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Colonization Factor Antigens I and II and Type 1 Somatic Pili in Enterotoxigenic *Escherichia coli*: Relation to Enterotoxin Type

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Enterotoxigenic *Escherichia coli* (ETEC) isolates from 36 persons with acute traveler's diarrhea from whom no other pathogens were recovered were tested (after no more than three subcultures) for the presence of colonization factor antigens I and II (CFA/I and CFA/II) and type 1 somatic pili. CFA/I or CFA/II was identified in 7 of 10 strains with heat-labile and heat-stable enterotoxins (LT^+/ST^+), but in only 2 of 12 LT^-/ST^+ ($P < 0.05$) and 0 of 14 LT^+/ST^- ($P < 0.02$) strains. CFA pili were not found among 74 non-enterotoxigenic *E. coli* strains. Type 1 somatic pili were demonstrable in 42% of the 36 ETEC and in 49% of the 74 non-enterotoxigenic *E. coli* isolates. The nine ETEC isolates bearing a CFA were serially subcultured on 10 consecutive days and retested for CFA and toxin. After five subcultures only one strain had lost a CFA, but after 10 passages three strains were negative; two lost CFA/I and one lost CFA/II. The strain that lost CFA/II became negative for both LT and ST as well and was found to lack a 48- and a 60-megadalton plasmid. The two strains that lost CFA/I also became negative for ST, but plasmid analysis revealed no plasmid loss. Disappearance of the CFA/I phenotype without loss of a plasmid can be explained by phase variation, as exhibited by type 1 somatic pili, or by rearrangement of base sequences in the CFA/I plasmid genome. If purified pili vaccines are to provide broad-spectrum protection against ETEC diarrhea, the search must be intensified to identify the antigens responsible for adhesion to intestinal mucosa in the many ETEC strains that lack CFA/I and CFA/II.

Enterotoxigenic *Escherichia coli* (ETEC) strains are important pathogens that cause travelers' diarrhea (14, 22), infant diarrhea in less-developed countries (3), and neonatal enteric colibacillosis in piglets (15) and calves (42). Earlier veterinary work with piglets showed that to cause diarrhea, ETEC strains must possess accessory virulence properties in addition to heat-labile (LT) or heat-stable (ST) enterotoxin or both (17, 24, 27, 41, 42). The best-characterized accessory virulence properties are adherence or colonization factors, which permit the attachment of ETEC to the mucosa of the small intestine (17, 24, 27, 41, 42). These colonization factors counter the potent peristaltic defense of the host and allow the release of toxin closer to the reactive sites. Several antigens responsible for ETEC colonization in animals have been characterized as hair-like organelles (pili or fimbriae) on the surface of the bacterium (17, 24, 27, 41, 42). Some pili, such as K88 and K99, cause mannose-resistant hemagglutination (MRHA) of certain erythrocytes (5, 18), whereas other adhesion pili, such as those of porcine *E. coli* 987, do not manifest hemagglutination (24). Type 1 somatic ("common") pili cause mannose-sensitive hemagglutination of guinea pig erythrocytes and manifest adhesion properties for epithelial cells in vitro (16, 29, 39). However, since type 1 somatic pili occur as frequently in normal flora as in pathogenic ETEC, their role, if any, as a virulence factor is unclear (20).

Reports of animal studies indicate that adhesion pili may have a role in immunoprophylaxis against ETEC (1, 25, 28, 35). Purified K88, K99, and strain 987 pili have been used to parenterally immunize pregnant gilts and cows (1, 25, 28, 35). Piglets and calves suckled by immunized mothers were significantly protected against otherwise lethal challenge with ETEC strains bearing the homologous pilus antigen, in contrast to piglets and calves suckled by nonimmunized

mothers (1, 25, 28, 35). If the colonization factors associated with human ETEC strains could be identified, purified, and shown to be immunogenic, then immunoprophylaxis of human ETEC disease would be feasible.

Adhesion pili analogous to K88 and K99 have been identified in some human ETEC strains and have been termed colonization factor antigens I and II (CFA/I and CFA/II) (9–11, 13, 30); strains possessing these pili have characteristic MRHA patterns (9, 10, 31). We examined the frequency of occurrence of CFA/I, CFA/II, and type 1 somatic pili in ETEC strains from cases of travelers' diarrhea after minimal subculturing and related their presence to enterotoxin type and serotype. Such information is critical since it will dictate the antigenic composition of a multivalent, purified pilus vaccine. To date, the available reports have varied greatly in their conclusions about the prevalence and significance of these antigens in human ETEC strains (11, 15, 20, 31, 34).

MATERIALS AND METHODS

Bacterial strains. ETEC strains were isolated from Peace Corps volunteers with acute diarrheal disease in the course of travelers' diarrhea studies in Kenya, Morocco, Honduras, Korea, Zaire, and Colombia (36, 38, 40). Specimens were collected as previously described (36, 38, 40). During episodes of diarrhea, stool specimens were collected daily, transported in modified Stewart medium, plated onto MacConkey agar, and incubated overnight at 37°C. Five lactose-positive colonies resembling *E. coli* were picked; a pool was also made of 10 other *E. coli*-like colonies. Lactose-positive colonies were confirmed as *E. coli* by standard biochemical techniques (8) and serotyped by the method of Ørskov and Ørskov (30). All isolates were tested for production of LT by Y1 adrenal cell assay (37) and for ST by the infant mouse assay (6). Enterotoxigenic strains were tested for the presence of pili by hemagglutination and agglutination with antisera (20). Seventy-four non-enterotoxigenic strains isolated from Peace Corps volunteers without diarrhea by means of surveillance coprocultures were also examined for CFA/I and CFA/II pili.

