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Genetic Control and Properties of Coli Surface Antigens of Colonization Factor Antigen IV (PCF8775) of Enterotoxigenic *Escherichia coli*

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Enterotoxigenic *Escherichia coli* producing coli surface antigen 4 (CS4), CS5, and CS6 of colonization factor antigen IV were examined. This factor was originally called putative colonization factor 8775 (PCF8775). All of the coli surface antigens were plasmid coded and were usually carried on the same plasmid as the genes coding for heat-stable toxin (ST) or heat-labile toxin (LT); thus, CS5-CS6-ST, CS6-ST, and CS6-LT plasmids were found. In strains of serotype O25:H42, the genes coding for CS4 and CS6 were on a plasmid separate from that containing the genes coding for ST and LT. CS4 and CS5 were fimbrial antigens with a subunit molecular mass of about 17.0 and 21.0 kilodaltons (kDa), respectively. CS6 was found as a single polypeptide with a molecular mass of about 14.5 kDa in strains of serotypes O25:H42, O27:H7, and O27:H20 when heated extracts were run on sodium dodecyl sulfate-polyacrylamide gels. CS6-positive extracts of strains of serogroups O148, O159, and O167 showed two bands with molecular masses between 14.5 and 16.0 kDa.

Adherence of pathogenic bacteria to the gut epithelial surfaces plays an important role in their ability to colonize and multiply in intestines. In some enterotoxigenic *Escherichia coli* strains (ETEC) adhesion has been shown to be mediated by a number of fimbrial antigens which often cause mannose-resistant hemagglutination (MRHA) of a variety of erythrocytes. These antigens are specific for the host; K88, K99, F41, and 987P are found on strains of animal origin, and colonization factor antigen I (CFA/I), CFA/II, and CFA/III and putative colonization factor 8775 (PCF8775) on strains of human origin (for reviews, see references 6 and 22). Two fimbrial adhesive factors on *E. coli* strains of human origin have been named CFA/III (3, 12). The CFA/III of Honda et al. (12) is distinct from the other adhesive factors, whereas the CFA/III described by Darfeuille et al. (3) is CFA/I (17; S. M. Scotland, personal communication). Recently putative colonization factors have been described on ETEC of serotypes O148:H28 (13) and O159:H4 (PCF159) (30).

CFA/I, CFA/III, and PCF159 are fimbrial antigens with a diameter of 6 to 8 nm (9, 12, 30). CFA/II is composed of three coli surface antigens: CS1, CS2, and CS3 (2, 27). CS1 and CS2 have a morphology similar to that of CFA/I, CFA/III, and PCF159 (21, 28), whereas CS3 consists of fine fibrils about 2 to 3 nm in diameter (16). PCF8775 also consists of two fimbrial antigens, CS4 and CS5, with a diameter of about 6 nm, whereas a structure has not been detected for the third antigen, CS6 (32). Two other apparently nonfimbrial adhesins have been described on human ETEC: 2230 on an *E. coli* serotype O25:H16 strain which attaches to human duodenal enterocytes and a factor mediating attachment of strains of serogroup O27 to intestinal 407 cells (4; L. V. Thomas, Ph.D. thesis, C.N.A.A., London, United Kingdom, 1985). The serological relationship of 2230 to CS6 was not examined (4). The attachment of strains of serotype O27 to intestinal 407 cells was not mediated by CS6, an antigen commonly found in strains of this serotype (20; Thomas, Ph.D. thesis).

The fimbrial antigens CFA/I and CS1 to CS5 cause MRHA of specific erythrocytes, whereas CFA/III and PCF159 are nonhemagglutinating (2, 12, 32). Strains producing CS6, 2230, and the intestinal 407 attachment factor do not cause MRHA of calf, human, or guinea pig erythrocytes (4, 32; Thomas, Ph.D. thesis).

Each human colonization factor is associated with a limited number of ETEC serogroups. CFA/I is found on strains of serogroups O4, O15, O25, O63, O78, O90, O110, O114, O126, O128, and O153 (2, 10, 31). CS1 or CS2 is found on strains of serotype O6:H16 or O6:H– and one strain of serotype O139:H28 in association with CS3, whereas CS3 alone is found on strains of serogroups O8, O78, O80, O85, O115, O139, and O168 (2, 8, 25). The prototype CFA/III strain of Honda et al. (12) belongs to serotype O25:H–. (L. V. Thomas, unpublished data). CS4 and CS6 occur together on strains of serotype O25:H42, CS5 and CS6 occur on strains of serogroups O6, O92, O115, and O167, whereas CS6 alone is found on strains of serogroups O25, O27, O92, O148, O153, O159, and O169 (19, 20, 31). Recently the colonizing ability of PCF8775 strains of serotype O25:H42 carrying CS4 and CS6 or CS6 only and O167:H5 carrying CS5 and CS6 has been demonstrated in the reversible intestinal adult rabbit diarrhea model (29).

Adhesion and enterotoxin genes have been shown to be coded for by the same plasmid in some human *E. coli* strains. CFA/I and CFA/II are coded for by nonautotransferring plasmids which also code for production of heat-stable enterotoxin (ST) only or ST and heat-labile enterotoxin (LT) (11, 18, 21, 23, 26). A nonautotransferring plasmid has been mobilized from an O167:H5 strain which codes for CS5, CS6, and ST (33).

