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Characterization of CS4 and CS6 Antigenic Components of PCF8775, a Putative Colonization Factor Complex from Enterotoxigenic *Escherichia coli* E8775

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PCF8775 is a putative colonization factor complex present on the surface of 10 to 20% of enterotoxigenic *Escherichia coli* strains and has been reported to be composed of antigen CS6 (morphology undefined) expressed alone or together with either of the rigid fimbrial antigens CS4 and CS5. To better define the individual components of this complex and the determinants of their expression, we prepared antiserum to the PCF8775 complex as it was expressed on prototype strain E8775 and then used the antiserum to identify the subunit structure of the antigens, to study their morphology, and to detect expression of individual components of the complex after transfer of plasmids into laboratory strain HB101. CS4 was purified from strain E8775, confirmed to be fimbrial by electron microscopy, and found to be composed of a 22-kilodalton protein subunit whose N-terminal amino acid sequence (1 to 20) was similar to that of colonization factor antigen I. Transconjugants that express CS6 but not CS4 were obtained by mating prototype strain E8775 with HB101. CS6 expression was mediated by a 61-megadalton plasmid. Expression of CS6 in the transconjugants correlates with expression of a 16-kilodalton cell surface protein. The CS6 antigen was confirmed to be present on the cell surface by immunogold labeling, but its morphology was beyond the limits of resolution by electron microscopy.

Enterotoxigenic *Escherichia coli* (ETEC) strains are an important cause of traveler's diarrhea in visitors to developing countries and an important cause of diarrheal dehydration in infants in these countries (14). All ETEC strains produce heat-labile or heat-stable enterotoxin or both, and most have fimbrial (or fibrillar or both) colonization factor antigens (CFAs) on their surface that promote attachment of the bacteria to the intestinal mucosa (9, 14, 24). Heat-labile and heat-stable enterotoxins are well defined biochemically and genetically, but of the putative CFAs so far identified on the surface of ETEC only CFA/I (8, 9) and the CFA/II complex (10, 16) have been well characterized. Vaccines against ETEC strains are being developed that contain protective antigens derived from the toxins and the CFAs (13). Successful vaccination against porcine ETEC with surface antigens suggests that human vaccines containing CFAs may be efficacious (9). Indeed, volunteer studies suggest that CFA/II antigens are protective against challenge with ETEC strains from different O serotypes bearing CFA/II (14–16). For broad protection, vaccines should contain all of the recognized CFAs. Hence, it is important to characterize all ETEC CFAs preliminary to determining their usefulness as vaccine components.

Well-characterized CFA/I has a fimbrial structure composed of 15-kilodalton (kDa) subunits, and its amino acid sequence is known (8). CFA/II actually represents a complex of three distinct antigens. All strains bearing CFA/II express CS3, and they may in addition express either CS1 or CS2 (2, 26). CS1 and CS2 are rigid fimbriae 6 to 7 nm in diameter (27), while CS3 consists of thin, flexible, wiry fibrillar fimbriae 2 to 3 nm in diameter (16). CS1 and CS3 have been purified. The subunit of CS1 has an apparent molecular mass of 16.8 kDa, and subunits of 14.5 and 15.5 kDa are present in purified preparations of CS3 (16). The molecular mass of the subunit of CS2 is 17 kDa (10).

PCF8775 is a putative CFA which was detected on 24% of ETEC isolates from Bangladesh (18). PCF8775 is analogous to CFA/II in that it is a complex composed of three antigens designated CS4, CS5, and CS6 which have been identified by immunodiffusion (30). Although its role in humans has not been defined, the role of PCF8775 antigens for colonization, disease, and protective immunogenicity in rabbits has been described (28). CS6 in particular was found to be important. A single plasmid has been shown to code for CS5, CS6, heat-stable enterotoxin, and colicin Ia (31). All strains bearing PCF8775 express CS6, and some express CS4 or CS5 as well. Both CS4 and CS5 are rigid fimbriae 6 to 7 nm in diameter and promote mannose-resistant hemagglutination of human and bovine erythrocytes. CS5 also promotes mannose-resistant hemagglutination of guinea pig erythrocytes. In contrast, CS6 does not promote mannose-resistant hemagglutination, and heretofore electron microscopic studies have failed to identify the morphology of CS6 (30). The nature of CS4, CS5, and CS6 antigens has not been further characterized.

As a first step toward production of a vaccine strain effective against ETEC expressing PCF8775, we mobilized a plasmid which mediates expression of the CS6 antigen into a laboratory strain of *E. coli*. This allowed identification and characterization of the structure of the CS6 component of PCF8775 and, by comparison with the proteins of E8775, that of CS4.

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MATERIALS AND METHODS

Bacterial strains and growth conditions. *E. coli* E8775 Tox$^-$, a derivative of E8775 which no longer expresses heat-labile or heat-stable enterotoxin, was obtained from A. Cravioto, Instituto Nacional de Ciencias y Tecnologia de la Salud del Niño, Mexico City, Mexico. A nalidixic acid-resistant (Nal^r) derivative was obtained by passing cultures in increasing amounts of nalidixic acid. *E. coli* HB101 (1) and HU735 were obtained from Dennis Kopecko, Department of Bacterial Immunology, Walter Reed Army Institute of Research, Washington, D.C. A nalidixic acid-resistant derivative of HB101 was obtained as for E8775. HU735 contains F'*ts*114*lac*::Tn*5* (25). *E. coli* M424C1 was from the collection of the Department of Gastroenterology, Walter Reed Army Institute of Research. M424C1 is O6:H16 and expresses the CS1 and CS3 components of CFA/II (16). *E. coli* B7A (5) was obtained from Samuel Formal, Department of Enteric Diseases, Walter Reed Army Institute of Research. E11881 (28, 30), E23666, E24133, E32018, and E17018 (19a) derivatives were from the Division of Enteric Pathogens, Central Public Health Laboratory Service, London, England.

Antibiotics were added to L broth supplemented with agar (20) as follows. Kanamycin (Sigma Chemical Co., St. Louis, Mo.) was added, when appropriate, at 50 μg/ml. Streptomycin sulfate (Pfizer Inc., New York, N.Y.) was used at 25 μg/ml, and nalidixic acid (Sigma) was used at 20 μg/ml. Lactose utilization was scored after growth on MacConkey agar plates (Difco Laboratories, Detroit, Mich.). Penassay broth was from Difco (Antibiotic Medium 3). CFA plates were prepared as previously described (29). Growth was at 37°C, save for the following exceptions. Strains were incubated at 30 to 32°C when maintenance of F'*ts*114*lac*::Tn*5* was desired and at 20 to 22°C when indicated to determine temperature-dependent expression of CS4 and CS6.

Preparation of antisera. E8775 Tox$^-$ and B7A cells from CFA plates incubated overnight at 37°C were mixed with Freund complete adjuvant (Difco) and inoculated subcutaneously into adult male New Zealand White rabbits. Rabbits were boosted weekly with cells in Freund incomplete adjuvant. A sample of E8775 antiserum was absorbed with E8775 Tox$^-$ cells grown on CFA plates at 22°C and with HB101 cells grown on CFA plates at 37°C. This absorbed antiserum should be depleted of antibodies that recognize surface antigens of E8775 that are not regulated by growth temperature and any cross-reacting surface antigens of HB101 but should retain antibodies to PCF8775 antigens. The B7A antiserum was absorbed with B7A cells grown on CFA plates at 22°C.

