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Plasmids Coding for Colonization Factor Antigen I and Heat-Stable Enterotoxin Production Isolated from Enterotoxigenic *Escherichia coli*: Comparison of Their Properties

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We examined seven enterotoxigenic *Escherichia coli* strains which produced colonization factor antigen I (CFA/I). Four of these strains were from South Africa (three serotype O78:H12 and one serotype O63:H−), one was from Ethiopia (O78:H12), and two were from Bangladesh (O78:H11 and O78:H12). Plasmids coding for CFA/I were mobilized from six of these strains by using resistance or enterotoxin factors. No plasmid was mobilized from the serotype O78:H12 Bangladesh strain. The transconjugants obtained from crosses with the O78 strains also produced heat-stable enterotoxin (ST), and additional investigations showed that CFA/I and ST were coded for by a single non-autotransferring plasmid. These plasmids were fertility inhibition negative, did not restrict any of the coliphages with which they were tested, and were incompatible with each other. Four had molecular weights of approximately 60×10^6, and one had a molecular weight of 52×10^6. Like the other CFA/I plasmids, the CFA/I plasmid transferred from the O63:H− strain coded for ST, but this plasmid also coded for heat-labile enterotoxin. In most other respects the properties of this plasmid were similar to those of the CFA/I-ST plasmids previously described. The molecular weight of this plasmid was 65×10^6. The IncT R-factor RtsI was marked with a transposon for tetracycline resistance and then transferred into the two Bangladesh wild-type strains. Plasmids which coded for tetracycline resistance, CFA/I, and ST were transferred from these strains. These plasmids were incompatible with RtsI and with the CFA/I-ST plasmids described above and were recombinants between RtsI and a CFA/I-ST plasmid. Their properties are also described.

Enterotoxigenic *Escherichia coli* strains produce diarrheal disease in both humans and animals by colonizing the small intestines and producing enterotoxins. Hairlike surface structures known as fimbriae or pili are thought to be important in mediating the adhesion of the bacteria to the intestinal epithelia (12, 18, 23, 34). Whereas common pili, which are found on nonpathogenic *E. coli*, cause mannose-sensitive hemagglutination of erythrocytes (8), the fimbriae associated specifically with the ability of a strain to adhere cause mannose-resistant hemagglutination (MRHA) (10, 19, 24, 25). The adhesion factors from enterotoxigenic *E. coli* strains of human and animal origin appear to be species specific, affect different types of erythrocytes, and are antigenically distinct. Factors K88 and 987P are associated with porcine strains (23, 26), factor K99 is associated with bovine strains (25), and colonization factor antigen I (CFA/I) and CFA/II are associated with human strains (5, 9, 11, 12, 13, 15, 24).

The strains producing CFA/I belong to several serogroups, including O15, O25, O63, O78, O114, O128, and O153 (5, 11, 15, 24, 29, 31). Like K88 and K99 production, CFA/I production is plasmid controlled. Evans et al. showed that a derivative of strain H10407 which had lost the ability to produce CFA/I had also lost a plasmid having a molecular weight of 60×10^6 (12). This CFA/I$^-$ strain (strain H10407-P) also lost the ability to produce heat-stable enterotoxin (ST), although it still produced heat-labile enterotoxin (LT) (9). In volunteer experiments strain H10407-P was incapable of adhering to intestines and causing disease (30).

There seems to be a close association between CFA/I and ST, since all of the strains which have been identified as CFA/I$^+$ are also ST$^+$ (5, 9, 11, 29, 32). CFA/I$^-$ derivatives which are ST$^-$ have lost a plasmid (9, 32). A CFA/I-ST plasmid has been transferred from an O78:H12 strain to *E. coli* K-12 (32), and a similar plasmid has been transferred from an O128:H12 strain (28). In this study we examined a number of other O78 strains and one O63:H− strain to determine

whether CFA/I-ST plasmids were found commonly and compared the properties of the plasmids which we found.

MATERIALS AND METHODS

Wild-type bacterial strains. Six strains of serogroup O78 and one strain of serotype O63:H− were examined. These strains were isolated from patients with diarrheal diseases. Their countries of isolation and properties are shown in Table 1. The O78 strains have been described previously (5, 15, 20, 32).

Standard plasmids. The plasmids used to mobilize the CFA/I-ST plasmids and for incompatibility and other tests are listed in Table 2.

Enterotoxin tests. ST was detected by the infant mouse test (6). The production of LT was tested with Y1 adrenal cell (7) and CHO cell (16) tissue culture assays.