In this study, we examined strains of a variety of serotypes that produce PCF8775 to look at the genetic control of production of CS4, CS5, and CS6. The antigenic identity of CS6 produced by these strains was confirmed by immu-

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nodiffusion tests, and the strains were tested in an enzyme-linked immunosorbent assay (ELISA). The molecular weights of the polypeptides of the adhesion antigens were determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

MATERIALS AND METHODS

Bacterial strains. The *E. coli* strains used formed part of the culture collection of the Division of Enteric Pathogens. They have been previously identified as possessing PCF8775 (see Tables 1 and 2) (20, 31, 32).

Enterotoxin production. ST production was detected by the infant mouse test (5), and LT production was detected by the use of Y-1 adrenal cells (7).

Mannose-sensitive hemagglutination and MRHA. The strains were grown overnight on CFA agar and tested for mannose-sensitive hemagglutination and MRHA, respectively, of calf, human, and guinea pig erythrocytes as previously described (2).

Preparation of antisera. Antisera to CS4 and CS5 were prepared by using a Formalinized suspension of the appropriate vaccine strains, E17374A and E17018A (see Table 1), to inject the rabbits intraperitoneally as previously described (31). The antisera were absorbed first with an MRHA-negative variant of the same strain that lacked CS4 and CS6 or CS5 and CS6 (E17374B and E17018B, respectively). These antisera were reabsorbed with a CS6-producing strain of a different serotype, E17018A or E17374A, to give antisera which reacted only against CS4 or CS5. Antisera were also made against CS6-positive strains of serotypes O27:H20 (E24133A) and O148:H28 (E519/66A) (see Table 2) and a derivative of a strain of serotype O25:H42 which had lost the ability to produce CS4 and only produced CS6 (E11881C) (see Table 1). The antisera were absorbed with the appropriate CS6-negative strains, E24133B, E519/66B, and E11881D, to make them specific for CS6.

Cross-absorptions were performed by using the absorbed antisera made against CS6 with E24133A, E519/66A, and E11881C and against CS5 and CS6 with E17018A by absorbing each antiserum again, as appropriate with the CS6-positive strains of serotypes O25:H42, O27:H20, O148:H28, and O167:H5 (E11881C, E24133A, E519/66A, and E17018A). Additionally, the antisera were also absorbed with a CS6-positive strain of serotype O25:H−, E19475A, and a CS6-positive strain of serotype O159:H20, E18519A (see Table 2).

Immunodiffusion. The antisera were tested by the Ouchterlony gel immunodiffusion technique as previously described (2). Antigens were prepared by suspending cultures from 15.24-cm-diameter CFA agar plates in 1.5-ml saline and heating the suspensions for 30 min at 60°C.

ELISA. Specific G immunoglobulins (IgGs) were precipitated from the absorbed antisera with ammonium sulfate at 50% saturation. Conjugates were prepared by labeling these CS4, CS5, and CS6 IgGs with horseradish peroxidase. The anti-CS6 IgG and conjugate were prepared from the antisera made against E11881C and E519/66A (see Tables 1 and 2). The techniques for preparing conjugates and performing the immunoassays have been described previously (19).

Isolation of CS- and enterotoxin-negative colonies. Colonies which were negative for CS4 and CS5 production were selected by picking MRHA-negative colonies and checking them for CS4, CS5, and CS6 by immunodiffusion or ELISA. Colonies which did not produce CS6 were isolated by picking single colonies from MacConkey plates onto CFA agar slopes. The cultures were washed off the slope into 1 ml of saline, and the suspensions were heated at 60°C for 30 min. These heated extracts were tested by ELISA. The CS-negative colonies were checked for enterotoxin production. Up to 100 colonies from each strain were examined to find these variants. To obtain enterotoxin-negative colonies of E11881, 40 single colonies of the appropriate parent strain were tested for LT production and negative colonies were tested for ST production.

Plasmid DNA studies. Plasmid DNA was prepared by the method of Birnboim and Doly (1) and separated by electrophoresis on 0.6% (wt/vol) agarose gels as described previously (34). Molecular masses of the plasmids were determined relative to standard plasmids run on the same gel.

DNA hybridization. Plasmids encoding production of CS6, ST, or LT were identified by hybridization with specific gene probes. Plasmid DNAs of wild-type strains and CS6-negative variants were separated by agarose gel electrophoresis and blotted to nylon membrane (Hybond N; Amersham Corp.). Gels were acid treated (0.25 N HCl) before denaturation of the DNA. DNA was bound to the filter by baking (80°C, 2 h). The probe for CS6 sequences was a 3-kilobase *Eco*RI fragment derived by molecular cloning from a plasmid, pDEP3, that encoded production of CS5 and CS6 and had been transferred to *E. coli* K-12 from strain E10703A of serotype O167:H5 (33). Experiments describing the construction and use of this probe have been described separately (36). The probe was specific in identifying strains that produced CS6 detectable by ELISA and did not cross-hybridize with strains producing CFA/I, CS1, CS2, or CS3. The probe was labeled with deoxyadenosine 5′-α-[^{35}S]thiotriphosphate, and hybridization was under stringent conditions (37). In a few cases, strains were tested by colony hybridization with this probe (37).