Conjugation and characterization of plasmids. Conjugations were performed on filters. Recipient and donor strains were grown overnight in Penassay broth at 37°C (HU735 containing F'*ts*114*lac*::Tn*5* was grown at 32°C). Selection was on MacConkey agar plates supplemented with kanamycin and nalidixic acid for E8775 Tox$^-$ Nal^r or HB101 Nal^r with F'*ts*114*lac*::Tn*5* or with kanamycin and streptomycin to select HB101 transconjugants. Transconjugants were checked for sensitivity to kanamycin, nalidixic acid, and streptomycin and scored for lactose utilization after growth on MacConkey agar plates.

Strains were cured of F'*ts*114*lac*::Tn*5* by growth at 45°C, followed by dilution and plating on MacConkey agar. Lac$^-$ derivatives were screened for Km^r and plasmids.

Strains were screened for plasmid composition by an alkaline lysis procedure (3). Digestion with restriction enzyme *Eco*RI (Bethesda Research Laboratories, Inc., Gaithersburg, Md.) was by manufacturer specifications. Plasmids were visualized by staining with ethidium bromide after electrophoresis on 0.6% agarose gels in Tris-borate buffer (17). For hybridization experiments, the DNA was transferred to Nylon 66 membranes (Pall Ultrafine Filtration Corp., Glen Cove, N.Y.) by the Southern transfer technique (17), with the following modifications. The gel was soaked in 0.25 M HCl for 30 min before denaturation, neutralization was achieved by soaking the gel in 3 M sodium acetate, pH 5.5, and 20× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) was used for the transfer.

For DNA hybridization, the 61-megadalton (MDa) plasmid from strain M56 (M16 cured of F'*ts*114*lac*::Tn*5*; see Results) was purified by a modification of the method of Helinski (17). The plasmid was labeled with [α-^{32}P]CTP (Du Pont Co., Wilmington, Del.) by nick translation as recommended by the manufacturer (Bethesda Research Laboratories). Hybridization was as previously described (17). The blot containing plasmids of E8775, M16, and M17 digested with *Eco*RI was prehybridized for 1 h at 65°C in 10 ml of hybridization solution (0.1% Ficoll [Sigma], 0.1% polyvinylpyrrolidone [Sigma], 0.1% bovine serum albumin, 0.9 M NaCl, 0.05 M sodium phosphate [pH 8.3], 0.05 M EDTA, 0.2% sodium dodecyl sulfate, 500 μg of salmon sperm DNA). Denatured ^{32}P-labeled plasmid DNA was added to the blot and incubated at 65°C overnight. The blot was washed three times for 30 min each time at room temperature in wash buffer (5 mM sodium phosphate [pH 7.0], 1 mM EDTA, 0.2% sodium dodecyl sulfate) and used to expose X-Omat AR film (Eastman Kodak Co., Rochester, N.Y.).

Detection of E8775 antigens by peroxidase-labeled antibody. Transconjugants expressing surface antigens from E8775 were detected by reaction of peroxidase-conjugated antibody with colonies blotted on nylon membranes. Km^r Sm^r transconjugants were transferred to CFA plates, incubated overnight at 37°C, and transferred to Nylon 66 membranes (Pall Ultrafine Filtration). The blots were dried at 80°C for 1 h and stored until use. Blots were blocked by incubation overnight at 45 to 50°C in phosphate-buffered saline containing 10% bovine serum albumin (Sigma). The absorbed antiserum to E8775 was diluted 1:100 in 50 ml of phosphate-buffered saline–2% bovine serum albumin and incubated with the 21 filters with agitation for 1 h at room temperature. Excess antiserum was removed by four washes with phosphate-buffered saline–0.1% Triton X-100 buffer. A peroxidase conjugate of goat anti-rabbit immunoglobulin G (Organon Teknika, Malvern, Pa.) was added and incubated as described above. The filters were then washed as before. ATBS [2,2'-azino,di-(3-ethylbenzthiazoline sulfonate)] reagent was prepared as suggested by the manufacturer (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, Md.).

Transmission electron microscopy. Negatively stained specimens of whole cells for electron microscopy were prepared as follows. Cell suspensions were made by picking 5 to 7 colonies from CFA plates (grown at either 18 or 37°C) and suspending them in 1 ml of a 1% (wt/vol) sodium phosphotungstate (pH 6.8) staining solution. A drop of the cell suspension was then placed on a 0.25% Formvar carbon-coated copper grid (200 mesh) for 1 min. Excess stain was removed from the grid with filter paper, and the grid was air dried before the specimen was examined in a Siemens IA transmission electron microscope operating at an acceleration voltage of 80 kV.

Immunogold labeling. Immunogold labeling and electron microscopy of *E. coli* E8775 and M17 were performed as described by Levine et al. (16), except that unwashed cell

suspensions and 0.5% phosphotungstic acid (pH 6.8) were used. The goat anti-rabbit immunoglobulin G colloidal gold conjugate (Janssen Pharmaceutical, Piscataway, N.J.) used contained 10-nm-diameter gold particles. Grids incubated without primary antisera or with nonimmune rabbit antiserum were used as controls. Specimens were examined in a Siemens IA transmission electron microscope operating at an acceleration voltage of 80 kV.

Preparation of heat-saline extracts. Heat-saline extracts were prepared as described by Ørskov et al. (22). Lawns of each strain of bacteria were grown on five CFA plates, and the bacteria were scraped from the plates and suspended in 2 ml of 0.8% NaCl. The suspensions were incubated at 60°C for 20 min and then subjected to centrifugation at 8,000 × *g* for 20 min. Supernatants were saved as the heat-saline extracts and stored at −70°C.

Purification and N-terminal sequence determination of CS4. E8775 cells were homogenized, and fimbriae were collected after ammonium sulfate precipitation as described by Isaacson for K99 (7). A 1-nmol sample of CS4 pilus protein was subjected to N-terminal amino acid sequence analysis by Edman degradation with a gas phase protein sequenator (6).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blots (immunoblots). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed as described by Laemmli (12). Identical sets (50 μl per well) were applied to each half of a 4% stacking-15% resolving gel. Proteins were transferred to nitrocellulose paper (Bio-Rad Laboratories, Rockville Centre, N.Y.). One section was stained with amido black to visualize all of the transferred proteins, and the other half was immunoblotted as recommended by the manufacturer (Bio-Rad). E8775 antiserum was used at a dilution of 1:1,000. The second antibody was goat anti-rabbit immunoglobulin G conjugated to peroxidase (Organon Teknika), and the substrate was 4-chloro-1-naphthol.

Immunodiffusion. Antigens in the heat-saline extracts were detected by reaction with antisera by the method of Ouchterlony and Nelsson (23). Undiluted antiserum or heat-saline extracts containing the antigen were added to the wells.

RESULTS

Preparation of antiserum to surface antigens of E8775 Tox⁻. Antiserum was raised in rabbits against whole cells of E8775 Tox⁻ grown under conditions known to promote expression of CS4 and CS6 antigens. This unabsorbed antiserum (Fig. 1) reacted with a number of antigens present in heat-saline extracts of E8775 grown at 37°C (Fig. 1, well 1) or 22°C (Fig. 1, well 2) as indicated by immunodiffusion. To remove antibodies to E8775 antigens other than CS4 and CS6, the antiserum was absorbed with E8775 Tox⁻ cells grown at 22°C (Fig. 1). Absorption of the E8775 antiserum resulted in removal of antibodies that recognized many antigens, but an antibody that recognized two antigens remained (Fig. 1, well B), one antigen that migrated only a short distance from the well and another that migrated further. Since Thomas et al. (30) reported that CS4 migrates only a short distance, we provisionally designated this antigen CS4 and the other antigen CS6.

