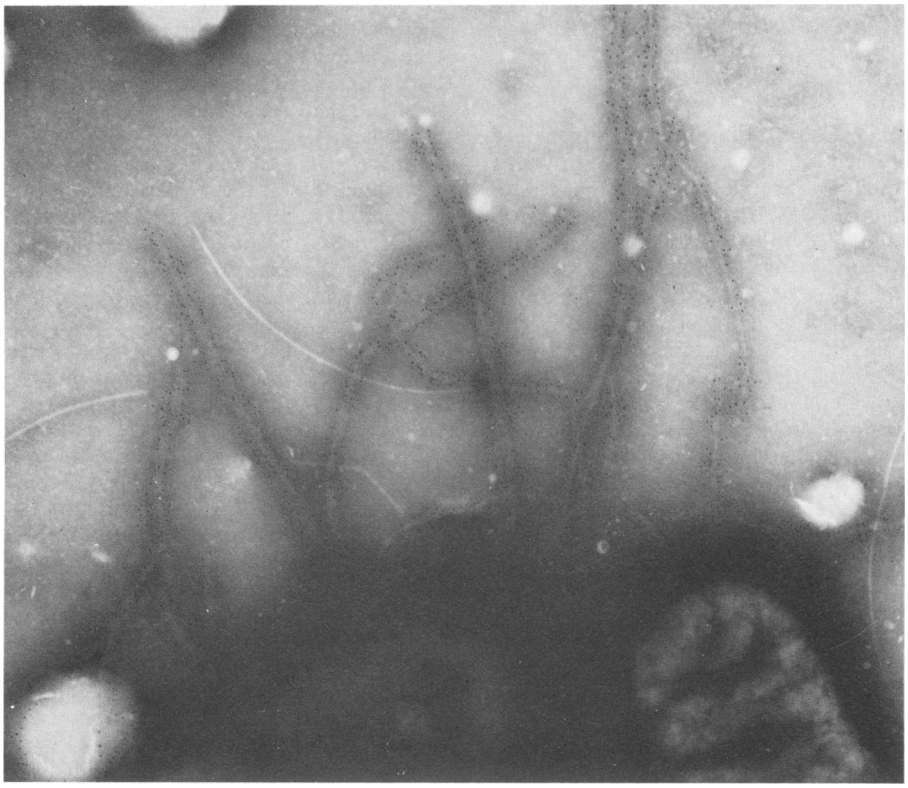

FIG. 4. ETEC O159:H4 labeled by the immunogold technique. Cells were incubated with rabbit anti-fimbriae serum and then with 10-nm-colloidal-gold-labeled goat anti-rabbit serum. Cells were negatively stained with 2% phosphotungstic acid. Fimbriae, not flagella or other structures, are clearly labeled with gold particles. Magnification, ×27,750.

Centre, Statens Serum institut, Copenhagen, Denmark), 6 reacted with specific absorbed antiserum against the fimbriae of strain 350C1 by both dot immunoblot of whole cells and Western blot of sheared-cell preparations. None of the eight ETEC O159 strains of other H subgroups and none of the three ETEC O27 strains reacted with this antiserum.

DISCUSSION

Studies of the human response to ETEC have shown that infection provides protection against subsequent challenge with the homologous strain (14). Antibodies to any of several products or structural components of *E. coli* might mediate this protection. Infection-derived antibodies to LT alone were not able to provide protection in studies with volunteers (14), and antibodies to ST are not produced after naturally occurring ETEC infection. The mechanism of protection does not involve bactericidal effects, since stool cultures are positive for ETEC in protected individuals after a peroral challenge (14).