Hemagglutination and immunodiffusion tests. The strains tested for CFA/I were grown overnight on slopes of CFA agar at 37°C (10). Lines producing CFA/I were detected by the ability to cause MRHA of calf erythrocytes at 4°C. Immunodiffusion with CFA/I antiserum was used to confirm the presence of the specific antigen, as previously described (15). The CFA/I antiserum was prepared in rabbits by using *E. coli* strain E6674 (O63:H−) as a vaccine. This strain possesses CFA/I that has been shown by immunodiffusion tests to be identical to the CFA/I first described in strain H10407 (15). To prepare an antiserum specific for CFA/I, this crude antiserum was absorbed with a variant of strain E6674 known to lack CFA/I. This antiserum showed no cross-reactivity with type 1 pili when strains causing mannose-sensitive hemagglutination were examined by immunodiffusion tests. The *E. coli* K-12 transconjugants tested for the presence of CFA/I were streaked onto CFA agar either singly or in pools of five and tested by hemagglutination.

The strains to be used for electron microscopy were grown on CFA agar for two successive 24-h periods to give maximum expression of CFA/I and suppress the production of type 1 fimbriae (9). These strains were tested for type 1 fimbriae by making a thick suspension (approximately 10^{10} cells per ml) in saline and spotting onto a tile with guinea pig erythrocytes to determine whether the strains could cause mannose-sensitive hemagglutination (8).

Direct transfer of plasmids. Donor and recipient cultures were grown in nutrient broth (Difco Laboratories) to a density of 2×10^8 cells per ml at 37 or 28°C. For bacterial crosses lasting 1 or 2 h the ratio of donor cells to recipient cells was 1:10, and for overnight crosses this ratio was 1:1. Mating mixtures were plated onto MacConkey agar containing the appropriate drugs. The *E. coli* K-12 recipient strains were 14R519, a *lac* nalidixic acid-resistant strain, and 21R868, which was *lac*$^+$ and resistant to streptomycin. When drug-sensitive wild-type *E. coli* strains were used as recipients, the *E. coli* K-12 donor was eliminated with colicin E2 (1).

Transfer of plasmids by mobilization. (i) Cotransfer of CFA/I-ST plasmids. The derepressed F-like R factors R1-19K$^-$ and R1-19 and the LT plasmid TP236-Tc were transferred into wild-type *E. coli* strains. One transconjugant from each of these crosses was mated with strain 14R519, and nalidixic acid-resistant colonies carrying the R factor were selected; 9 to 200 transconjugants were then examined for cotransfer of MRHA.

(ii) Test for transfer factor ability. Broth cultures of the strains tested and of the strains carrying the non-autotransferring plasmid NTP2 or NTP107 were mixed at a ratio of 1:1 and incubated overnight. To 2 ml of each suspension, 1 ml of the final recipient culture was added, and again overnight mating was allowed to occur. This mixture was then plated onto MacConkey agar containing the appropriate drugs (1).

Formation of CFA/I-ST-Rts1 recombinants. The R-factor Rts1 carrying the tetracycline resistance transposon Tn*10* (17) was transferred from *E. coli* K-12 to strains H10407 and E9505. Transconjugants resistant to kanamycin and tetracycline were tested for MRHA, and MRHA$^+$ lines were stored at room temperature. These strains were examined after 10 months. Broth cultures were grown at 37°C to a density of approximately 5×10^8 cells per ml, diluted, and plated onto nutrient agar containing tetracycline. Suitable plates were replicated onto media containing kanamycin. Colonies which were tetracycline resistant and kanamycin sensitive were tested for MRHA.

Fertility inhibition. Broth cultures of strains carrying the CFA/I-ST plasmids and a derepressed F-like factor were tested with phage $\mu 2$ in surface spot tests. Plasmids that are fertility inhibition positive (*fi*$^+$) inhibit the synthesis of F fimbriae by derepressed F-like factors and therefore reduce or abolish visible lysis of the culture by phage $\mu 2$ (2).

Phage testing of *E. coli* K-12 strains carrying CFA/I-ST plasmids. (i) Phage propagation. The phages $\mu 2$ and fd were used in propagation experiments to detect F fimbriae (2).

(ii) Phage restriction. The ability of *E. coli* K-12 strains carrying CFA/I-ST plasmids to restrict *E. coli* phages was tested by using surface spot tests, as previously described (20). The phages used were T1, T3, T4, T5, T7, BF23, $\phi 8400$, $\phi 109$, $\phi 2$, and λvir.