Cultivation of strains. Strains found to be enterotoxigenic were cultivated (only one to three passages after primary isolation) so as to promote piliation. Isolates were cultured both on modified Casamino Acids-yeast extract agar (CFA agar) (10) and in Mueller-Hinton broth (15 ml) (20). CFA agar cultures were incubated aerobically for 24 h at 37°C before being tested for pili. Mueller-Hinton broth cultures were incubated aerobically for 48 h at 37°C, subcultured into Mueller-Hinton broth for another 48 h, and then tested (20). The tubes of broth were centrifuged and decanted, and the pellets were suspended in saline to a concentration of 10^{10} organisms per ml.

Growth on solid agar (especially CFA agar) is favorable for the expression of pili associated with MRHA (such as CFA/I and CFA/II) and less favorable for type 1 somatic pili, whereas static broth cultures offer optimal growth conditions for the expression of type 1 somatic pili and suboptimal conditions for CFA/I and CFA/II. In a separate experiment, the ETEC strains found to bear CFA (I or II) were subcultured daily for 10 days to determine the stability of CFA expression. The initial, fifth, and tenth cultures were plated on CFA agar; the intervening subcultures were inoculated on tryptic soy agar (which is less optimal for CFA production because of its glucose concentration). After the fifth and tenth passages the strains were retested for CFA, LT, and ST.

Hemagglutination. CFA/I and CFA/II were identified by MRHA of human type A and bovine erythrocytes, and type 1 somatic pili were indicated by mannose-sensitive hemagglutination of guinea pig erythrocytes (9, 20, 39). Guinea pig, bovine, and human type A erythrocytes were freshly obtained, washed twice in 0.85% NaCl, and suspended to 3% in NaCl with and without mannose (0.1%); each *E. coli* strain was characterized with all three species of erythrocytes. Hemagglutination was carried out on glass slides at 24 and 4°C with human and guinea pig cells and at 4°C with bovine erythrocytes. Several bacterial colonies were harvested with a sterile wooden applicator stick and mixed on the slide with 0.025 ml of the erythrocyte suspension. Agglutination was graded from 0 to 4^+, depending on the rapidity and strength of the reaction. Strains causing MRHA of both human and bovine erythrocytes were putative CFA/I strains (10), and those that manifested MRHA of bovine but not human cells were putative CFA/II strains (9). Strains that showed mannose-sensitive hemagglutination of guinea pig erythrocytes after cultivation in broth were considered to possess type 1 somatic pili (39).

Agglutination with specific antisera. Antisera to CFA/I and CFA/II were prepared by absorption by the method of Evans et al. (13). Albino rabbits weighing 2.5 kg were repeatedly inoculated intravenously with *E. coli* strains possessing CFA/I (H10407) or CFA/II (A346C1). The rabbits (two per strain) were exsanguinated after 21 days, when the serum agglutinated the immunizing strains at titers >1:512. The respective sera were absorbed with *E. coli* H10407-P (which lacks CFA/I) or *E. coli* A346C1-P grown at 18°C (which lacks CFA/II) until the sera failed to agglutinate these strains. The sera continued to strongly agglutinate 37°C-grown strains H10407 and A346C1, respectively.

Electron microscopy. Strains with typical HA patterns for CFA/I and CFA/II that were agglutinated by specific antisera were inoculated onto paired CFA agar plates and incubated at 37 or 18°C. Bacterial cells were harvested from these plates with phosphate-buffered saline (approximately 10^9 bacteria per ml) and examined under the electron microscope. One drop of bacterial suspension was placed on a Formvar-coated 300-mesh copper grid, and after 1 min the excess fluid was withdrawn with a piece of filter paper. The bacteria were immediately stained with 1% sodium phosphotungstate (pH 7.3) for 1 min. Excess stain was removed, and the grid was air dried before being examined with a Siemens Elmiskop 1A electron microscope operated at 80 kV.

Plasmid analysis. Certain *E. coli* strains were analyzed for plasmid content by an alkaline extraction procedure (2) followed by agarose gel electrophoresis (23). Known molecular weight standards were run concomitantly to allow sizing of the plasmids. Purified

TABLE 1. Prevalence of CFA/I, CFA/II, and type 1 somatic pili in ETEC strains isolated from patients with acute travelers' diarrhea: relationship with toxin type[a]

Enterotoxin phenotype (no. of isolates)	No. of strains with indicated class of pilus/total (%)		
	CFA/I	CFA/II	Type 1 somatic
LT^+/ST^+ (10)	3/10 (30)	4/10 (40)	1/10 (10)
LT^-/ST^+ (12)	1/12 (8)	1/12 (8)	4/12 (33)
LT^+/ST^- (14)	0/14	0/14	10/14 (71)

[a] Patients with infections that yielded an ETEC strain of only one serotype and toxin type were included.

plasmid DNA was also digested with the restriction endonucleases *Hin*dIII and *Eco*RI (Bethesda Research Laboratories) according to the manufacturer's instructions and examined by agarose gel electrophoresis.

DNA hybridization. The *E. coli* strains examined for plasmid content were also analyzed for the presence of LT and porcine ST genes by colony hybridization with cloned LT and porcine ST gene probes, respectively (26, 44).