Local secretory IgA to colonization factor antigens may be the key to protection. Purified K88, K99, and 987P fimbriae from animal-derived ETEC have been used as successful vaccines against colibacillosis in farm animals (21, 23). Studies of the human response to colonization factor antigens have shown that when volunteers ingest organisms with colonization factors, significant increases in secretory IgA antibody and serum antibody to the colonization factor are stimulated, indicating their expression in vivo and immune recognition by the host (6, 13a, 16). Volunteers given a single peroral dose of ST^- LT^- $CS1^+$ $CS3^+$ ETEC O6:H16 as a live vaccine developed intestinal secretory IgA against CS1 and CS3 and were significantly protected when challenged with an ST^+ LT^+ $CS1^+$ $CS3^+$ strain of serotype O139:H28 (13a), suggesting that antibodies to the only common antigens, CS1 and CS3 fimbriae, were adequate for protection. Stool cultures were positive for vaccinees and controls. However, jejunal fluid cultures were positive for only 1 of 12 vaccinees but were positive for five of six controls ($P < 0.004$), indicating that the site of protection was the jejunal mucosa in which colonization was prevented (13a; M. Levine, R. Black, M. L. Clements, J. G. Morris, G. Losonsky, E. Boedeker, and B. Rowe, Abstr. 21st Joint Conference on Cholera, p. 36, 1985).

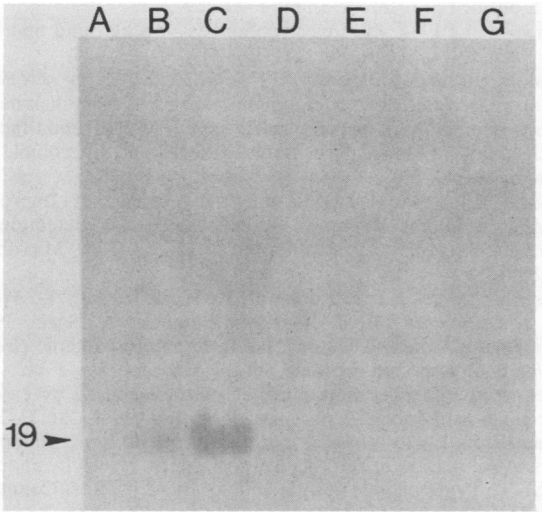

FIG. 5. Western immunoblot of crude and purified ETEC O159:H4 fimbriae. Lanes A through G correspond to those in Fig. 1. Only lanes B and C containing crude and purified fimbrial subunits reacted with monospecific anti-fimbria serum.

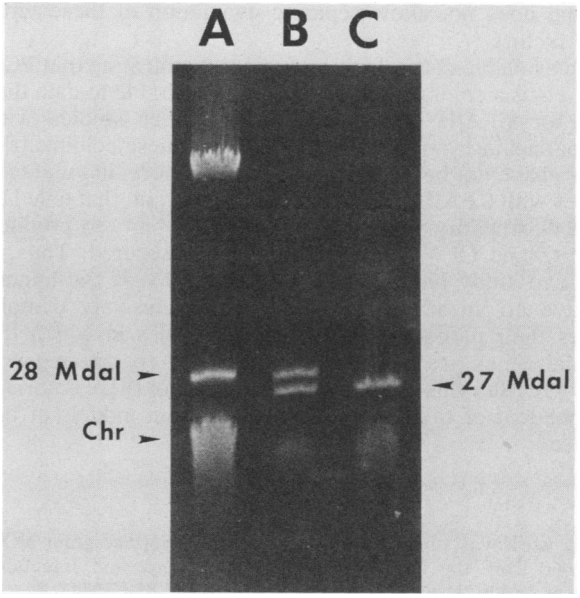

FIG. 6. Agarose gel electrophoresis of plasmid DNA of ETEC O159:H4 strain 350C1. Lanes: B, naturally occurring strain with two plasmids of 27 and 28 MDa; C, plasmid of the derivative cured of the 28-MDa plasmid (this strain produced ST, LT, and fimbriae); A, plasmids of the derivative cured of the 27-MDa plasmid, which did not produce ST, LT, or fimbriae. The new, large-molecular-weight plasmid in lane A was introduced during incompatibility mating.