Probes for ST and LT genes were synthetic oligonucleotides labeled with alkaline phosphatase and supplied in kit form (SNAP system; DuPont, NEN Research Products). The ST probe contained both STA1- and STA2-specific sequences. Hybridization to blotted plasmid DNA was by the methods recommended by the manufacturer.

SDS-PAGE. Heated extracts were prepared from growth on CFA agar as described for immunodiffusion. They were centrifuged in a microcentrifuge, and the supernatants were tested. SDS-PAGE was performed by the method of Laemmli (15) with vertical slab gels. Extracts were run on 13.5% polyacrylamide gels overnight at 40 V. Proteins of known molecular mass were run on the same gel. The polypeptides were also detected by immune precipitation with protein A–Sepharose CL-4B (Pharmacia) (24). Supernatants (150 μl each) from the heated extracts in lysis buffer containing approximately 50 μg of protein were mixed with 10 μl of IgG and left for 1 h at 4°C. A 20-μl volume of protein A-Sepharose suspension was added, and the mixture was kept at 4°C for at least 30 min. After centrifugation, the pellet was washed five times with a buffer containing 10 mM sodium phosphate buffer (pH 7.2), 150 mM NaCl, and 1% Nonidet P-40 and twice with distilled water. The precipitate was suspended in 50 μl of sample buffer (0.0625 M Tris hydrochloride [pH 6.8], 2% SDS, 10% glycerol, 5% 2-mercaptoethanol, 0.001% bromophenol blue) and heated for 5 min at 100°C before being loaded on the gel.

RESULTS

Plasmids coding for CS antigens. Table 1 shows the properties of variants of two strains of serotype O25:H42, E17374

TABLE 1. Properties of PCF8775-positive strains of serotypes O25:H42 and O167:H5 and their CS-negative derivatives

Serotype and strain	PCF8775 component			Enterotoxin production		Molecular masses of plasmids present (MDa)[a]	Hybridization with CS6 probe[b]
	CS4	CS5	CS6	ST	LT		
O25:H42							
E17374A	+	−	+	+	+	53, 48, 45,[c,d] 4.5	+
E17374B	−	−	−	+	+	53, 45, 4.5	−
E11881A[e]	+	−	+	+	+	52, 50,[c,d] 2.1, <2, <2	+
E11881C[e]	−	−	+	+	+	52, 49, 2.1, <2, <2	+[f]
E11881D[e]	−	−	−	+	+	49,[c,d] 2.1, <2, <2	−[f]
E11881E	+	−	+	−	−	52, 44, 2.1, <2, <2	+[f]
E11881F	−	−	+	−	−	52, 44, 2.1, <2, <2	+[f]
E11881B	−	−	−	−	−	44, 2.1, <2, <2	−
O167:H5							
E17018A[g]	−	+	+	+	−	88,[d] 47, 44, 3.6	+
E17018B[g]	−	−	−	−	−	67, 47, 44, 3.6	−

[a] Values of <10 MDa correspond to bands detected on agarose gels and may not represent individual plasmids. Underlined plasmids code for CS6.
[b] Results show hybridization of a 3-kilobase CS6 probe to Southern blots of plasmid DNA prepared as described in Materials and Methods.
[c] Plasmid hybridized with LT probe.
[d] Plasmid hybridized with ST probe.
[e] E11881A is E11881, E11881C is E11881/16 and E11881D is E11881/2 (32).
[f] These strains were tested by colony hybridization with the CS6 probe.
[g] Both of these strains produced colicin Ia.

and E11881, which originally coded for CS4, CS6, ST, and LT. These strains were drug sensitive. The MRHA-negative derivatives were relatively easy to find. In some cases, up to 80% of colonies examined had lost MRHA. In general MRHA-negative derivatives failed to produce either CS4 or CS6. Strain E11881C was an MRHA-negative variant that still produced CS6 and was enterotoxigenic. Enterotoxin production was lost from this strain to give E11881F.

It seemed likely that CS4 and CS6 were coded for by the same plasmid, since these properties were usually lost together. E17374B, E11881B, and E11881D had all lost a plasmid of 48 or 52 megadaltons (MDa). This was the plasmid to which the CS6 probe hybridized (Table 1). When only the ability to produce CS4 was lost, there was no alteration in the plasmid content of strain E11881C or E11881F, suggesting either that there was a mutation in the

TABLE 2. Properties of CS6-positive strains and their CS6-negative derivatives

Serotype and strain	CS6	Enterotoxin production		Drug resistance[a]	Molecular masses of plasmid(s) present (MDa)[b]	Hybridization with CS6 probe[c]
		ST	LT			
O27:H7						
E3135A	+	+	−	Tc	90,[d] 37, 5.3, 3.6	+
E3135B	−	−	−	Tc	35, 4.9, 3.6	−
O27:H20						
E24133A	+	+	−	Cm, Tc	100,[d] 64, 61, 5.4	+
E24133B	−	−	−	Cm, Tc	100, 64, 60, 5.4	−
O27:H20						
E32018A	+	+	−	—	100,[d] 9.6, 4.4, 2.9	+
E32018B	−	−	−	—	9.6, 4.4, 2.9	−
O148:H28						
E519/66A	+	+	−	—	50,[d] 33, 30, 6.8, 3.3	+
E519/66B	−	−	−	—	33, 30, 6.8, 3.3	−
O148:H28						
E25117A	+	+	−	—	49,[d] 4.0, <2	+
E25117B	−	−	−	—	<2	−
O148:H28						
B7aA	+	+	+	—	49,[d] 39,[d,e] 31, 2.6	+
B7aB	−	+	+	—	39,[d,e] 31, 2.6	−
O159:H20						
E18519A	+	+	−	—	88,[d] 9.5, 5.2, 4.4	+
E18519B	−	−	−	—	5.2, 4.4	−
O25:H−						
E19475A	+	−	+	—	58,[e] 38, 3.8, 2.6	+
E19475B	−	−	−	—	35, 3.8, 2.6	−