Insertion of transposons into the CFA/I-ST plasmid NTP113. Plasmid NTP113 was marked with kanamycin or ampicillin resistance for use in transfer experiments and incompatibility tests. The kanamycin resistance transposon Tn*5* (4) was inserted into NTP113 (Table 3) from F'*pro AB lac*:Tn*5*. The F-T plasmid carrying an ampicillin resistance transposon (Tn*A*) was used for transposition of Tn*A* to NTP113. This Tn*A* transposon was derived from a non-autotransferring plasmid (NTP21) (21). A derivative of NTP113 which coded for production of ST but not for CFA/I was obtained by transposition of Tn*A*.

Surface exclusion and incompatibility tests. Surface exclusion is interference by a resident plasmid with the entry of genetic material via conjugation, and incompatibility is the inability of two different plasmids to coexist stably in the same host cell. In tests for these properties, we used derivatives of NTP113 marked with drug resistance as described above. These derivatives were then tested for the ability to transfer into and coexist stably in *E. coli* K-12 strains containing other CFA/I-ST plasmids or F-like R factors, as previously described (21).

TABLE 1. *Enterotoxigenic strains of E. coli*

Strain	Serotype	Origin	Enterotoxin production		CFA/I production	Drug resistance[a]	Mol wt of plasmids ($\times 10^6$)
			ST	LT			
H10407	O78:H11	Bangladesh (ca. 1971)	+	+	+		56, 42, 3.8
E9505	O78:H12	Bangladesh (1977)	+	−	+		57, 42, 4.1
E7464	O78:H12	South Africa (1977)	+	+	+		60, 55, 34
E7473	O78:H12	South Africa (1977)	+	+	+	Sm Su	61, 54, 42, 4.4
E7479	O78:H12	South Africa (1977)	+	−	+	Ap Sm Su Tc	63, 51, 44, 3.4
E9570	O78:H12	Ethiopia (1977)	+	−	+		60, 44
E11938	O63:H-	South Africa (1978)	+	+	+	Sm Su	68, 61, 4.5, 2.9, 2.5

[a] Abbreviations for drug resistance markers: Ap, ampicillin; Sm, streptomycin; Su, sulfathiazole; Tc, tetracycline.

TABLE 2. *Standard plasmids*

Plasmid[a]	Marker(s)[b]	Incompatibility group	Reference
F-T	Tc	FI	3
TP160 Ap Cm Sm Su^-	Tc	FI	3
R1-19	Ap Cm Km Sm Su	FII	17
R1-19K^-	Ap Cm Sm Su	FII	17
R100-1	Cm Sm Su Tc	FII	17
240 *drp*	Tc	FII	14
TP236-Tc	LT Tc	Enterotoxin plasmid group 1	20
TP237-Tc	LT Tc	Enterotoxin plasmid group 3	20
TP273	Cm Sm Su Tc	ND[c]	20
Rts1-Tc	Km Tc	T	37
NTP2	Sm Su	2	33
NTP107	Km	MP10	21

[a] The prefix TP indicates autotransferring plasmids, and the prefix NTP indicates non-autotransferring plasmids.

[b] Abbreviations for drug resistance markers: Cm, chloramphenicol, Km, kanamycin; Ap, ampicillin; Sm, streptomycin; Su, sulfathiazole; Tc, tetracycline.

[c] ND, Not determined.

Preparation and agarose gel electrophoresis of plasmid DNA. The methods used for partial purification of plasmid deoxyribonucleic acid (DNA) and agarose gel electrophoresis have been described previously (39). Wild-type *E. coli* strains and *E. coli* K-12 derivatives grown in 50 ml of nutrient broth were lysed with a mixture of 1% (wt/vol) Brij 58 and 0.4% (wt/vol) sodium deoxycholate. Plasmid DNA was purified partially from the cleared lysates by phenol extraction and ethanol precipitation. Samples were subjected to electrophoresis on vertical 0.6% (wt/vol) agarose gels at 140 V for 4 h in buffer (pH 8.0) containing 89 mM tris(hydroxymethyl)aminomethane, 89 mM boric acid, and 2.5 mM ethylenediaminetetraacetate. The molecular weights of the plasmids were measured by comparing the mobility of the plasmid DNA with the mobilities of plasmids of known molecular weights (range 4.7×10^6 to 78×10^6) run on the same gel.