The following O serogroups have been repeatedly associated with ETEC-induced diarrhea in humans throughout the world: O6, O8, O25, O27, O63, O78, O80, O85, O115, O128, O148, O159, and O167. These are the serogroups representing the majority of the ETEC strains isolated from patients with the most severe (e.g., requiring hospitalization) cases of diarrhea. Conspicuously, serogroups O27, O115, O148, O159, and O167 were not associated with CFA/I or CFA/II. The recently described PCF8875 complex of CS4, CS5, and CS6 fimbrial antigens (32) has been found to occur in serogroups O27, O115, O148, and O167. Before this study, O159 was the last common and important serogroup with no known colonization factor. We describe here a distinct fimbrial colonization factor for this serogroup. The way is clear, in theory, to prepare a fimbrial antigen vaccine of truly broad spectrum that will prevent illness caused by all of the O serogroups associated with more severe forms of ETEC-induced diarrhea. Preliminary studies with volunteers suggest that perorally administered purified fimbriae may undergo proteolysis in the stomach or intestine or may be less potent immunogens than fimbriae attached to living organisms (Levine et al., 21st JCC). A live, peroral vaccine strain(s) engineered to express several colonization factor antigens might provide protection against a broad spectrum of ETEC strains; an attenuated *Salmonella typhi* strain such as Ty21a might be a useful host strain (36).

We propose the designation putative colonization factor O159:H4 (PCF O159:H4) for this newly characterized fimbria. The definitive role of PCF O159:H4 in the pathogenesis of diarrhea in humans needs to be evaluated, as does the role of CFA/II and PCF8775 fimbriae. In the naturally occurring O159:H4 strain, genes for the toxins and fimbriae are carried on the same plasmid, so simple curing of the

plasmid does not allow separate evaluation of these virulence factors.

The available data, albeit preliminary, indicating that PCF O159:H4 is a colonization factor are comparable to data that exist for CFA/II and PCF8775. Volunteer studies with fimbria-lacking variants of strains having these colonization factors have not been performed. Furthermore, in volunteer studies with CFA/I, the plasmid-lacking variant that failed to cause diarrhea in volunteers had lost the ability to produce both ST and CFA/I after the plasmid was cured. Thus, a definitive study has not been done for any of the human putative colonization factors which conclusively demonstrates their pathogenic role. Future studies in which the fimbrial genes are inactivated by genetic engineering will allow precise evaluation of the importance of these fimbriae, independent of toxin, in animal and human models of the disease.