RESULTS

Piliation and toxin type. Forty diarrheal episodes in 40 patients yielded ETEC isolates. The infections in 36 patients were apparently due to a single serotype manifesting one enterotoxin phenotype; further characterizations were confined to these 36 ETEC strains.

A distinct relationship was apparent between toxin type and piliation. Ten infections were due to LT^+/ST^+, 12 to LT^-/ST^+, and 14 to LT^+/ST^- strains (Table 1). Overall, 9 of the 36 (25%) infecting ETEC strains elaborated CFA/I or CFA/II and 15 (42%) possessed type 1 somatic pili. CFA/I or CFA/II pili occurred in 7 of 10 LT^+/ST^+ strains but in none of 14 LT^+/ST^- strains, a statistically significant difference ($P = 0.02$, two-tailed Fischer's exact test). In contrast, 10 of 14 LT^+/ST^- strains produced type 1 somatic pili versus 1 of the 10 LT^+/ST^+ strains. CFA/I and CFA/II in LT^-/ST^+ strains (2 of 12) occurred significantly less frequently than they did in LT^+/ST^+ strains ($P = 0.05$), and type 1 somatic pili were uncommon. CFA/I and CFA/II pili were never encountered simultaneously within the same strain.

Confirmation of piliation. The nine ETEC strains that showed MRHA patterns characteristic of CFA/I or CFA/II and that were also agglutinated by specific anti-CFA antisera were retested after cultivation on CFA agar at 37 and 18°C. Incubation at the lower temperature is known to suppress production of CFA pili. Bacteria grown at 37°C showed typical MRHA patterns, were strongly agglutinated by the homologous CFA antiserum, and were heavily piliated when observed under the electron microscope (Fig. 1A). In contrast, after incubation at 18°C, these nine strains no longer hemagglutinated human or bovine erythrocytes, did not react with anti-CFA/I or -CFA/II antiserum, and were nonpiliated when directly visualized under the electron microscope (Fig. 1B).

Serotypes. The *E. coli* strains comprised a wide array of serotypes; some were nontypable (Table 2). The most frequently encountered O serogroups from 19 isolates included O159 (6 isolates; Kenya, Korea), O6 (4 isolates; Morocco, Honduras, Zaire), Ox2 (4 isolates; Morocco), O27 (3 isolates; Morocco, Honduras, Kenya), and O20 (2 isolates; Morocco). The remaining 17 isolates were spread among a wide variety of serogroups or were untypable.

In two of the four strains with CFA/I (Table 2), the O group could not be determined because of spontaneous agglutination; the other strains were type O20. Four of the five CFA/II-positive ETEC strains were type O6 (Table 2).

Stability of CFA expression. CFA expression in CFA-positive strains appeared to remain rather stable for five passages, with only one of nine strains appearing negative at that point despite cultivation on a suboptimal medium (tryptic soy agar) (Table 3). However, by the 10th subculture, three of the nine strains were negative (Table 3). The strains that became CFA negative after serial passage included two CFA/I and one CFA/II strain. The two CFA/I strains lost ST as well as CFA/I but retained LT, whereas the CFA/II strain lost both ST and LT in addition to CFA/II. Plasmid analysis revealed the loss of two plasmids approximately 60 and 48 megadaltons (Md) in size from the CFA/II strain, but no change in plasmid profile for the CFA/I strains (Fig. 2). DNA hybridization studies with the cloned LT gene showed the disappearance of genes homologous to the probe concomitant with the observed plasmid loss. Further hybridization studies with a porcine ST gene probe were not helpful, since the ST genes in these strains did not hybridize with this particular ST probe. The two ETEC strains that lost the CFA/I phenotype without losing a plasmid were inoculated into 5.0-cm ligated loops of rat ileum and incubated for 18 h in an attempt to restore CFA/I piliation by in vivo passage. Examination of 100 clones growing on CFA agar after culture of the rat ileal loop fluid failed to reveal any colonies that had regained the CFA/I phenotype.

CFA and type 1 somatic pili in non-enterotoxigenic *E. coli*. Of the 74 non-enterotoxigenic *E. coli* strains tested, none gave an MRHA pattern typical of CFA/I. Seven strains showed MRHA of bovine cells at 4°C, characteristic of CFA/II. None of these strains, however, were agglutinated by our CFA/II antiserum. One additional