[a] Cm, Chloramphenicol; Tc, tetracycline; —, no resistance.
[b] Values of <10 Mda correspond to bands detected on agarose gels and may not represent individual plasmids. Underlined plasmids code for CS6.
[c] Results show hybridization of a 3-kilobase CS6 probe to Southern blots of plasmid DNA prepared as described in Materials and Methods.
[d] Plasmids hybridized with ST probe.
[e] Plasmids hybridized with LT probe.

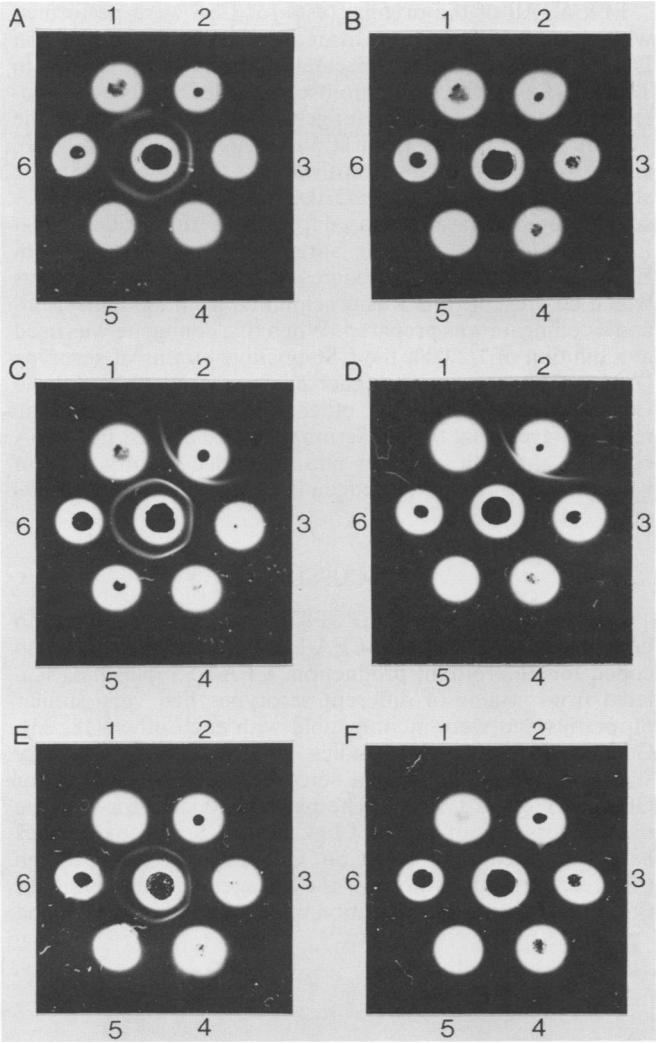

FIG. 1. Immunodiffusion tests with heated extracts of CS6-positive strains with absorbed antisera. The following antigens were used (wells): 1, E11881C (O25:H42); 2, E17018A (O167:H5); 3, E24133A (O27:H20); 4, E25117A (O148:H28); 5, E18519A (O159:H20); 6, E19475A (O25:H−). The antisera used were prepared against the following strains (panels): A, E11881C, absorbed with E11881D; B, E11881C, absorbed with E11881D and E17018A; C, E17018A, absorbed with E17018B; D, E17018A, absorbed with E17018B and E11881C; E, E519/66A, absorbed with E519/66B; F, E519/66A, absorbed with E519/66B and E11881C.

CS4 gene(s) or that a small deletion involving this region had occurred but was not detectable on our gels. Alternatively, the CS4-, CS6-positive strains could carry two distinct plasmids of similar size, only one of which is lost in the CS4-negative derivative. LT and ST probes both hybridized to the plasmids with molecular masses of 53 and 50 MDa in E17374A and E11881A, respectively. In variants E11881E, E11881F, and E11881B, in which loss of enterotoxin production had occurred, a plasmid with a molecular mass of 44 MDa was seen. When E11881B was examined with the LT and ST probes, no hybridization to plasmids occurred. The 44-MDa plasmid was probably a deletion derivative of the 50-MDa plasmid seen in the enterotoxin-positive strains.

Examination of strains of serotype O167:H5 has previously been described (33), and it was found that E17018A, which produced CS5, CS6, ST, and colicin Ia, had a large plasmid with a molecular mass of 88 MDa. E17018B, which did not produce CS5, CS6, and ST, did not contain this plasmid; instead, a plasmid with a molecular mass of 67 MDa was present (Table 1). Hybridization of plasmid DNA with the CS6 and ST probes (Table 1) confirmed that the 88-MDa plasmid in E17018A coded for CS6 and ST production and had undergone deletion to give a smaller plasmid in E17018B that did not hybridize. E17018B retained the ability to produce colicin Ia.