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LITERATURE CITED

1. **Batteiger, B., W. J. Newhall, and R. B. Jones.** 1982. The use of Tween-20 as a blocking agent in the immunological detection of proteins transferred to nitrocellulose membranes. J. Immunol. Methods **55**:297–307.
2. **Birnboim, H. C., and J. Doly.** 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. **7**:1513–1523.
3. **Davis, B.** 1964. Disc electrophoresis—method and application to human serum proteins. Ann. N.Y. Acad. Sci. **121**:404–427.
4. **Evans, D. G., and D. J. Evans, Jr.** 1978. New surface-associated heat-labile colonization factor antigen (CFA/II) produced by enterotoxigenic *Escherichia coli* of serogroups O6 and O8. Infect. Immun. **21**:638–647.
5. **Evans, D. G., D. J. Evans, Jr., W. S. Tjoa, and H. L. DuPont.** 1978. Detection and characterization of colonization factor of enterotoxigenic *Escherichia coli* isolated from adults with diarrhea. Infect. Immun. **19**:727–736.
6. **Evans, D. G., T. K. Satterwhite, D. J. Evans, Jr., and H. L. DuPont.** 1978. Differences in serological responses and excretion patterns of volunteers challenged with enterotoxigenic *Escherichia coli* with and without the colonization factor antigen. Infect. Immun. **19**:883–888.
7. **Evans, D. G., R. P. Silver, D. J. Evans, Jr., D. G. Chase, and S. L. Gorbach.** 1975. Plasmid-controlled colonization factor associated with virulence in *Escherichia coli* enterotoxigenic for humans. Infect. Immun. **12**:656–667.
8. **Faulk, W. P., and C. M. Taylor.** 1971. An immunocolloid method for the electron microscope. Immunochemistry **8**:1081–1083.
9. **Gorbach, S. L., B. H. Kean, D. G. Evans, D. J. Evans, and D. Bessudo.** 1975. Travelers' diarrhea and toxigenic *Escherichia coli*. N. Engl. J. Med. **292**:933–936.
10. **Gordon, J. E.** 1971. Diarrheal disease of early childhood—worldwide scope of the problem. Ann. N.Y. Acad. Sci. **176**:9–15.
11. **Gothefors, L., C. Ahren, B. Stoll, D. K. Barua, F. Ørskov, M. A. Salek, and A.-M. Svennerholm.** 1985. Presence of colonization factor antigens on fresh isolates of fecal *Escherichia coli*: a prospective study. J. Infect. Dis. **152**:1128–1133.
12. **Honda, T., M. Arita, and T. Miwatani.** 1984. Characterization of new hydrophobic pili of human enterotoxigenic *Escherichia coli*: a possible new colonization factor. Infect. Immun. **43**:959–965.
13. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
13a. **Levine, M. M., J. G. Morris, G. Losonsky, E. Boedeker, and B. Rowe.** 1986. Fimbriae (pili) as vaccines, p. 143–145. *In* D. Lark, S. Normark, and E. Brent-Uhlin (ed.), Protein-carbohydrate interactions in biological systems. Academic Press, London.
14. **Levine, M. M., D. R. Nalin, D. L. Hoover, E. J. Bergquist, R. B. Hornick, and C. R. Young.** 1979. Immunity to enterotoxigenic *Escherichia coli*. Infect. Immun. **23**:729–736.
15. **Levine, M. M., M. B. Rennels, V. Daya, and T. P. Hughes.** 1980. Hemagglutination and colonization factors in enterotoxigenic and enteropathogenic *Escherichia coli* that cause diarrhea. J. Infect. Dis. **141**:733–737.
16. **Levine, M. M., P. Ristaino, G. Marley, C. Smyth, S. Knutton, E. Boedeker, R. Black, C. Young, M. L. Clements, C. Cheney, and R. Patnaik.** 1984. Coli surface antigens 1 and 3 of colonization factor antigen II-positive enterotoxigenic *Escherichia coli*: morphology, purification, and immune responses in humans. Infect. Immun. **44**:409–420.
17. **Levine, M. M., P. Ristaino, R. B. Sack, J. B. Kaper, F. Ørskov, and I. Ørskov.** 1983. Colonization factor antigens I and II and type 1 somatic pili in enterotoxigenic *Escherichia coli*: relation to enterotoxin type. Infect. Immun. **39**:889–897.
18. **Lugtenberg, B. J., J. Meijers, R. Peters, P. van der Hoek, and L. van Alphen.** 1979. Electrophoretic resolution of the "major outer membrane protein" of *Escherichia coli* K12 into four bands. FEBS Lett. **58**:254–258.
19. **McConnell, M. M., L. V. Thomas, N. P. Day, and B. Rowe.** 1985. Enzyme-linked immunosorbent assays for the detection of adherence factor antigens of enterotoxigenic *Escherichia coli*. J. Infect. Dis. **152**:1120–1127.
20. **Merson, M. H., G. K. Morris, D. A. Sack, J. G. Wells, J. C. Feeley, R. B. Sack, W. M. Creech, A. Z. Kapikian, and E. J. Gangarosa.** 1976. Traveler's diarrhea in Mexico: a prospective study of physicians and family members attending a conference. N. Engl. J. Med. **294**:1299–1305.
21. **Morgan, R. L., R. E. Isaacson, H. W. Moon, C. C. Brinton, and C.-C. To.** 1978. Immunization of suckling pigs against enterotoxigenic *Escherichia coli*-induced diarrheal disease by vaccinating dams with purified 987 or K99 pili: protection correlates with pilus homology of vaccine and challenge. Infect. Immun. **22**:771–777.
22. **Moseley, S. L., I. Huq, A. R. M. A. Alim, M. So, M. Samadpour-Motalebi, and S. Falkow.** 1980. Detection of enterotoxigenic *Escherichia coli* by DNA colony hybridization. J. Infect. Dis. **142**:892–898.
23. **Nagy, B., H. W. Moon, R. E. Isaacson, C.-C. To, and C. C. Brinton.** 1978. Immunization of suckling pigs against enteric enterotoxigenic *Escherichia coli* infection by vaccinating dams with purified pili. Infect. Immun. **21**:269–274.
24. **Ornstein, L.** 1964. Disc electrophoresis—background and theory. Ann. N.Y. Acad. Sci. **121**:321–349.
25. **Ørskov, I., and F. Ørskov.** 1977. Special O:K:H serotypes among enterotoxigenic *Escherichia coli* strains from diarrhea in adults and children: occurrence of the CF (colonization factor) antigen and of hemagglutinating abilities. Med. Microbiol. Immunol. **163**:99–110.
26. **Ristaino, P. A., M. M. Levine, and C. R. Young.** 1983. Improved GM1-enzyme-linked immunosorbent assay for detection of *Escherichia coli* heat-labile enterotoxin. J. Clin. Microbiol. **18**:808–815.
27. **Rosenberg, M. L., J. P. Koplan, I. K. Wachsmuth, J. G. Wells, E. J. Gangarosa, R. L. Guerrant, and D. A. Sack.** 1977. Epidemic diarrhea at Crater Lake from enterotoxigenic *Escherichia coli*. Ann. Intern. Med. **86**:714–718.
28. **Sack, R. B.** 1975. Human diarrheal disease caused by enterotoxigenic *Escherichia coli*. Annu. Rev. Microbiol. **29**:333–353.
29. **Shapiro, A. L., E. Vinuela, and J. B. Maizel.** 1967. Molecular weight estimation of the polypeptide chains by electrophoresis in SDS polyacrylamide gels. Biochem. Biophys. Res. Commun. **28**:815–820.
30. **Taylor, W. R., W. L. Schell, J. G. Wells, K. Choi, D. E. Kinnunen, P. T. Heiser, and A. G. Helstad.** 1982. A foodborne outbreak of enterotoxigenic *Escherichia coli* diarrhea. N. Engl. J. Med. **306**:1093–1095.