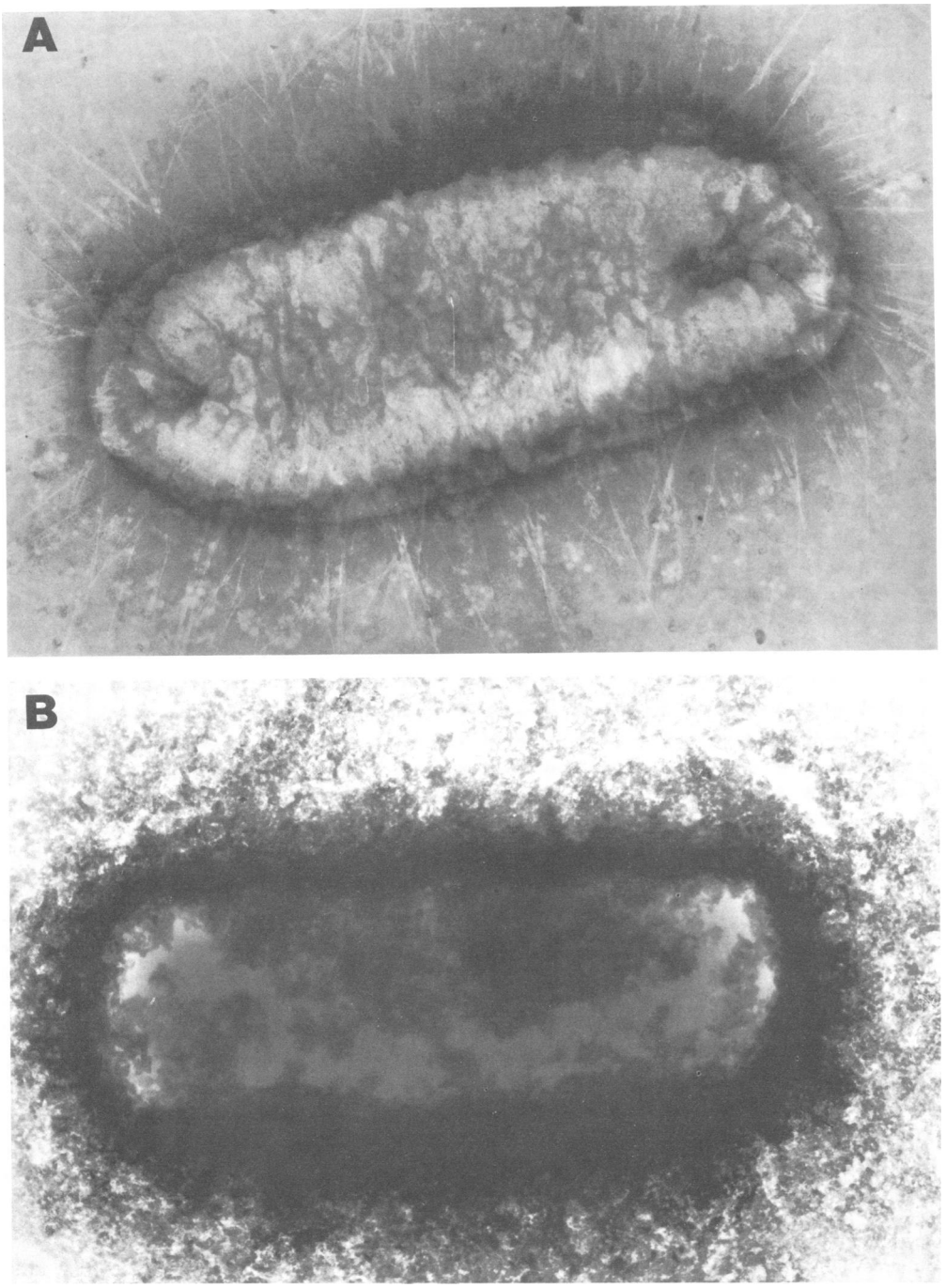

FIG. 1. (A) Electron micrograph of *E. coli* M424C1, which is piliated when grown at 37°C. (B) Electron micrograph of *E. coli* M424C1, which becomes nonpiliated when grown at 18°C.

strain caused MRHA of human but not bovine erythrocytes. Type 1 somatic pili were detected in 36 of the 74 (49%) non-enterotoxigenic *E. coli* strains.

DISCUSSION

CFA/I and CFA/II in human ETEC strains are analogous to the K88 antigen of porcine ETEC

TABLE 2. ETEC isolates from 36 volunteers with travelers' diarrhea due to infection with a single strain

Site	Patient designation	Clone	Serotype[a]	Enterotoxin phenotype	Pilus type present		
					CFA/I	CFA/II	Type 1 somatic
Morocco	8	408C1	O6:K15:H16	LT/ST	−	+	−
Morocco	11	411C1	O25:K?:H42	LT/ST	−	−	−
Morocco	24	424C1	O6:K15:H16	LT/ST	−	+	−
Morocco	33	633C1	O20:H$^-$	LT/ST	+	−	−
Morocco	45	145C2	O128:H SA	LT/ST	−	+	+
Morocco	52	452C1	O20:H$^-$	LT/ST	+	−	−
Honduras	10	410C1	SA:H$^-$	LT/ST	+	−	−
Kenya	19	350C1	O159:H4	LT/ST	−	−	−
Korea	10/18	C2	O159:H4	LT/ST	−	−	−
Honduras	18	218C5	O6:K15:H SA	LT/ST	−	+	−
Morocco	7	407C4	O27:K?:H20	ST	−	−	−
Morocco	9	109C2	SA:H12	ST	+	−	−
Morocco	15	415C1	Ox2:K?:H$^-$	ST	−	−	−
Morocco	21	421C1	SA:K83:H$^-$	ST	−	−	+
Morocco	26	526CB	Ox2:K?:H$^-$	ST	−	−	−
Morocco	43	443C1	Ox2:K?:H$^-$	ST	−	−	−
Morocco	6	406C1	Ox2:K?:H$^-$	ST	−	−	−
Honduras	46	446C3	O27:H20	ST	−	−	+
Honduras	49	449C3	O?:H$^-$	ST	−	−	+
Kenya	13	338C5	O27:H7	ST	−	−	+
Zaire	K	Z102C6A	O6:H16	ST	−	+	−
Zaire	L	Z105C3	O110(O18a,b):H27	ST	−	−	−
Morocco	3	403C3	O3,O7:H?	LT	−	−	+
Honduras	15	415C3	O?:HSA	LT	−	−	+
Honduras	26	426C2	O?:K25:H$^-$	LT	−	−	−
Honduras	29	1029C2	O8:H9	LT	−	−	+
Honduras	39	439C3	O25,O68:K?:H$^-$	LT	−	−	+
Honduras	40	440C5	O114:K:H49	LT	−	−	+
Honduras	41	441C1	O132:K12:H52	LT	−	−	−
Honduras	48	448C2	SA:H SA	LT	−	−	−
Honduras	49	449C2	O15:H$^-$	LT	−	−	+
Honduras	56	456C1	O?:H$^-$	LT	−	−	−
Kenya	4	330C1	O159:H34	LT	−	−	−
Kenya	7	375C4	O159:H4	LT	−	−	+
Kenya	14	336C4b	O159:H4	LT	−	−	+
Kenya	16	334C1	O159:H4	LT	−	−	+