CS6-negative variants of strains of a variety of other serotypes were isolated (Table 2), and the identity of the plasmid coding for CS6 and ST or LT was determined by DNA hybridization with the appropriate probe. In most cases, loss of CS6 was accompanied by loss of enterotoxin production and of a plasmid. Thus, two strains of serogroup O27, two of serogroup O148, and one of serogroup O159 lost CS6 and ST, whereas the strain of serotype O25:H− became CS6 and LT negative. When the pair of E24133 strains (O27:H20) was examined, no plasmid differences were seen. Presumably, the loss of the ability to produce CS6 and ST by E24133B was due to deletion in the plasmid with a molecular mass of 100 MDa.

B7a differed from the other two strains of serotype O148:H28 described in Table 2 in that it produced LT as well as ST. A plasmid with a molecular mass of 48 MDa coded for CS6; this was similar in size to the CS6-ST plasmids in E519/66A and E25117A. However, the CS6-negative derivative of B7a still produced ST as well as LT. Hybridization tests with the ST and LT probes showed that B7a contained an ST-LT plasmid as well as the CS6-ST plasmid.

Immunodiffusion tests. When heated extracts of strains of six different serotypes producing CS6 were tested by immunodiffusion with antisera prepared against three CS6-positive strains and absorbed with the appropriate CS6-negative derivative, a single, continuous precipitin line around the antiserum well was seen (Fig. 1A, C, and E). There appeared to be no antigenic differences between the CS6s produced by different strains, since no spurs were seen. A similar result was seen with an absorbed antiserum prepared against a strain of serotype O27:H20, E24133A (data not shown). The line close to the antigen well (Fig. 1C, well 2) was due to the reaction between CS5 fimbriae and CS5 antibodies in the serum prepared against E17018A.

To confirm the antigenic identity of CS6, absorptions were performed with the four antisera described above. The antisera were initially absorbed with the CS6-negative variant of the strain used to make the antiserum, and then each absorbed antiserum was divided for reabsorption of separate portions with CS6-positive strains of different serotypes (O25:H42, O167:H5, O27:H20, O148:H28, and O159:H20). In all cases, the CS6 immunoglobulin was completely absorbed by the CS6-positive strains so that immunodiffusion lines were not visible, thus confirming the antigenic identity of CS6. Figure 1D shows the reaction of six strains with antisera absorbed with E17018B and E11881C. CS5 antibodies remained in the antiserum and reacted with the heated extract of E17018A, whereas CS6 antibodies were absorbed.

SDS-PAGE. Heated extracts of the CS-positive strains and the CS-negative variants obtained from them were run on polyacrylamide gels. Examples of these pairs are shown in Fig. 2. A prominent polypeptide with a molecular mass of 17.0 kDa was seen in E17374A and not in E17374B (Fig. 2, lanes 1 and 2). Similarly, the heated extract from E17018A had a prominent band with a molecular mass of 21.0 kDa which was missing from E17018B (Fig. 2, lanes 3 and 4). These bands were identified as CS4 and CS5, respectively,

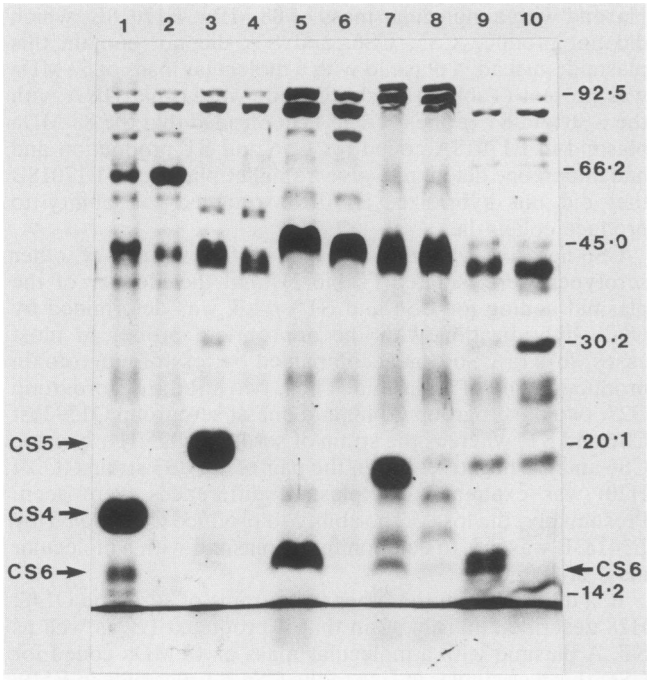

FIG. 2. SDS-PAGE patterns of protein in heated extracts of pairs of PCF8775-positive and -negative strains. Lanes: 1, E17374A; 2, E17374B; 3, E17018A; 4, E17018B; 5, E24133A; 6, E24133B; 7, E25117A; 8, E25117B; 9, E18519A; 10, E18519B. The electrophoresis was run for 20 h at 40 V on a 13.5% (wt/vol) polyacrylamide gel. The gel was stained with Coomassie blue. The positions of the protein standards are shown by the numbers (kilodaltons) on the right. The positions of the CS polypeptides are indicated. The CS6 polypeptides vary in molecular mass as described in the text.