31. **Thomas, L. V., A. Cravioto, S. M. Scotland, and B. Rowe.** 1982. New fimbrial antigenic type (E8775) that may represent a colonization factor in enterotoxigenic *Escherichia coli* in humans. Infect. Immun. **35**:1119–1124.
32. **Thomas, L. V., M. M. McConnell, B. Rowe, and A. M. Field.** 1985. The possession of three novel coli surface antigens by enterotoxigenic *Escherichia coli* strains positive for the putative colonization factor PCF8775. J. Gen. Microbiol. **131**:2319–2326.
33. **Thomas, L. V., and B. Rowe.** 1982. The occurrence of colonization factors (CFA I, CFA II, and E8775) in enterotoxigenic *Escherichia coli* from various countries in South East Asia. Med. Microbiol. Immunol. **171**:85–90.
34. **Thompson, M. R., H. Brandwein, M. LaBine-Racke, and R. A. Giannella.** 1984. Simple and reliable enzyme-linked immunosorbent assay with monoclonal antibodies for detection of *Escherichia coli* heat-stable enterotoxins. J. Clin. Microbiol. **20**:59–64.
35. **Towbin, H., T. Staehelin, and J. Gordon.** 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA **76**:4350–4354.
36. **Yamamoto, T., Y. Tamura, and T. Yokota.** 1985. Enteroadhesion fimbriae and enterotoxin of *Escherichia coli*: genetic transfer to a streptomycin-resistant mutant of the *galE* oral-route live-vaccine *Salmonella typhi* Ty21a. Infect. Immun. **50**: 925–928.