[a] SA, Spontaneous agglutination.

strains. All three are clearly pilus structures morphologically when seen in electron photomicrographs; they preferentially appear after cultivation at 37°C on solid agar, but are not expressed after culture at 18°C; and they cause MRHA of certain erythrocytes.

Veterinary experience with purified K88, K99, and strain 987 purified pili vaccines has been so promising that it is now believed that up to 90% of ETEC diarrhea in piglets in North America may be prevented by a trivalent pilus antigen vaccine (25, 28, 35). Since CFA/I and CFA/II represent accessory virulence properties in those strains that possess them, there is great expectation that purified CFA/I and CFA/II pili vaccines may stimulate protective immunity in humans against ETEC strains possessing the homologous antigen. Indeed, preliminary studies in laboratory animals have shown that purified CFA/I and CFA/II vaccines administered orally or enterally stimulate intestinal secretory immunoglobulin A antibody in rabbits and provide protection against challenge with ETEC strains bearing the homologous pilus antigen (7; M. M. Levine, R. E. Black, M. L. Clements, C. R. Young, C. C. Brinton, Jr., P. Fusco, S. Wood, E. C. Boedeker, C. Cheney, P. Schadl, and H. Collins, *in* E. C. Boedeker, ed., *Attachment of Microorganisms to the Gastrointestinal Mucosal Surface*, in press). However, the ultimate human benefit to be derived from a CFA/I and CFA/II vaccine, regardless of its efficacy, will depend on the frequency with which these antigens occur in the ETEC strains that cause diarrhea in humans. There has been considerable controversy on this point. Evans et al. (11)

TABLE 3. Effect of multiple passage on expression of CFA pili and enterotoxins

Strain (phenotype and CFA)	Expressed in subculture:								
	Initial			Fifth			Tenth		
	CFA	ST	LT	CFA	ST	LT	CFA	ST	LT
M452C1 (LT^+/ST^+, CFA/I)	+	+	+	+	+	+	+	+	+
M109C2 (LT^-/ST^+, CFA/I)	+	+	−	+	+	−	+	+	−
M633C1 (LT^+/ST^+, CFA/I)	+	+	+	−	−	+	−	−	+
M410C1 (LT^+/ST^+, CFA/I)	+	+	+	+	+	+	−	−	+
M424C1 (LT^+/ST^+, CFA/II)	+	+	+	+	+	+	+	+	+
M408C1 (LT^+/ST^+, CFA/II)	+	+	+	+	+	+	+	+	+
M145C2 (LT^+/ST^+, CFA/II)	+	+	+	+	+	+	+	+	+
Z102C6 (LT^-/ST^+, CFA/II)	+	+	−	+	+	−	+	+	−
H218C5 (LT^+/ST^+, CFA/II)	+	+	+	+	+	+	−	−	−

reported that CFA/I occurs in 89% of ETEC strains, whereas Gross et al. (15), Thomas et al. (46), and Ørskov and Ørskov (31) identified these antigens in only a small percentage of ETEC strains causing human diarrhea. It could be argued that the studies reporting low prevalence included a preponderance of strains that had lost their CFA plasmids (9, 11, 32). However, a previous report of ours as well as the current data argue strongly against this (20). We previously showed that many ETEC strains lacking CFA/I and CFA/II nevertheless cause diarrhea in volunteers, colonize the intestine, and result in brisk immune responses (20). From those observations we concluded that many ETEC strains must possess colonization factors distinct from CFA/I and CFA/II or other adhesive factors that promote attachment to the mucosa of the proximal small intestine (20). The data reported in the current study support this concept. Although CFA/I or CFA/II occurred in 7 of 10 (70%) LT^+/ST^+ strains, such strains represented the causative agents in approximately only one-fourth of the 36 single-strain ETEC infections. Of the 12 LT^-/ST^+ and 14 LT^+/ST^- isolates, CFA/I or CFA/II was found in only two, both LT^-/ST^+.