Generation of transconjugants expressing surface antigens from E8775. F′*ts*114*lac*::Tn*5* was transferred into E8775 Tox⁻ Nal^r by conjugation, yielding E8775 derivative M13. M13 was then mated with HB101, and transconjugants were obtained. All transconjugants were Lac$^+$, indicating that the

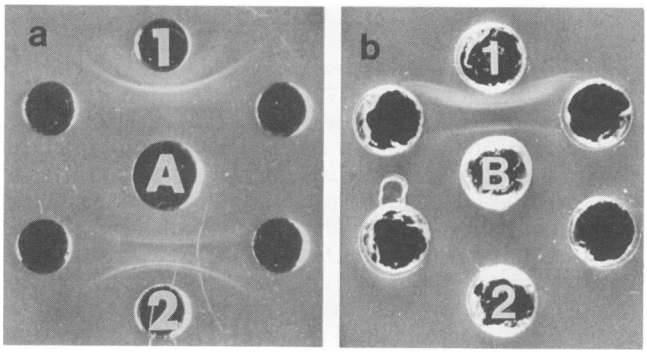


FIG. 1. Immunodiffusion of membrane extracts. Wells: 1, membrane extract of E8775 grown at 37°C; 2, membrane extract of E8775 grown at 22°C; A, unabsorbed antiserum to E8775 grown at 37°C; B, absorbed antiserum ($E8775_{abs}$) to E8775 grown at 37°C and absorbed with both E8775 grown at 22°C and HB101 grown at 37°C. The unlabeled wells were empty.

F′*ts*114*lac*::Tn*5* factor was present. Expression of antigens from E8775 cells was detected after blotting of colonies to nylon membranes, binding of the absorbed antiserum to E8775 Tox⁻, and reaction of peroxidase-conjugated antibody. Of 1,000 colonies, 87 were positive for expression of E8775 antigens. Ten were screened for plasmid composition, and two transconjugants with distinct plasmid profiles (see below and Fig. 6) were designated M16 and M17 and chosen for characterization.

Characterization of antigens by immunodifusion. Heat-saline extracts prepared from M16 and M17 yielded identical immunodiffusion patterns but different patterns from that seen with E8775 Tox⁻ extracts. Extracts of M16 (Fig. 2A, well 1) and M17 (Fig. 2A and B, wells 3) both contained an antigen which migrated far from the well, but the major antigen in the E8775 Tox⁻ extract (Fig. 2A and B, wells 2) remained near the antigen well. To confirm the identities of these surface antigens and the specificity of the absorbed antiserum, we compared the immunodiffusion patterns of ETEC strains bearing known PCF8775 components with those of E8775 Tox⁻ and transconjugant M17. Figure 2B shows a line of identity of the antigen in M17 with those of strains reported to express CS6 (E11881C in well 5 and E11881E in well 6), but a derivative strain that does not express CS6 (E11881B in well 4) lacks the antigen. An

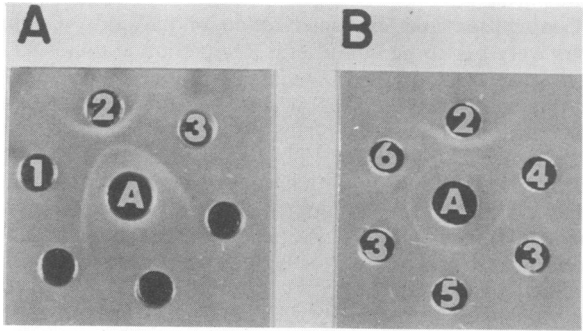


FIG. 2. Immunodiffusion of membrane extracts from *E. coli* strains expressing PCF8775 and derivatives (outer wells) against $E8775_{abs}$ antiserum (center wells). Wells: A, $E8775_{abs}$ antiserum; 1, M16; 2, E8775 Tox⁻; 3, M17; 4, E11881B; 5, E11881C; 6, E11881E. The unlabeled wells were empty.

TABLE 1. Antigens detected in membrane extracts of strains bearing PCF8775

Strain	Serotype	PCF8775 expressed[a]	Antigen(s) detected by:	
			Immunodiffusion[b]	Western blot[c] (kDa)
E8775 Tox$^-$	O25:H42	CS4$^+$ CS6$^+$	CS4, CS6	16, 22
M16			CS6	16
M17			CS6	16
E11881B	O25:H42	CS4$^-$ CS6$^-$	ND[d]	ND
E11881C	O25:H42	CS4$^-$ CS6$^+$	CS6	16
E11881E	O25:H42	CS4$^+$ CS6$^+$	CS4, CS6	16, 22
E23666A	O27:H20	CS4$^-$ CS6$^+$	CS6	16
E23666B	O27:H20	CS4$^-$ CS6$^-$	ND	ND
E24133A	O27:H20	CS4$^-$ CS6$^+$	CS6	16
E24133B	O27:H20	CS4$^-$ CS6$^-$	ND	ND
E32018A	O27:H20	CS4$^-$ CS6$^+$	CS6	16
E32018B	O27:H20	CS4$^-$ CS6$^-$	ND	ND
E17018A	O167:H5	CS5$^+$ CS6$^+$	CS6	16
E17018B	O167:H5	CS5$^-$ CS6$^-$	ND	ND
B7A	O148:H28		CS6	16

[a] From references 18, 19a, 28, and 30.
[b] Identified by lines of identity with heat-saline extract of E8775 Tox$^-$.
[c] In Western blots with E8775$_{abs}$ antiserum at a dilution of 1:1,000.
[d] ND, None detected.