Electron microscopy. The strains were grown on CFA agar for two 24-h periods. A thick suspension of each culture (approximately 10^{10} cells per ml) was made in peptone water containing 4% formaldehyde. This suspension was diluted 1:2 in distilled water and 1 drop was applied to a Formvar carbon-coated electron microscope grid (400 mesh) for 3 min. Excess fluid was removed by blotting with filter paper, and 1 drop of 1% phosphotungstic acid (pH 6.4) was placed on the grid for 1 min before blotting as described above.

For electron microscopy of anti-CFA/I serum, cultures were suspended in peptone water without formaldehyde, and 1 drop of each suspension diluted 1:2 in distilled water was applied to the grid as described above. Excess fluid was removed by blotting, and the grid was floated sample side down on 1 drop of antiserum diluted 1:30 in phosphate-buffered saline. The serum drops with grids were incubated in a humidified chamber for 15 min at room temperature. The grids were removed from the drops, blotted, washed four times with distilled water, stained for 1 min with 3% phosphotungstic acid, and finally blotted.

RESULTS

Properties of enterotoxigenic *E. coli*. The wild-type strains were selected for this study because they were all positive for CFA/I. All of the strains produced ST, and four also produced LT (Table 1). The O78 strains have been described previously, and the plasmid contents of CFA/I^+ and CFA/I^- colonies of all of these

TABLE 3. *Properties of plasmids coding for CFA/I and ST*

Plasmid	Strain from which plasmid was isolated	LT	Phage restriction	Incompatibility with:		Mol wt ($\times 10^6$)	No. of MRHA$^+$ colonies/no. of R1-19K$^-$ or R1-19 transconjugants tested from K-12-K-12 crosses
				NTP113-Km or NTP113-Ap	Rts1		
NTP118	H10407	−	−[a]	+	NT[b]	56	10/20
NTP117	E7464	−	−	+	NT	58	2/20
NTP113	E7473	−	−	NT	NT	58	5/10
NTP115	E7479	−	−	+	NT	52	11/20
NTP114	E9570	−	−	+	NT	56	3/20
NTP116	E11938	+	T4, T5, BF23	+	NT	65	1/20
TP268[c]	H10407	−	−	+	+	ca. 100	NT
TP267[c]	E9505	−	T4, BF23	+	+	>100	NT

[a] —, No phage restriction.
[b] NT, Not tested.
[c] Autotransferring recombinant plasmids coding for tetracycline resistance.

strains except E7479 have been analyzed (5, 15, 20, 32).

The strains were tested initially for transfer factors. As previously described, strain E7473 contained a transfer factor, and the autotransferring plasmid from strain E7464 coded for LT production (20). Mobilization experiments showed that strains E9505 and E11938 carried factors which could transfer the non-autotransferring resistance plasmid NTP2. Neither of these plasmids coded for MRHA or for enterotoxin production.

Strain E7479 was resistant to ampicillin, streptomycin, sulfathiazole, and tetracycline. Transconjugants from mating experiments with *E. coli* K-12 were resistant to all four of these drugs or to only ampicillin and tetracycline. Additional crosses and gel electrophoresis showed that strain E7479 contained an autotransferring ampicillin-tetracycline resistance plasmid having a molecular weight of 63×10^6 and a non-autotransferring streptomycin-sulfathiazole resistance plasmid having a molecular weight of 4.4×10^6. Again, no MRHA or enterotoxin production was detected. Strains H10407 and E9570 did not contain transfer factors. No CFA/I-ST plasmids could be transferred directly from any of these seven wild-type strains, so we attempted to mobilize them.

Transfer of plasmids coding for CFA/I and ST. (i) Mobilization. Some of the strains described here had been examined previously for possession of an LT plasmid (20). We were not able to transfer such a plasmid from strain E7473, but during attempts to do this a derepressed R factor (R1-19K$^-$) was introduced into the wild-type strain. This strain was then mated with strain 14R519, and transconjugants which carried the R factor were selected. These transconjugants were examined to determine whether they were *fi*$^+$; 9 of 30 transconjugants had this property, and five of the nine colonies produced CFA/I and ST. After the loss of the R factor from one transconjugant, we showed by gel electrophoresis that a single plasmid having a molecular weight of 60×10^6 was present (NTP113) (32). When R1-19K$^-$ or F-T was transferred back into a transconjugant carrying NTP113, there was no inhibition of lysis of phage $\mu 2$, so that the plasmid was *fi*$^-$. Additional experiments showed that in some colonies of the wild-type strain, the R-factor R1-19K$^-$ was repressed.