The nine strains bearing CFA/I or CFA/II pili were serially passaged on solid agar. After five subcultures only one strain had lost the CFA, but after 10 passages three strains had become CFA negative. Plasmid analysis indicated that loss of CFA/II, ST, and LT in strain H218C5 was accompanied by the loss of two plasmids, approximately 60 and 48 Md in size. In contrast, the two CFA/I strains lost both CFA/I and ST without the discernible loss of a plasmid. Previous investigators have shown that the loss of ST is accompanied by the loss of CFA and was apparently due to the loss of a plasmid approximately 60 Md in size (9, 13, 14, 21, 34). We confirmed these observations with the CFA/II-producing strain, but our results with the two CFA/I strains indicated that another genetic mechanism could be responsible for the loss of phenotypic expression of CFA/I. This could imply that CFA pili undergo a phenotypic phase variation similar to that of type 1 somatic pili (45). If phase variation does occur with CFA/I pili (D. P. Taylor, submitted for publication), we do not yet know how to manipulate the environment to favor the return of CFA/I expression in turned-off cells. In vivo passage in a ligated loop of rat ileum for 18 h failed to restore CFA/I piliation in the two strains possessing CFA plasmids that ceased expression after multiple passage on solid agar. Alternatively, the loss of CFA expression without the loss of a plasmid may indicate a chromosomal mutation, as suggested by Evans et al. (12), who observed no loss of plasmids in some strains from which CFA/I or CFA/II but not ST was lost. The simultaneous loss of both ST and CFA/I suggests a single event that adversely affects the expression of both CFA and ST. The genetics of CFA/I production are not well understood, but Smith and co-workers (43) determined that two regions of a CFA/I-encoding plasmid are required for expression and that the two sites were separated by some 38 kilobases of DNA not involved with CFA production. Interestingly, these investigators found the position of the ST genes in the plasmid to be very close to the CFA/I genes. At least one ST gene has been determined to be a transposon (Tn*1681*) which is flanked by inverted repeats of IS*1* (44). Insertion elements have been shown to be responsible for a variety of deletion, duplication, inversion, and transposition events, and the presence of IS*1* within an operon leads to as much as a 1,000-fold increase in deletion formation frequency in the region (19). A single recombination or deletion event could affect the expression of adjacent genes either through deletion of DNA from both genes or by a simple frame shift that could affect the termination signal for one gene, with subsequent interference of initiation for the adjacent gene. Preliminary restriction endonuclease di-

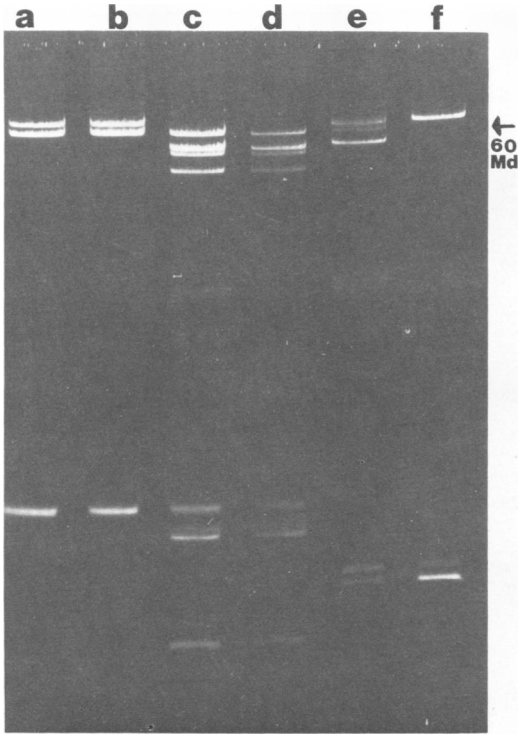

FIG. 2. Plasmid analysis of *E. coli* strains examined for stability of CFA expression. Electrophoresis of extracted plasmid DNA was performed in 0.7% agarose. Lanes: a, *E. coli* strain M633C1 after first subculture, possessing plasmids of 70, 58, and 2.6 Md; b, *E. coli* strain M633C1 after 10th subculture; c, strain H410C1 after first subculture, possessing plasmids of 58, 40, 36, 30, 2.6, 2.3, and 1.5 Md; d, strain H410C1 after 10th subculture; e, strain H218C5 after first subculture, possessing plasmids of 70, 60, 48, and 1.8 Md; f, strain H218C5 after 10th subculture, now only possessing plasmids of 70 and 1.8 Md.

gestions of total plasmid DNA from strains M633C1 and H410C1 after 1 and 10 subcultures had been done revealed no discernible difference in restriction fragments greater than 2 kilobases long (data not shown). Further investigations with different restriction enzymes under conditions that allow the detection of much smaller differences are in progress.

Most ETEC strains that bear CFA/I or CFA/II have been shown by numerous investigators to fall within a restricted number of O serogroups. These include O15, O20, O25, O63, O78, O128, and O153 for CFA/I and O6 and O8 for CFA/II. This pattern was also seen in the present study. Our observation of the rarity of CFA in LT^+/ST^- strains was also noted by Reis et al. (34). Thus, the antigens involved in attachment to the mucosa must be identified in the remaining majority of strains that lack CFA/I or CFA/II (46); these would have to be included in a future pilus vaccine for it to provide broad-spectrum prophylaxis. At least one new putative adhesin has been reported (46).

The prevalence of type 1 somatic pili in the LT^+/ST^+ and LT^-/ST^+ strains in this study was lower than that reported by Brinton (4) or by us previously (20). The reasons for this are unclear, but may be related to the fact that in many instances the cultures originated from a single clone. The precise role, if any, of type 1 somatic pili as an adhesive factor mediating the attachment of ETEC organisms to the small intestine mucosa is unclear. Although type 1 somatic pili promote the adhesion of ETEC cells to epithelial cells in vitro and the attachment of ETEC cells can be prevented by specific anti-pilus antibody (16), the observation that the 74 nonpathogenic colonic flora *E. coli* strains possessed type 1 somatic pili with approximately the same prevalence (49%) as did the 36 pathogenic ETEC strains (42%) makes these pili unlikely candidates for protective immunogens. Efforts must be intensified to identify the antigens responsible for adhesion in the ETEC pathogens that lack CFA/I and CFA/II. A multidisciplinary approach, including bacterial genetic techniques, should be employed to optimize this search, and wherever possible the ETEC strains studied should be minimally passaged and of known pedigree.