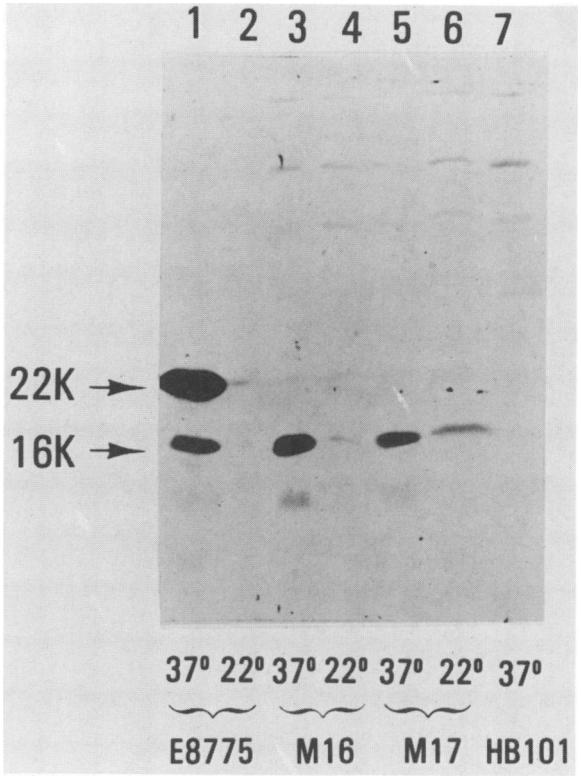


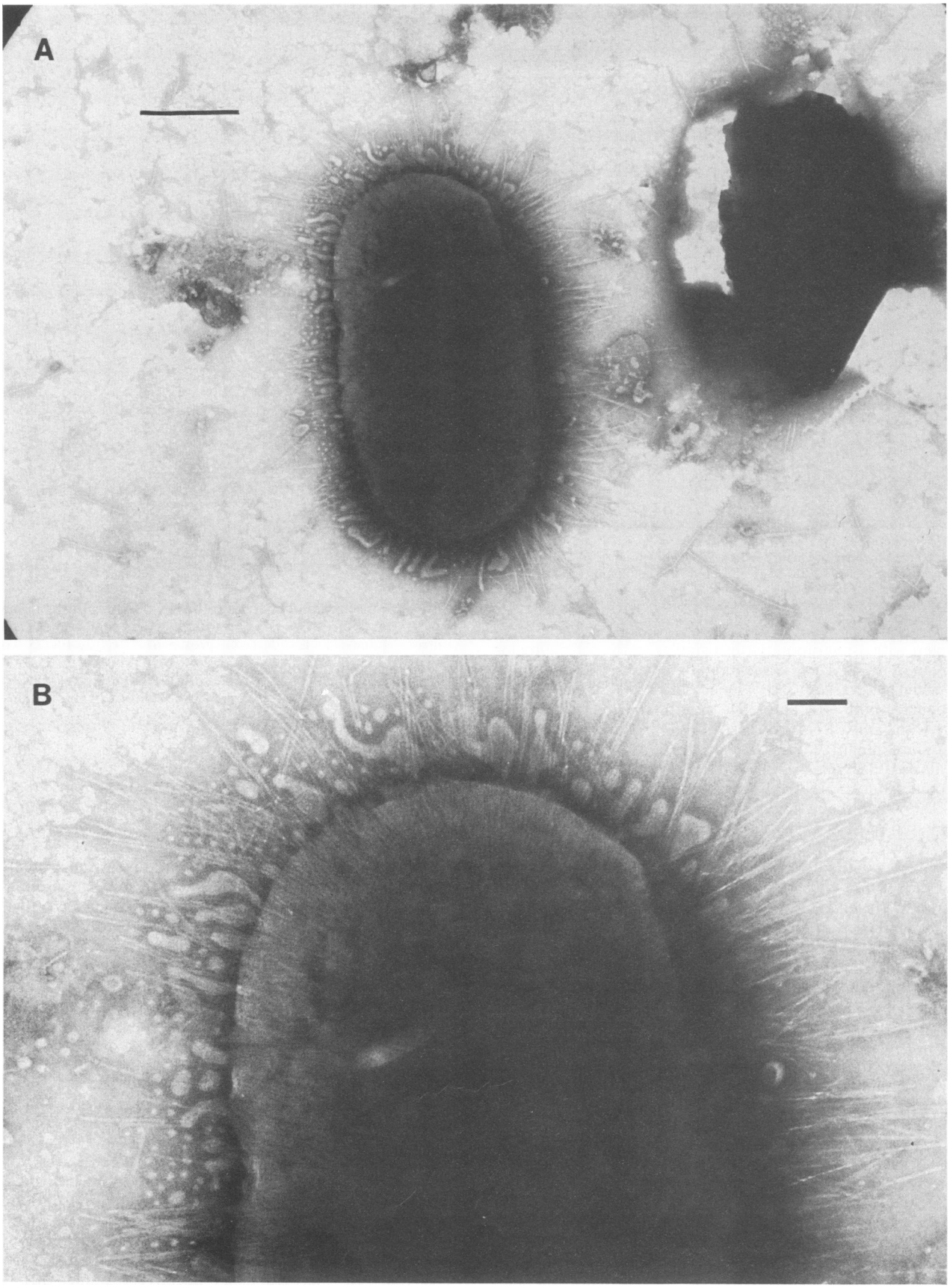
FIG. 3. Western blot analysis of CS4 and CS6 antigens in membrane extracts of E8775 Tox$^-$, transconjugants M16 and M17, and controls. Lanes: 1, E8775 Tox$^-$ grown at 37°C; 2, E8775 Tox$^-$ grown at 22°C; 3, M16 grown at 37°C; 4, M16 grown at 22°C; 5, M17 grown at 37°C; 6, M17 grown at 22°C; 7, HB101 grown at 37°C. E8775$_{abs}$ antiserum was used at a 1:1,000 dilution. The positions of the 16- and 22-kDa fragments are shown.

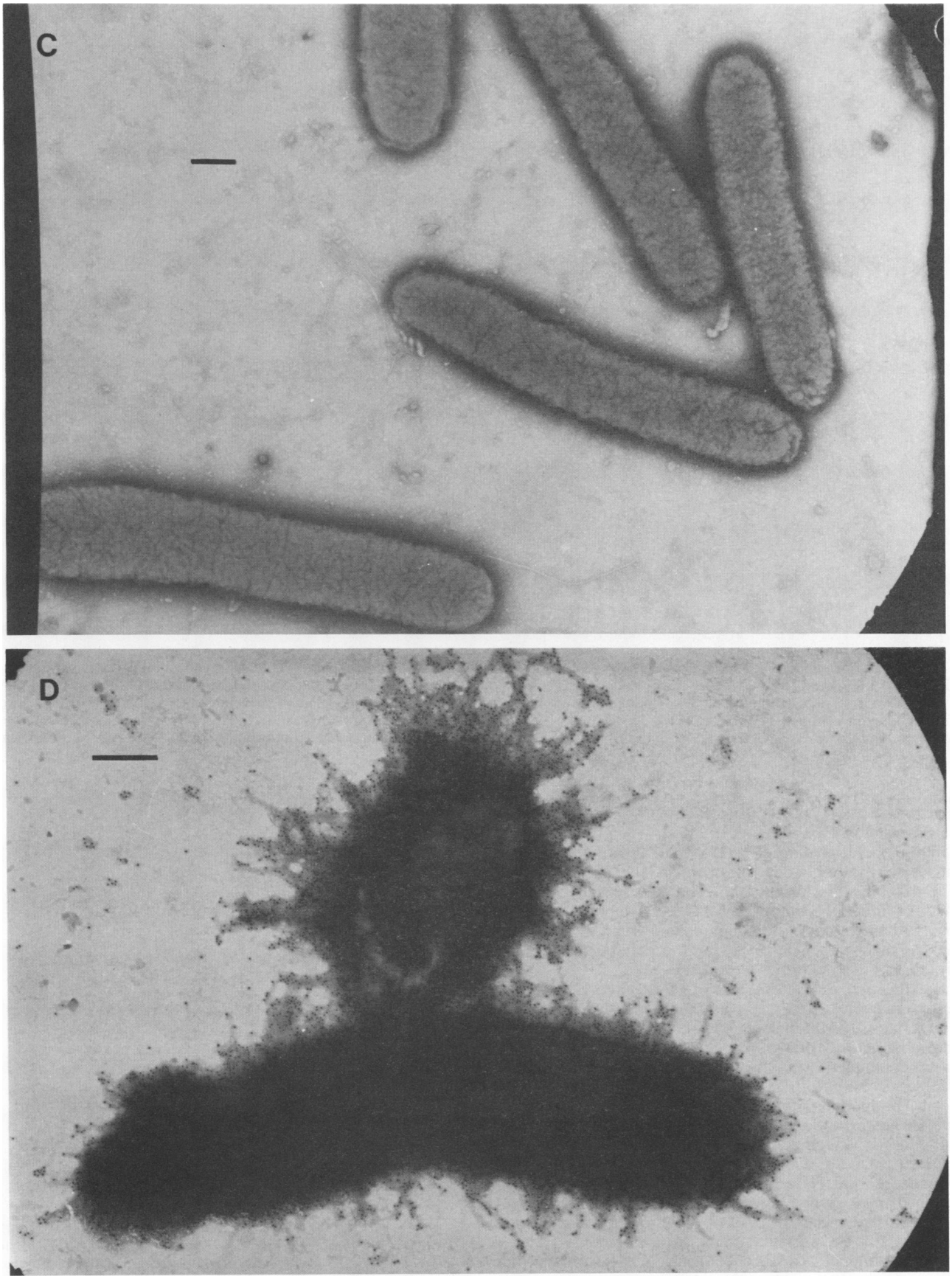
antigen with a migration pattern similar to that of the major antigen in E8775 Tox$^-$ is seen faintly in strain E11881E (well 6), which expresses CS4. Hence, the absorbed antiserum recognizes both CS6 and CS4, the transconjugants express only CS6, and E8775 expresses both CS4 and CS6. These data are summarized in Table 1.