by immunoprecipitation with specific IgG and protein A-Sepharose. Fainter bands of polypeptides with molecular masses of 14.5 to 16.0 kDa were also seen only in the CS6-positive variants (Fig. 2, lanes 1 and 3). Bands at similar positions were seen in other CS6-positive strains (Fig. 2, lanes 5, 7, and 9). The putative CS6 band of E24133A was particularly strong. The polypeptides were identified by immunoprecipitation with specific anti-CS6 IgG and protein A-Sepharose (Fig. 3). Two polypeptides from the extracts of E17018A (Fig. 3, lane 4), E18519A (lane 6), and E25117A (lane 9) reacted with CS6 IgG. The bands from E17018A and E25117A were of similar molecular masses, 14.5 and 16.0 kDa, whereas the CS6 bands from E18519A were about 14.5 and 15.0 kDa. Only one polypeptide, with a molecular mass of about 14.5 kDa, in the heated extracts from E11881C (Fig. 3, lane 1) and E24133A (lane 11) reacted with CS6 IgG. When other CS6-positive strains of these serogroups were tested, it was found that all of the strains of serogroups O167, O148, and O159 had two polypeptides reacting with CS6 IgG, whereas strains of O25:H42, O27:H7, and O27:H20 had only one polypeptide. The CS5-CS6-ST plasmid from a strain of O167:H5 has been transferred into *E. coli* K-12 (33). When a heated extract of this strain was tested with anti-CS6 IgG, two polypeptide bands of molecular masses 14.5 and 16.0 kDa were identified (data not shown). E25117A (Fig. 2, lane 7), a strain of serotype O148:H28, also had a prominent band of protein material with a molecular mass of about 19.0 kDa not visible in the CS6-ST-negative derivative E25117B or the other CS6-only strains. This band was not picked out with CS6 IgG (Fig. 3, lane 9).

ELISA. All of the original tests for CS6 were performed with a conjugate prepared from anti-CS6 IgG made against E11881C (O25:H42). Representative readings are shown in Table 3. Extracts giving an absorbance of <0.1 were considered negative for the antigen. The absorbance in the ELISA with extracts from strains of O25:H−, O148:H28, and O167:H5 were consistently lower than those with the strains of serotypes O25:H42, O27:H20, and O159:H20. A second conjugate was prepared from antiserum made against E519/66A (O148:H28). The antiserum was absorbed with E519/66B so that only antibodies to plasmid-coded antigens would be present. IgG was precipitated from the antiserum, and a conjugate was prepared. When this conjugate was used at a dilution of 1/2,000, the CS6-positive strains of serotype O148 gave a very much higher positive result than did the CS6-positive strains of all other serotypes (Table 3). The results suggest that the antiserum prepared against E519/66A contains antibodies to two plasmid-coded factors, one of which is CS6. The other antigen is coded for only by strains of serotype O148:H28.

DISCUSSION

Like CFA/I and CFA/II, PCF8775 antigens were found to be coded for by plasmids. CFA/I and CFA/II plasmids also coded for enterotoxin production. CFA/I-ST plasmids isolated from strains of different serotypes had very similar properties and were incompatible with each other (18, 35). Genetic and molecular studies also suggested that very similar plasmids in different serotypes of ETEC coded for CFA/II, ST, and LT (21). The plasmids coding for CS6 are more diverse. Four types of linkage of CS and toxin genes have been identified: CS4-CS6, CS5-CS6-ST, CS6-ST, and CS6-LT. Particularly novel is the linkage of genes coding for CS6 and LT and the separation of ST and LT and CS4 and

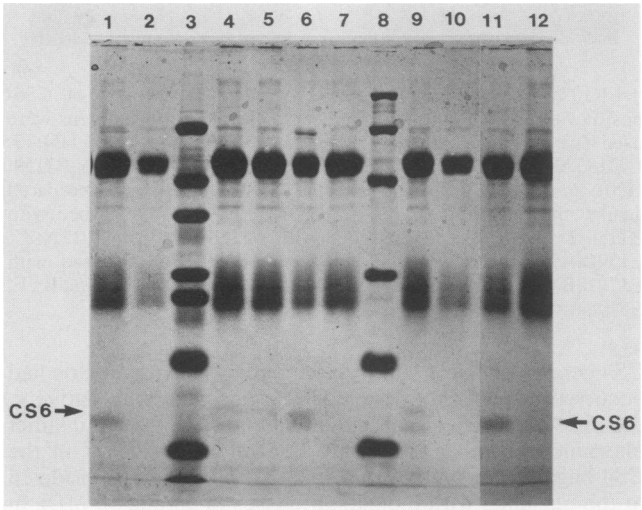

FIG. 3. SDS-PAGE patterns of immunoprecipitates in heated extracts of pairs of PCF8775-positive and -negative strains. Lanes: 1, E11881C; 2, E11881D; 4, E17018A; 5, E17018B; 6, E18519A; 7, E18519B; 9, E25117A; 10, E25117B; 11, E24133A; 12, E24133B. The electrophoresis was run for 20 h at 40 V on a 13.5% (wt/vol) polyacrylamide gel. The gel was stained with Coomassie blue. The protein standards (top to bottom) in lane 3 were 66.0, 45.0, 36.0, 29.0, 24.0, 20.1, and 14.2 kDa, and those in lane 8 were 92.5, 66.2, 45.0, 31.0, 21.5, and 14.4 kDa. The positions of the CS6 immunoprecipitates are indicated. The molecular masses are given in the text.