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LITERATURE CITED

1. **Acres, S. D., R. E. Isaacson, L. A. Babiuk, and R. A. Kapitany.** 1979. Immunization of calves against enterotoxigenic colibacillosis by vaccinating dams with purified K99 antigen and whole cell bacterins. Infect. Immun. **25**:121–125.
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. **Black, R. E., M. H. Merson, I. Huq, A. R. M. A. Alim, and M. Yunus.** 1981. Incidence and severity of rotavirus and *Escherichia coli* diarrhea in rural Bangladesh. Implications for vaccine development. Lancet **i**:41.
4. **Brinton, C. C., Jr.** 1977. The piliation phase syndrome and the uses of purified pili in disease control, p. 33. *In* Proceedings of the 13th Joint U.S.-Japan Conference on Cholera. Department of Health, Education, and Welfare publication no. 78-1590. National Institutes of Health, Bethesda, Md.
5. **Burrows, M. R., R. Sellwood, and R. A. Gibbons.** 1976. Hemagglutinating and adhesive properties associated with the K99 antigen of bovine strains of *Escherichia coli*. J. Gen. Microbiol. **96**:269–275.
6. **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.

7. **de la Cabada, F. J., D. G. Evans, and D. J. Evans, Jr.** 1981. Immunoprotection against enterotoxigenic *Escherichia coli* diarrhea in rabbits by peroral administration of purified colonization factor antigen I (CFA/I). FEMS Microbiol. Lett. **11**:303–307.
8. **Edwards, P. R., and W. H. Ewing.** 1972. Identification of enterobacteriaceae, 3rd ed. Burgess Publishing Co., Minneapolis, Minn.
9. **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.
10. **Evans, D. G., D. J. Evans, Jr., and W. S. Tjoa.** 1977. Hemagglutination of human group A erythrocytes by enterotoxigenic *Escherichia coli* isolated from adults with diarrhea: correlation with colonization factor. Infect. Immun. **18**:330–337.
11. **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.
12. **Evans, D. G., M. E. Penaranda, L. Selvidge, and D. J. Evans, Jr.** 1981. A novel effect of anti-CFA antibody on CFA-positive enterotoxigenic *Escherichia coli*: selective growth of CFA-negative variants, p. 96–101. *In* T. Holme, J. Holmgren, M. H. Merson, and R. Molley (ed.), Acute enteric infections in children. Elsevier/North-Holland Biomedical Press, Amsterdam.
13. **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.
14. **Gorbach, S. L., B. H. Kean, D. G. Evans, D. J. Evans, Jr., and D. Bessudo.** 1975. Travelers' diarrhea and toxigenic *Escherichia coli*. N. Engl. J. Med. **292**:933–936.
15. **Gross, R. J., A. Cravioto, S. M. Scotland, T. Cheasty, and B. Rowe.** 1978. The occurrence of colonization factor (CF) in enterotoxigenic *Escherichia coli*. FEMS Lett. **3**:231–233.
16. **Isaacson, R. E., P. C. Fusco, C. C. Brinton, and H. W. Moon.** 1978. In vitro adhesion of *Escherichia coli* to porcine small intestinal epithelial cells: pili as adhesive factors. Infect. Immun. **21**:392–397.
17. **Jones, G. W., and J. M. Rutter.** 1972. Role of the K88 antigen in the pathogenesis of neonatal diarrhea caused by *Escherichia coli* in piglets. Infect. Immun. **6**:918-927.
18. **Jones, G. W., and J. M. Rutter.** 1974. The association of K88 antigen with hemagglutinating activity in porcine strains of *Escherichia coli*. J. Gen. Microbiol. **84**:135–144.
19. **Kopecko, D. J. H.** 1980. Specialized genetic recombination systems in bacteria: their involvement in gene expression and evolution, p. 135–234. *In* F. Hahn (ed.), Progress in molecular and subcellular biology, vol. 7. Springer-Verlag Press, Heidelberg.
20. **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.
21. **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.
22. **Merson, M. H., G. K. Morris, D. A. Sack, J. G. Wells, J. C. Feeley, R. B. Sack, W. B. Creech, and A. Z. Kapikian.** 1976. Travelers' diarrhea in Mexico: a prospective study of physicians and family members attending a congress. N. Engl. J. Med. **294**:1299–1305.
23. **Meyers, J. A., D. Sanchez, L. P. Elwell, and S. Falkow.** 1976. Simple agarose gel electrophoresis method for the identification and characterization of plasmid deoxyribonucleic acid. J. Bacteriol. **127**:1529–1537.
24. **Moon, H. W., B. Nagy, R. E. Isaacson, and I. Ørskov.** 1977. Occurrence of K99 antigen on *Escherichia coli* isolated from pigs and colonization of pig ileum by K99$^+$ enterotoxigenic *E. coli* from calves and pigs. Infect. Immun. **15**:614–620.
25. **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 challenge. Infect. Immun. **22**:771–777.
26. **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.