Characterization of antigens by Western blots. Heat-saline extracts from E8775 Tox$^-$ contained a prominent protein at 22 kDA which reacted with the absorbed antiserum and was expressed in cells grown at 37°C (Fig. 3, lane 1) but not in cells grown at 22°C (Fig. 3, lane 2). The absorbed antiserum also reacted with a 16-kDA protein. There was no reaction of the antiserum with M424C1 (data not shown), a strain bearing CS1 and CS3 (16), or with a CFA/II vaccine preparation composed of about 80% CS3 and 20% CS1 (data not shown; unpublished data). In contrast, the prominent antigen in transconjugants M16 and M17 is 16 kDa (Fig. 3, lanes 3 and 5). There was weak reactivity with a number of larger proteins, but these were also present in extracts prepared from HB101 (Fig. 3, lane 7); hence, they are not associated with the plasmids from E8775. The antigens of M17 were identical to those of M16 (Fig. 3, lanes 3 and 5). Only the 16-kDa antigen was expressed in the transconjugants, not the 22-kDa protein seen in E8775. The 16-kDa protein (Fig. 3, lane 1) was seen in heat-saline extracts from E8775 but stained much less intensely than the 22-kDa protein.

To confirm that the CS4 antigen is 22 kDa and to determine whether the 16-kDa antigen correlated with CS6, we analyzed clinical isolates that express PCF8775. The results are summarized in Table 1. We analyzed heat-saline extracts of clinical isolates that express PCF8775, including strains that express both CS4 and CS6 (E8775 Tox$^-$ and E11881E), strains that do not express either (E11881B), and strain pairs which were characterized as to their ability to express CS6 (E23666A, E24133A, and E32018A) or their inability to do so (E23666B, E24133B, and E32018B). We also examined a strain that expresses CS5 together with CS6 (E17018A) and its derivative (E17018B), which expresses neither (19a, 30), and B7A, which is a classic ETEC strain of serogroup O148 first isolated in British troops in Aden and later found in Vietnam (5) but whose surface antigens had not been defined. The 22-kDa antigen was found in all strains known to express CS4. In each strain reported to have CS6, we found expression of the 16-kDa antigen. Strain B7A also expressed the 16-kDa antigen, and immunodiffusion showed a line of identity when a heat-saline extract of B7A was run along with a heat-saline extract of M17 against E8775$_{abs}$ antiserum (data not shown). To prove that B7A expresses the 16-kDa antigen, we obtained antiserum to B7A and absorbed it with B7A grown at 22°C to obtain B7A$_{abs}$ antiserum specific for surface antigens whose expression is temperature dependent. This B7A$_{abs}$ antiserum recognized the 16-kDa antigen of M17 and E8775 (data not shown). These data suggest that the 16-kDa antigen of B7A and E8775 is CS6.

Characterization of CS4 and CS6 by electron microscopy. Negative staining of E8775 Tox$^-$ revealed fimbrial structures when the cells were grown at 37°C (Fig. 4A) but no fimbriae if the cells were grown at room temperature (data not shown). This agrees with the data reported by Thomas et al. (29). Electron micrographs of negatively stained M17 revealed no discernible surface structures (Fig. 4C), except for