TABLE 3. ELISAs against PCF8775 strains of different serotypes with a conjugate prepared from serum made against E17374A and E519/66A

Strain	Serotype	A_{490}[a] with:	
		Anti-E11881C	Anti-E519/66A
E17374A	O25:H42	0.991	0.127
E19475A	O25:H−	0.567	0.172
E24133A	O27:H20	1.305	0.158
E519/66A	O148:H28	0.445	1.454
E25117A	O148:H28	0.346	1.253
E35580	O148:H28	0.401	1.317
E37571	O148:H28	0.290	1.183
E18519A	O159:H20	0.922	0.112
E17018A	O167:H5	0.313	0.153
Negative control[b]		0.048	0.031

[a] The A_{490} was determined with IgG conjugates. The test strains were measured against blanks prepared in the same way, except that the antigen was replaced by saline. Each strain was tested in duplicate, and an average A_{490} is given. Extracts giving an A_{490} of <0.1 were considered negative for the antigen. Results of a typical experiment are shown. All observations were repeated two or more times, and similar data were obtained each time.

[b] The CS6-negative derivatives of E17374, E19475, E24133, E18519, E519/66, and E17018 were used as controls. The results given are average values.

CS6 on two plasmids. Genes coding for another adhesive factor, CFA/III (12), have been shown to be separate from toxin genes. The strain of serotype O25:H− in which this adhesion factor was identified has been shown to contain a plasmid coding for CFA/III and a second coding for CS6 and LT (M. McConnell, unpublished data). Strains of several different serogroups (O27, O148, and O159) carried CS6-ST plasmids which differed considerably in molecular mass. However, DNA hybridization with the CS6 probe showed that CS6-positive strains of the different serotypes had closely related gene sequences.

The plasmids coding for CS6 may code for other antigens besides CS4 and CS5. For example, the ST plasmid in strains of serotype O148:H28 codes for CS6 and another antigen, as demonstrated by ELISA. The relationship of this antigen to the 19.0-kDa polypeptide seen in extracts from E25117A and to the factor mediating adhesion to the human intestinal mucosa of strains of serotype O148:H28 described by Knutton et al. is being investigated (13). Two of three of the CS6-positive strains of serotype O27 examined here also adhered to intestinal 407 cells (Thomas, Ph.D. thesis). This adhesion ability seems to be coded for by the CS6-ST plasmid present in these strains, since CS6-ST-negative derivatives did not adhere to intestinal 407 cells (S. M. Scotland, personal communication).

The fimbrial antigens CS4 and CS5 were similar in morphology to previously described adhesion fimbriae (32) and, like CS1 and CS2, have been shown to attach to human duodenal enterocytes (S. Knutton, personal communication). The molecular masses of CS4 and CS5 polypeptides, about 17.0 and 21.0 kDa, respectively, were similar to those of CFA/I, CS1, CS2, CS3, CFA/III, and PCF159 (15.0, 16.3, 15.3, 14.8, 18.0, and 19.0 kDa) (12, 22, 27, 30). The molecular masses of the polypeptides reacting with CS6 antisera, 14.5 to 16.0 kDa, were in the same size region as those of fimbrial subunits, although a fimbrial structure has not been seen for CS6 (32). The nonfimbrial adhesive factor 2230 was also seen by SDS-PAGE as a polypeptide with a molecular mass of 16.0 kDa (4). We have not been able to examine the serological relationship between 2230 and CS6, since the 2230 strain is not available.

When strains of different serotypes were examined for CS6, one or two polypeptides were found which reacted with CS6 antisera, but no antigenic differences were observed when CS6 strains were examined by immunodiffusion with cross-absorbed antisera. A similar phenomenon has been found in *E. coli* from urinary tract infections when fimbrial subunits of different molecular masses but the same antigenicity have been found (14). In that case, it was thought that two types of fimbriae which differ in subunit size were found on the cells. CS6 is apparently nonfimbrial, but the polypeptides might be assembled to form a polymeric structure, as in 2230 (4). In that case, in strains in which two polypeptides were shown to react with CS6 antiserum either two polymers which differ in polypeptide size or one polymer made up of polypeptides of different sizes might be present.

The recent finding that strains possessing CS4 and CS6, CS5 and CS6, or CS6 alone colonize rabbit intestines (29) and the evidence of similarity with other colonization factors described here show that PCF8775 is a colonization factor. We therefore propose to change the name of PCF8775 to CFA/IV, in conformity with the nomenclature used for other human colonization factors. The term CFA/IV would cover the three coli surface antigens CS4, CS5, and CS6 in the same way that CFA/II comprises CS1, CS2, and CS3.