R1-19K$^-$ was transferred into wild-type strains E9505, E9570, E7464, and E7479, and R1-19 was transferred into strains H10407 and E11938. These strains were then mated with strain 14R519, and transconjugants which carried the R factor were selected. We examined a maximum of 200 colonies per cross for the ability to cause MRHA of calf erythrocytes. MRHA$^+$ colonies were obtained from crosses involving strains E9570 (1 of 40), E7479 (4 of 140), E11938 (1 of 60), and H10407 (1 of 10). No transfer of MRHA$^+$ ability was obtained from strain E7464 (0 of 200) or E9505 (0 of 200).

Strain E7464 carries an autotransferring LT plasmid (20). Experiments showed that a derivative of this plasmid marked with drug resistance (TP236-Tc) was able to mobilize NTP113-Km efficiently in *E. coli* K-12 to *E. coli* K-12 crosses (see below). TP236-Tc was introduced into the wild-type strain E7464. After matings with strain 14R519 were performed, we selected transconjugants for tetracycline resistance and examined for cotransfer of MRHA; 3 of 50 colonies were positive.

One or two MRHA$^+$ colonies from each cross were tested by immunodiffusion with anti-CFA/I serum and for ST production. All colonies tested gave positive results in immunodiffusion tests and produced ST when tested in infant mice. An *E. coli* K-12 strain carrying a CFA/I-ST plasmid from strain E7473 was tested for ST by using a weaned pig gut loop, and a positive result was obtained again (R. J. Bywater, personal communication).

(ii) Recombination with Rts1. When cultures of strains H10407 (Rts1-Tc) and E9505 (Rts1-Tc) were tested for loss of kanamycin resistance, we found 7 of 1,086 and 2 of 1,684 kanamycin-sensitive colonies, respectively. Lines that were tetracycline resistant and MRHA$^+$ were tested for transfer of tetracycline resistance to *E. coli* K-12. Three of four lines from strain H10407 (tetracycline resistant, MRHA$^+$) transferred tetracycline resistance and MRHA production, and one of two lines from strain E9505 (tetracycline resistant, MRHA$^+$) transferred these two properties. Immunodiffusion showed that MRHA was caused by CFA/I. The *E. coli* K-12 transconjugants were also positive in infant mouse ST tests.

Isolation of *E. coli* K-12 lines carrying only CFA/I-ST plasmids. Drug-resistant transconjugants coding for CFA/I and ST production were examined for LT production and for the ability to restrict coliphages. The DNA contents of these transconjugants were examined by gel electrophoresis.

The transconjugant obtained by mobilization with R1-19 from strain H10407 was LT$^-$, but it restricted coliphages T4, T5, and BF23. After a short cross to another *E. coli* K-12 strain, we obtained some CFA/I$^+$ ST$^+$ transconjugants which did not restrict coliphages. A comparison of the DNAs of the two types of transconjugants showed that a small plasmid having a molecular weight of 3.8×10^6 was absent from the strain which did not restrict coliphages.

A CFA/I$^+$ ST$^+$ transconjugant from strain E7464 obtained by using the LT plasmid TP236-Tc as the transfer factor did not restrict any coliphages. We showed by gel electrophoresis that this transconjugant contained two bands of plasmid DNA, corresponding to molecular weights of about 34×10^6 and 60×10^6. TP236-Tc has a molecular weight of about 60×10^6. After a short cross, we obtained a drug-resistant transconjugant which was CFA/I$^+$ ST$^+$. Only the band of DNA at a position corresponding to a molecular weight of 60×10^6 was visible. No properties have been assigned to the 34×10^6 plasmid yet.

The transconjugants obtained from crosses involving strains E7473, E7479, and E9570 did not produce LT or restrict coliphages. An examination of the DNAs showed plasmid bands at positions corresponding to the molecular weights of the R factor and the CFA/I-ST plasmids. The R factors were therefore lost from all of the CFA/I$^+$ ST$^+$ transconjugants, and their plasmid DNAs were examined again. The CFA/I-ST plasmids had molecular weights of approximately 60×10^6, except for the plasmid from strain E7479, which was smaller (molecular weight, 52×10^6) (Table 3).

The CFA/I$^+$ ST$^+$ transconjugant obtained by using R1-19 as a transfer factor in strain E11938 (O63H−) was LT$^+$ and restricted coliphages T4, T5, and BF23. The CFA/I property was mobilized to another *E. coli* K-12 strain when there was cotransfer of ST, LT, and coliphage restriction, suggesting that all of these properties were coded for by a single plasmid. After the loss of the R factor, DNA from this transconjugant was examined. A single band was observed at a position corresponding to a molecular weight of 65×10^6.