A
B


C
D

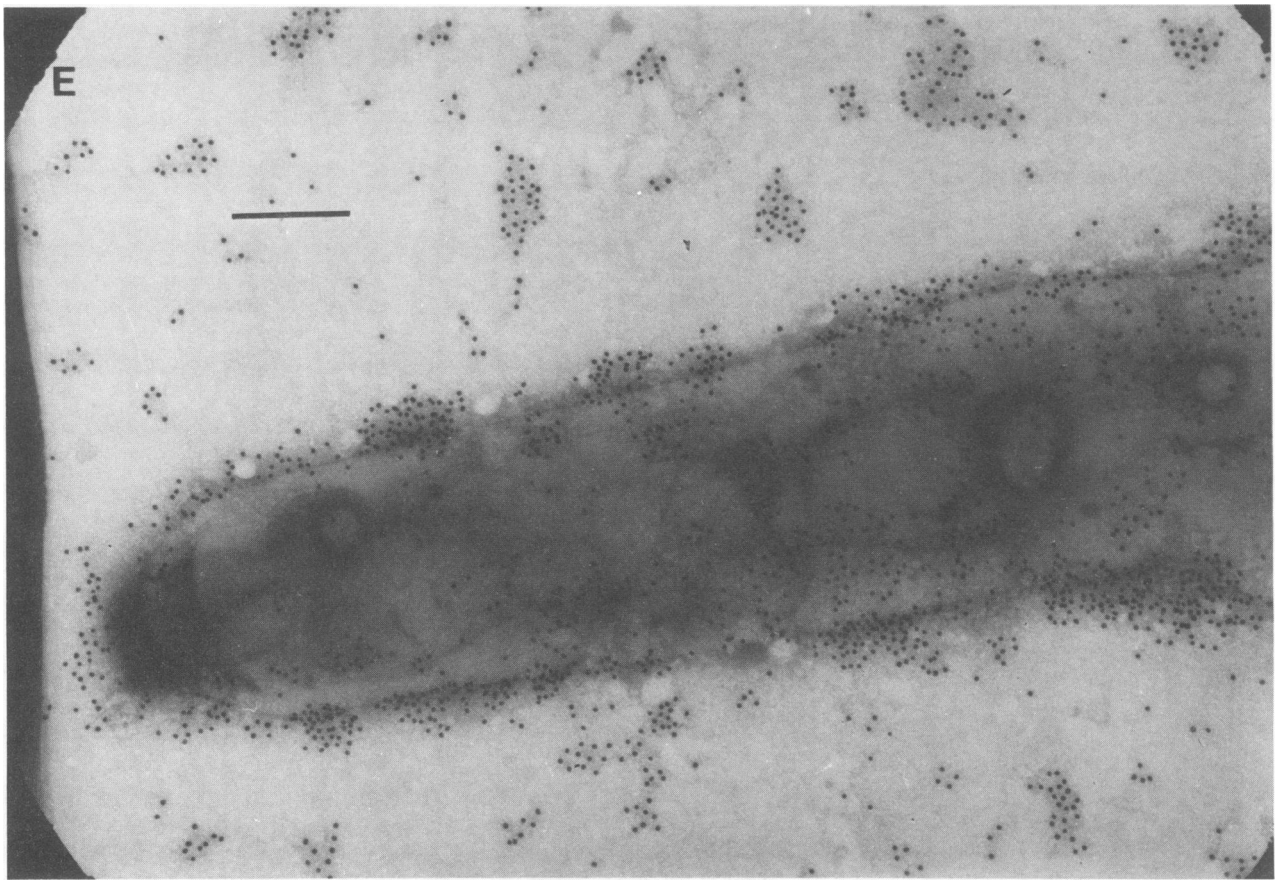


FIG. 4. Electron micrographs. Panels: A and B, *E. coli* E8775 Tox$^-$ negatively stained with 1% sodium phosphotungstate, pH 6.8 (bars, 0.5 μm [A] and 0.1 μm [B]); C, *E. coli* transconjugant M17 negatively stained with 1% sodium phosphotungstate, pH 6.8 (bar, 0.2 μm); D, *E. coli* E8775 Tox$^-$ labeled by the immunogold technique (bar, 0.5 μm); E, *E. coli* M17 labeled by the immunogold technique (bar, 0.2 μm). For immunogold labeling (D and E), cells were incubated with rabbit E8775$_{abs}$ antiserum (1:2 dilution), followed by colloidal gold-labeled goat anti-rabbit immunoglobulin G serum (1:6 dilution). Cells were stained with 0.5% sodium phosphotungstate, pH 6.8. All cells were grown at 37°C on CFA plates.

occasional solitary, large pilus structures which are probably the F sex pilus.

Immunogold labeling of E8775 Tox$^-$ cells with E8775$_{abs}$ antiserum resulted in deposition of the label on the fimbriae (Fig. 4D), showing that the absorbed antiserum recognizes the CS4 fimbrial structures. M17 cells labeled with the absorbed antiserum by the immunogold technique (Fig. 4E) showed much label on and near the cell surface, but no structures were definitively evident.

Isolation and characterization of CS4. When E8775 Tox$^-$ cells were subjected to a standard procedure for purification of fimbriae (see Materials and Methods), fimbrial structures were obtained, as shown by electron microscopy, and their major component was the 22-kDa protein. This protein reacted strongly with the absorbed antiserum in Western blots. The immunodiffusion pattern of this preparation developed against the absorbed antiserum was the same as that reported for CS4; i.e., the antigen migrated only a short distance from the well (data not shown). The amino acid sequence of the N terminus of the purified fimbriae was obtained and is given in Fig. 5, where it is compared with the published sequences of CFA/I (8) and the CS2 component of CFA/II (10). The fact that the CS4 preparation yielded a single N-terminal sequence confirms that it contains one major protein.

	CS4	CFA/I	CS2
1	val	val	ala
	glu	glu	glu
	lys	lys	lys
	asn	asn	asn
5	ile	ile	ile
	thr	thr	thr
	val	val	val
	cys	thr	thr
	ala	ala	ala
10	ser	ser	ser
	val	val	val
	asp	asp	asp
	pro	pro	pro
	thr	val	val
15	ile	ile	ile
	cys	asp	asp
	ile	leu	leu
	lys	leu	leu
	gln	gln	gln
20	ala	ala	ala

FIG. 5. N-terminal amino acid sequence of CS4. The published sequences of CFA/I (8) and CS2 (10) are given for comparison. Underlines indicate amino acids that differ from those of CS4.

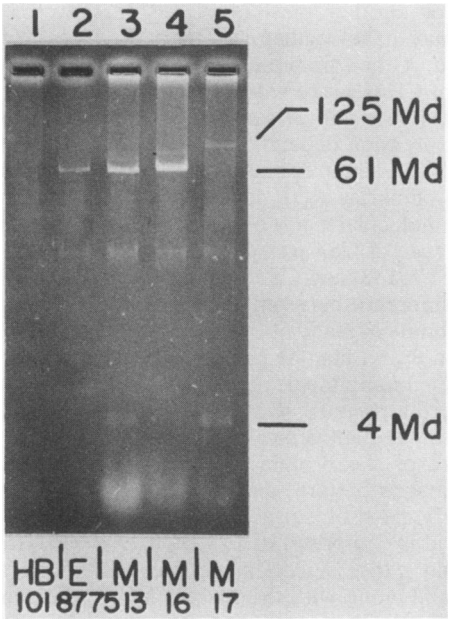


FIG. 6. Plasmid profiles of *E. coli* E8775 Tox$^-$ and transconjugants. Lanes: 1, HB101; 2, E8775 Tox$^-$; 3, M13 (E8775 Tox$^-$ containing the F′ from HU735); 4, M16; 5, M17. The positions of the 4-, 61-, and 125-MDa plasmids are shown.

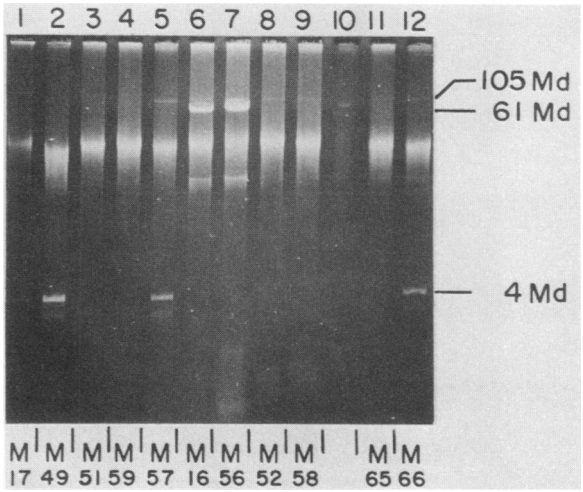


FIG. 7. Plasmid profiles of strains M16 and M17 and derivatives containing plasmids from them. Lanes: 1, M17; 2, M49 (M17 cured of the F′ hybrid); 3 and 4, M51 and M59 (transconjugants containing the F′ hybrid from M17); 5, M57 (spontaneous Lac$^-$ variant of M17); 6, M16; 7, M56 (M16 cured of the F′ factor); 8 and 9, M52 and M58 (transconjugants containing F′ from M16); 10, blank; 11, M65 (transconjugant containing F′ from HU735); 12, M66 (strain M49 containing F′ from HU735). The positions of the 4-, 61-, and 105-MDa plasmids are shown.

Temperature-dependent expression of CS6. Expression of the 16-kDa antigen is suppressed in E8775 Tox$^-$, M16, M17 (Fig. 3), or B7A (data not shown) cells grown at 22°C. Expression of the 22-kDa antigens is also suppressed at this temperature. Moreover, immunodiffusion patterns of heat-saline extracts from E8775 Tox$^-$ and M17 cells grown at 22°C lack the precipitin lines associated with CS4 and CS6 (data not shown). CS4 and CS6 precipitin lines were also lacking in immunodiffusion against antiserum that had not been absorbed with E8775. This confirms that the 16- and 22-kDa antigens are present on cells grown at 37°C but not on cells grown at 22°C. Therefore, we conclude that expression of CS6 and CS4 is temperature dependent. CS4 expression has previously been reported to be temperature dependent (29).