27. **Nagy, B., H. W. Moon, and R. E. Isaacson.** 1977. Colonization of porcine intestine by enterotoxigenic *Escherichia coli*: selection of piliated forms in vivo, adhesion of piliated forms to epithelial cells in vitro, and incidence of a pilus antigen among porcine enteropathogenic *E. coli*. Infect. Immun. **16**:344–352.
28. **Nagy, B., H. W. Moon, R. E. Isaacson, C.-C. To, and C. C. Brinton, Jr.** 1978. Immunization of suckling pigs against enteric enterotoxigenic *Escherichia coli* infection by vaccinating dams with purified pili. Infect. Immun. **21**:269–274.
29. **Old, D. C.** 1972. Inhibition of the interaction between fimbrial hemagglutinations and erythrocytes by D-mannose and other carbohydrates. J. Gen. Microbiol. **71**:149–157.
30. **Ørskov, F., and I. Ørskov.** 1975. *Escherichia coli* O:K:H serotypes isolated from human blood: prevalence of the K1 antigen with technical details of O and H antigenic determination. Acta Pathol. Microbiol. Scand. Sect. B. **83**:595-600.
31. **Ørskov, I., and F. Ørskov.** 1977. Special O+K+H serotypes among enterotoxigenic *E. coli* strains from diarrhea in adults and children. Occurrence of the CF (colonization factor) antigen and hemagglutinating abilities. Med. Microbiol. Immunol. **163**:99–110.
32. **Penaranda, M. E., M. B. Mann, D. G. Evans, and D. J. Evans, Jr.** 1980. Transfer of an ST:LT:CFA/II plasmid in *Escherichia coli* K12 strain RR1 by cotransformation with PSC301 plasmid DNA. FEMS Microbiol. Lett. **8**:251–254.
33. **Reis, H. L., M. H. T. Affonso, L. R. Tabulsi, A. J. Oh, R. Maas, and W. K. Maas.** 1980. Transfer of a CFA/I-ST plasmid promoted by a conjugative plasmid in a strain of *Escherichia coli* of serotype O128ac:H12. Infect. Immun. **29**:140–143.
34. **Reis, M. H. L., D. P. Matos, A. F. Pestana de Castro, M. R. F. Toledo, and L. R. Trabulsi.** 1980. Relationship among enterotoxigenic phenotypes, serotypes, and sources of strains in enterotoxigenic *Escherichia coli*. Infect. Immun. **28**:24–27.
35. **Rutter, J. M., and G. W. Jones.** 1973. Protection against enteric disease caused by *Escherichia coli*—a model for vaccination with a virulence determinant. Nature (London) **242**:531–532.
36. **Sack, D. A., D. C. Kaminskyu, R. B. Sack, J. N. Itotia, R. R. Arthur, A. Z. Kapikian, F. Ørskov, and I. Ørskov.** 1978. Prophylactic doxycycline for travelers' diarrhea. Results of a prospective double-blind study of Peace Corps volunteers in Kenya. N. Engl. J. Med. **298**:758–763.
37. **Sack, D. A., and R. B. Sack.** 1975. Test for enterotoxigenic *Escherichia coli* using Y-1 adrenal cells in miniculture. Infect. Immun. **11**:334–366.
38. **Sack, R. B., J. L. Froelich, A. W. Zulich, D. S. Hadi, A. Z. Kapikian, F. Ørskov, I. Ørskov, and H. B. Greenberg.** 1979. Prophylactic doxycycline for travelers' diarrhea. Results of a prospective double-blind study of Peace Corps volunteers in Morocco. Gastroenterology **76**:1368–1373.
39. **Salit, I. E., and E. C. Gotschlich.** 1977. Hemagglutination by purified type 1 *Escherichia coli* pili. J. Exp. Med. **146**:1169–1194.
40. **Santosham, M., R. B. Sack, J. Froelich, H. Greenberg, R. Yolken, A. Kapikian, C. Javier, C. Medina, F. Ørskov, and**

I. Ørskov. 1981. Biweekly prophylactic doxycycline for travelers' diarrhea. J. Infect. Dis. **143**:598–602.

41. **Smith, H. W., and M. A. Linggood.** 1971. Observations on the pathogenic properties of the K88, Hly and Ent plasmids of *Escherichia coli* with particular reference to porcine diarrhea. J. Med. Microbiol. **4**:467–485.
42. **Smith, H. W., and M. A. Linggood.** 1972. Further observations on *Escherichia coli* enterotoxins with particular regard to those produced by atypical piglet strains and by calf and lamb strains: the transmissible nature of these enterotoxins and of a K antigen possessed by calf and lamb strains. J. Med. Microbiol. **5**:243–250.
43. **Smith, H. R., G. A. Willshaw, and B. Rowe.** 1982. Mapping of a plasmid coding for colonization factor antigen I and heat-stable enterotoxin production isolated from an enterotoxigenic strain of *Escherichia coli*. J. Bacteriol. **149**:264–275.
44. **So, M., F. Heffron, and B. J. McCarthy.** 1979. The *E. coli* gene encoding heat stable toxin is a bacterial transposon flanked by inverted repeats of ISI. Nature (London) **277**:453–456.
45. **Swaney, L. M., Y.-P. Liu, C.-M. To, C.-C. To, K. Ippen-Ihler, and C. C. Brinton, Jr.** 1977. Isolation and characterization of *Escherichia coli* phase variants and mutants deficient in type 1 pilus production. J. Bacteriol. **130**:494–505.
46. **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.