LITERATURE CITED

1. **Birnboim, H. C., and J. Doly.** 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. **7**:1513–1523.
2. **Cravioto, A., S. M. Scotland, and B. Rowe.** 1982. Hemagglutination 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. **Darfeuille, A., B. Lafeuille, B. Joly, and R. Cluzel.** 1983. A new colonization factor antigen (CFA/III) produced by enterotoxigenic *Escherichia coli* O128:B12. Ann. Microbiol. Inst. Pasteur **134A**:53–64.
4. **Darfeuille-Michaud, A., C. Forestier, B. Joly, and R. Cluzel.** 1986. Identification of a non-fimbrial adhesive factor of an enterotoxigenic *E. coli* strain. Infect. Immun. **52**:468–475.
5. **Dean, A. G., Y.-C. Ching, R. G. Williams, and L. B. Harden.** 1972. Test for *Escherichia coli* enterotoxin using infant mice: application in a study of diarrhea in children in Honolulu. J. Infect. Dis. **125**:407–411.
6. **De Graaf, F. K., and F. R. Mooi.** 1986. The fimbrial adhesins of *Escherichia coli*. Adv. Microb. Physiol. **28**:65–143.
7. **Donta, S. T., H. W. Moon, and S. C. Whipp.** 1974. Detection of heat-labile *Escherichia coli* enterotoxin with the use of adrenal cells in tissue culture. Science **183**:334–336.
8. **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.
9. **Evans, D. G., D. J. Evans, Jr., S. Clegg, and J. A. Pauley.** 1979. Purification and characterization of the CFA/I antigen of enterotoxigenic *Escherichia coli*. Infect. Immun. **25**:738–748.
10. **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.
11. **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.
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. **Knutton, S., D. R. Lloyd, and A. S. McNeish.** 1987. Identifica-

tion 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.

14. **Korhonen, T. K., M. Rhen, V. Vaisanen-Rhen, and A. Pere.** 1985. Antigenic and functional properties of enterobacterial fimbriae, p. 319–354. *In* D. E. S. Stewart-Tull and M. Davies (ed.), Immunology of the bacterial cell envelope. John Wiley & Sons Ltd., Chichester.
15. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (*London*) **227**: 680–684.
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. **Manning, P. A., K. N. Timmis, and G. Stevenson.** 1985. Colonization factor antigen II (CFA/II) of enterotoxigenic *Escherichia coli*: molecular cloning of the CS3 determinant. Mol. Gen. Genet. **200**:322–327.
18. **McConnell, M. M., H. R. Smith, G. A. Willshaw, A. M. Field, and B. Rowe.** 1981. Plasmids coding for colonization factor antigen I and heat-stable enterotoxin production isolated from enterotoxigenic *Escherichia coli*: comparison of their properties. Infect. Immun. **32**:927–936.
19. **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.
20. **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.
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. **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, Inc., London.
23. **Penaranda, M. E., M. B. Mann, D. G. Evans, and D. J. Evans.** 1980. Transfer of an ST:LT:CFA/II plasmid into *Escherichia coli* K-12 strain RR1 by cotransformation with PSC301 plasmid DNA. FEMS Microbiol. Lett. **8**:251–254.
24. **Perbal, B.** 1984. A practical guide to molecular cloning, p. 540–543. John Wiley & Sons, Inc., New York.
25. **Scotland, S. M., M. M. McConnell, G. A. Willshaw, B. Rowe, and A. M. Field.** 1985. Properties of wild-type strains of enterotoxigenic *Escherichia coli* which produce colonization factor antigen II and belong to serogroups other than O6. J. Gen. Microbiol. **131**:2327–2333.
26. **Smith, H. R., A. Cravioto, G. A. Willshaw, M. M. McConnell, S. M. Scotland, R. J. Gross, and B. Rowe.** 1979. A plasmid coding for the production of colonisation factor antigen I and heat-stable enterotoxin in strains of *Escherichia coli* of serogroup O78. FEMS Microbiol. Lett. **6**:255–260.
27. **Smyth, C. J.** 1982. Two mannose-resistant haemagglutinins on enterotoxigenic *Escherichia coli* of serotype O6.H16 or H− isolated from travellers' and infantile diarrhoea. J. Gen. Microbiol. **128**:2081–2096.
28. **Smyth, C. J.** 1984. Serologically distinct fimbriae on enterotoxigenic *Escherichia coli* of serotype O6:K15:H16 or H−. FEMS Microbiol. Lett. **21**:51–57.
29. **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.
30. **Tacket, C. O., D. R. Maneval, and M. M. Levine.** 1987. Purification, morphology, and genetics of a new fimbrial putative colonization factor of enterotoxigenic *Escherichia coli* O159:H4. Infect. Immun. **55**:1063–1069.
31. **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. Immun. **171**:85–90.
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., 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.
34. **Willshaw, G. A., H. R. Smith, and E. S. Anderson.** 1979. Application of agarose gel electrophoresis to the characterization of plasmid DNA in drug-resistant enterobacteria. J. Gen. Microbiol. **114**:15–25.
35. **Willshaw, G. A., H. R. Smith, M. M. McConnell, E. A. Barclay, J. Krnjulac, and B. Rowe.** 1982. Genetic and molecular studies of plasmids coding for colonization factor antigen I and heat-stable enterotoxin in several *Escherichia coli* serotypes. Infect. Immun. **37**:858–868.
36. **Willshaw, G. A., H. R. Smith, M. M. McConnell, and B. Rowe.** 1988. Cloning of genes encoding coli-surface (CS) antigens in enterotoxigenic *Escherichia coli*. FEMS Microbiol. Lett. **49**: 473–478.
37. **Willshaw, G. A., H. R. Smith, S. M. Scotland, and B. Rowe.** 1985. Cloning of genes determining the production of Vero cytotoxin by *Escherichia coli*. J. Gen. Microbiol. **131**:3047–3053.