Plasmid composition of E8775 Tox$^-$ and transconjugants and identification of the plasmid mediating expression of CS6. E8775 Tox$^-$ contains plasmids of 4 and 61 MDa (Fig. 6, lane 2). M13 (lane 3), the E8775 Tox$^-$ derivative carrying the F′ factor, contained these plasmids, as well as two larger plasmids. These were probably derivatives of the F′ factor. Distinct plasmid patterns were detected in the transconjugants; M17 contained 4- and 125-MDa plasmids (Fig. 6, lane 5; Fig. 7, lane 1), and M16 contained a predominant 61-MDa plasmid and a less prominent 105-MDa plasmid (Fig. 6, lane 4; Fig. 7, lane 6). To determine the locations of the CS6 genes, strains containing single plasmids were constructed.

We cured M17 of the 125-MDa plasmid by growing the cells at 45°C and screening for loss of the Lac$^+$ phenotype carried on the F′ factor to obtain strain M49. This Lac$^-$ derivative also lost the kanamycin resistance phenotype and lacked the 125-MDa plasmid but retained the 4-MDa plasmid (Fig. 7, lane 2). M49 did not express CS6 when assayed by the colony blot assay.

Strains which contain only the 125-MDa plasmid were constructed by conjugation of M17 and a nalidixic acid-resistant derivative of HB101. These matings resulted in derivatives M51 and M59 (Fig. 7, lanes 3 and 4) that were Km^r Nal^r Lac$^+$ and reacted with the absorbed antiserum and, hence, expressed CS6. Therefore, CS6 expression in M17 is mediated by the 125-MDa plasmid. The Lac$^+$ Km^r phenotype of M51 and M59 indicated at least part of F′*ts*114*lac*::Tn*5* was present on the 125-MDa plasmid. To show that the 125-MDa plasmid is not the same plasmid used for mobilization, strains similar to M51 and M59 but carrying F′*ts*114*lac*::Tn*5* conjugated directly from HU735 were constructed. Introduction of this F′ from HU735 into either HB101 or HB101 containing the 4-MDa plasmid resulted in strains M65 and M66, respectively, and neither expressed CS6. The F′ in these two strains was smaller than the 125-MDa plasmid in M17 (Fig. 7, lanes 11 and 12); hence, the 125-MDa plasmid in M17 has additional DNA not present in F′*ts*114*lac*::Tn*5* and this DNA mediates expression of CS6. M57 is a spontaneous Lac$^-$ derivative of M17 (Fig. 7, lane 5).

The plasmid pattern of M16 is distinct from that of M17, but expression of CS6 in M17 and M16 is indistinguishable. Conjugation of M16 and the Nal^r derivative of HB101 resulted in transfer of the Lac$^+$ Km^r phenotype associated with F′*ts*114*lac*::Tn*5* to form strains M52 and M58. That phenotype correlated with the 105-MDa plasmid from M16 (Fig. 7, lanes 8 and 9), which is the size of F′*ts*114*lac*::Tn*5* (lanes 11 and 12). M52 did not express CS6; hence, the 105-MDa plasmid of M16 appears to be F′*ts*114*lac*::Tn*5*. M56, a derivative of M16 cured of the F′*ts*114*lac*::Tn*5*, was obtained. This derivative contained the 61-MDa plasmid (Fig. 7, lane 7) and reacted with the absorbed antiserum. Hence, CS6 expression in M16 is mediated by the 61-MDa plasmid.

To determine how much of the plasmid DNA from the 61-MDa plasmid was present in the large plasmid of M17, DNA hybridization was performed. Plasmid DNAs from E8775, M16, and M17 were digested with restriction enzyme *Eco*RI and hybridized with the 61-MDa plasmid from M56. The pattern of hybridization was the same for all strains (Fig.

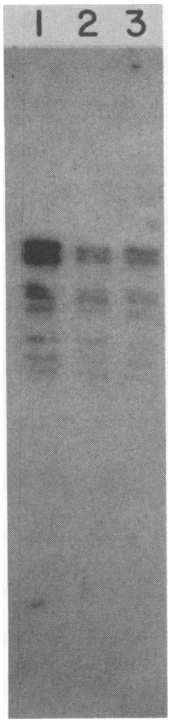


FIG. 8. Hybridization of *Eco*RI digests of plasmids from E8775, M16, and M17 with the 61-MDa plasmid from M16. Lanes: 1, E8775; 2, M16; 3, M17.

8); hence, the entire plasmid is present in both strains M16 and M17.

DISCUSSION

We prepared an antiserum that recognizes CS4 and CS6 antigens of PCF8775 and confirmed its specificity for CS4 and CS6 with strains known to express CS6 and CS4. From these data, we conclude that a 22-kDa protein is the CS4 subunit and a 16-kDa protein is the CS6 subunit. Expression of both antigens was dependent on growth temperature. By conjugation, we mobilized the genes that mediate expression of the CS6 antigenic component of PCF8775 from E8775 into HB101, a laboratory strain of *E. coli*. CS6 expression in the transconjugants correlates with the presence of a protein of 16 kDa in the HB101 derivatives. The morphology of CS6 remains undefined, but immunogold labeling suggests that it is strongly expressed as a surface antigen.

Our experience indicates that CS4 is the predominant antigen of PCF8775 expressed on E8775. This is in contrast to the report of Thomas et al. (30), who found CS6 to be the major PCF8775 antigen recovered from E8775. The difference may be strain variation. CS4 (the 22-kDa antigen) was never detected without the presence of at least a small amount of the 16-kDa antigen. This agrees with the findings of Thomas et al. (19, 30) that CS6 (the 16-kDa protein) defines PCF8775 but other antigens may be expressed as well in various concentrations. We did not find transconjugants expressing CS4, although we cannot rule out their presence. It is not known whether lack of CS4 expression in strains M16 and M17 is because CS4 cannot be expressed in strain HB101 or whether the genes for CS4 are not present on the 61-MDa plasmid of M16 or the cointegrate plasmid of M17. The genes for CS4 expression could be on the chromosome or another plasmid of E8775, but this 61-MDa plasmid may have regulatory functions necessary for CS4 expression. Other fimbrial CFAs, notably CS1 and CS2 antigens of CFA/II, are expressed only in certain serotypes of O6 and one strain of serogroup O139. In these strains, antigen expression depends on the biotype of the O6 strain (21). Analogous control may exist for CS4 expression.

The CS4 fimbriae were purified from E8775 cells and had a subunit molecular mass of 22 kDa. The N-terminal amino acid sequence of CS4 was determined to be very similar to those of CFA/I (8) and CS2 (10). Of the first 13 amino acids, the only difference between the sequences of CS4 and CFA/I is a threonine in place of cysteine at position 8. CS2 differs at position 8 and also at position 1. Neither CS4 nor CS2 cross-reacts immunologically with CFA/I (9, 18), suggesting that while the primary structures of these fimbriae may be related, they are antigenically distinct.

The size of the protein associated with CS6, the gold immunolabeling pattern, and the general analogy to CS3 in the CFA/II class of surface antigens suggest that CS6 is fibrillar and is composed of 16-kDa subunits. Extracts from strains that express CS6 sometimes contained a 12-kDa protein band along with the 16-kDa band. The same preparation showed various proportions of the 16- and 12-kDa bands on gels run on different days. Although the 12-kDa band reacted with the antiserum, it reacted less strongly than the 16-kDa band. It seems likely that the 12-kDa band is a form of the 16-kDa band rather than a minor subunit.

The antiserum to strain B7A recognizes the 16-kDa (CS6) antigen in both E8775 and B7A. In their studies on adherence of strain B7A to cultured epithelial cells, Knutton et al. (11) observed rare bacterial cells bearing a dense mass of curly fibrils about 3 nm in diameter. The identity of these fibrils is unknown, but it has been suggested that they are CS6. We did not observe such fibrils on B7A cells expressing CS6.

Darfeuille-Michaud et al. (4) have characterized a nonfimbrial antigen from ETEC strain 2230 (serotype O25:H16) that promotes attachment to human enterocytes in vitro. They described it as a 16-kDa surface protein which is extracted from cells by heat, does not promote hemagglutination, and is not expressed at 18°C. Despite these similarities, there is no evidence at present that the strain 2230 adhesin is CS6.

Svennerholm et al. (28) have reported that bacteria bearing CS6 are able to colonize and protect against rechallenge by ETEC expressing CS6 in the reversible-intestinal-tie adult rabbit diarrhea model. This suggests that CS6 is a colonization factor and may be an effective antigen for eliciting protective immunity. The plasmids described here and clones derived from them should be useful in producing strains to be used as a vaccine against diarrhea caused by ETEC bearing PCF8775 and in defining the role of PCF8775 antigens in virulence.

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LITERATURE CITED

1. **Boyer, H. W., and D. Roulland-Dussoix.** 1969. A complementation analysis of the restriction and modification of DNA in *Escherichia coli*. J. Mol. Biol. **41**:459–472.
2. **Cravioto, A., S. M. Scotland, and B. Rowe.** 1982. Hemaggluti-

nation activity and colonization factor antigens I and II in enterotoxigenic and non-enterotoxigenic strains of *Escherichia coli* isolated from humans. Infect. Immun. **36**:189–197.

3. **Crosa, J. H., and S. Falkow.** 1981. Plasmids, p. 266–282. *In* P. Gerhardt (ed.), Manual of methods for general bacteriology. American Society for Microbiology, Washington, D.C.
4. **Darfeuille-Michaud, A., C. Forestier, B. Joly, and R. Cluzel.** 1986. Identification of a nonfimbrial adhesive factor of an enterotoxigenic *Escherichia coli* strain. Infect. Immun. **52**:468–475.
5. **DuPont, H. L., S. B. Formal, R. B. Hornick, M. J. Snyder, J. P. Libonati, D. G. Sheahan, E. H. LaBrec, and J. P. Kalas.** 1971. Pathogenesis of *Escherichia coli* diarrhea. N. Engl. J. Med. **285**: 1–9.
6. **Hewick, R. A., M. W. Hunkapiller, L. E. Hood, and W. J. Dreyer.** 1981. A gas liquid solid phase peptide and protein sequenator. J. Biol. Chem. **256**:7990–7997.
7. **Isaacson, R.** 1977. K99 surface antigen of *Escherichia coli*: purification and partial characterization. Infect. Immun. **15**:272–279.
8. **Klemm, P.** 1982. Primary structure of the CFA1 fimbrial protein from human enterotoxigenic *Escherichia coli* strains. Eur. J. Biochem. **124**:339–348.
9. **Klemm, P.** 1985. Fimbrial adhesins of *Escherichia coli*. Rev. Infect. Dis. **7**:321–340.
10. **Klemm, P., W. Gaastra, M. M. McConnell, and H. R. Smith.** 1985. The CS2 fimbrial antigen from *Escherichia coli*, purification, characterization and partial covalent structure. FEMS Microbiol. Lett. **26**:207–210.
11. **Knutton, S., D. R. Lloyd, and A. S. McNeish.** 1987. 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. Infect. Immun. **55**:86–92.
12. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
13. **Levine, M. M.** 1984. *Escherichia coli* infections, p. 187–235. *In* R. Germanier (ed.), Bacterial vaccines. Academic Press, Inc., Orlando, Fla.
14. **Levine, M. M.** 1987. *Escherichia coli* that cause diarrhea: enterotoxigenic, enteropathogenic, enteroinvasive, enterohemorrhagic, and enteroadherent. J. Infect. Dis. **155**:377–389.
15. **Levine, M., J. G. Morris, G. Losonsky, E. Boedeker, and B. Rowe.** 1986. Fimbriae (pili) adhesins as vaccines, p. 143–145. *In* D. L. Lark (ed.), Protein-carbohydrate interactions in biological systems. Academic Press, London.
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. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
18. **McConnell, M. M., L. V. Thomas, N. P. Day, and B. Rowe.** 1985. Enzyme-linked immunosorbent assays for the detection of adhesion factor antigens of enterotoxigenic *Escherichia coli*. J. Infect. Dis. **152**:1120–1127.
19. **McConnell, M. M., L. V. Thomas, S. M. Scotland, and B. Rowe.** 1986. The possession of coli surface antigen CS6 by enterotoxigenic *Escherichia coli* of serogroups O25, O27, O148, and O159: a possible colonization factor? Curr. Microbiol. **14**:51–54.

19a. **McConnell, M. M., L. V. Thomas, G. A. Willshaw, H. R. Smith, and B. Rowe.** 1988. Genetic control and properties of coli surface antigens of colonization factor antigen IV (PCF8775) of enterotoxigenic *Escherichia coli*. Infect. Immun. **56**:1974–1980.

20. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
21. **Mullany, P., A. M. Field, M. M. McConnell, S. M. Scotland, H. R. Smith, and B. Rowe.** 1983. Expression of plasmids coding for colonization factor antigen II (CFA/II) and enterotoxin production in *Escherichia coli*. J. Gen. Microbiol. **129**:3591–3601.
22. **Ørskov, I., F. Ørskov, and A. Birch-Andersen.** 1980. Comparison of *Escherichia coli* fimbrial antigen F7 with type 1 fimbriae. Infect. Immun. **27**:657–666.
23. **Ouchterlony, O., and L. A. Nelsson.** 1978. Immunodiffusion and immunoelectrophoresis, p. 1–44. *In* D. M. Weir (ed.), Handbook of experimental immunology. Blackwell Scientific Publications, Ltd., Oxford.
24. **Parry, S. H., and D. M. Rooke.** 1985. Adhesins and colonization factors of *Escherichia coli*, p. 79–155. *In* M. Sussman (ed.), The virulence of *Escherichia coli*. Academic Press, London.
25. **Sansonetti, P. J., D. J. Kopecko, and S. B. Formal.** 1982. Involvement of a plasmid in the invasive ability of *Shigella flexneri*. Infect. Immun. **35**:852–860.
26. **Smyth, C. J.** 1982. Two mannose-resistant haemagglutinins on enterotoxigenic *Escherichia coli* of serotype O6:K15:H16 or H^- isolated from traveller's and infantile diarrhoea. J. Gen. Microbiol. **128**:2081–2096.
27. **Smyth, C. J.** 1984. Serologically distinct fimbriae on enterotoxigenic *Escherichia coli* of serotype O6:K15:H16 or H^-. FEMS Microbiol. Lett. **21**:51–57.
28. **Svennerholm, A.-M., Y. L. Vidal, J. Holmgren, M. M. McConnell, and B. Rowe.** 1988. Role of PCF8775 antigen and its coli surface subcomponents for colonization, disease, and protective immunogenicity of enterotoxigenic *Escherichia coli* in rabbits. Infect. Immun. **56**:523–528.
29. **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.
30. **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.
31. **Thomas, L. V., B. Rowe, and M. M. McConnell.** 1987. In strains of *Escherichia coli* O167 a single plasmid encodes for the coli surface antigens CS5 and CS6 of putative colonization factor PCF8775, heat-stable enterotoxin, and colicin Ia. Infect. Immun. **55**:1929–1931.