Properties of CFA/I-ST plasmids. Except for the transconjugant from strain E7464, the original CFA/I$^+$ ST$^+$ transconjugants carried a derepressed F-like R factor. All were lysed by phage μ2, and, therefore, none carried an *fi*$^+$ plasmid. R1-19K$^-$ was transferred into a strain carrying the CFA/I-ST plasmid NTP117, which was isolated from strain E7464. Again, no inhibition of μ2 lysis was observed, so this plasmid was also *fi*$^-$.

Strains carrying only CFA/I-ST plasmids did not propagate μ2 or fd phage. These strains were tested for transfer factor ability by using them to try to mobilize NTP107, and all were found to be negative. However, all of the plasmids were cotransferred at a high rate with R1-19K$^-$ or R1-19 in overnight crosses (Table 3).

The CFA/I-ST plasmids were tested for incompatibility. A mutant of NTP113 was obtained by transposition of ampicillin resistance onto the plasmid. This mutant plasmid did not code for CFA/I production but still coded for ST. This plasmid was transferred to *E. coli* K-12 strains carrying NTP114, NTP115, NTP116, NTP117, or NTP118, and ampicillin-resistant transconjugants were examined for the ability to cause MRHA. In all cases there was a high rate of displacement of MRHA (at least 8 of 10 colonies tested), showing that the plasmids were incompatible. In the test involving NTP116, we observed simultaneous losses of LT and MRHA. To confirm the linkage of the three pathogenic properties on a single plasmid, we looked for MRHA$^-$ segregants of the wild-type strain E11938. Two MRHA$^-$ colonies were found to be ST$^-$ and LT$^-$. An examination of the DNAs of

these colonies showed that a plasmid having a molecular weight of 65×10^6 was missing. Again, these results suggested that CFA/I, ST, and LT were coded for by a single plasmid.

Properties of MRHA$^+$ transconjugants containing Rts1. We examined CFA/I$^+$ ST$^+$, tetracycline-resistant transconjugants from the crosses of strains H10407 (Rts1-Tc) and E9505 (Rts1-Tc) with *E. coli* K-12. An examination of the DNA of the transconjugant from strain H10407 showed a single plasmid band having a molecular weight of about 100×10^6. Therefore, CFA/I production, ST production, and tetracycline resistance were coded for by a single plasmid. (TP268). The transconjugant from strain E9505 showed restriction of phages T4, ϕ2, and BF23. Strain E9505 contains a transfer factor which restricts T4 and ϕ2, whereas Rts1 restricts T4 and BF23 partially (36; A. Ashley, unpublished data).

A short cross was performed to separate the drug resistance plasmid from the transfer factor. Some tetracycline-resistant transconjugants were still CFA/I$^+$ ST$^+$ and partially restricted T4 and BF23 but had lost the ability to restrict ϕ2. An examination of the DNA of one such transconjugant showed a single plasmid, corresponding to a molecular weight of $>100 \times 10^6$ (TP267).

The sizes of the CFA/I-ST-Tc plasmids in both cases were greater than the size of any of the plasmids originally present in strain H10407 or E9505, suggesting that recombination probably occurred between Rts1 and the CFA/I-ST plasmids. Rts1 is temperature sensitive for transfer (37). Therefore, we tested plasmids TP267 and TP268 for the ability to transfer in crosses at 28 and 37°C. Both plasmids transferred better at 28 than at 37°C, showing that they possessed the transfer system of Rts1.

These plasmids were also tested for surface exclusion and incompatibility with Rts1. When one of these plasmids was introduced into *E. coli* K-12 carrying TP268, 10-fold exclusion was observed, and 10 of 10 colonies lost tetracycline resistance and MRHA, showing that the two plasmids were incompatible. When Rts1 was introduced into strains carrying TP267, very high exclusion was observed (10^5); 10 of 10 colonies lost tetracycline resistance but retained MRHA. Gel electrophoresis of one transconjugant showed that it carried two plasmids, one having a molecular weight of $>100 \times 10^6$ (which corresponded to Rts1) and one having a molecular weight of 60×10^6 (the size commonly found for CFA/I-ST plasmids). TP267 appeared to have dissociated into its component plasmids in this experiment.

TP267 and TP268 were both tested for incompatibility with the CFA/I-ST plasmid NTP113, which was marked with kanamycin resistance as described above. TP267 and TP268 were transferred into strains carrying NTP113-Km. No exclusion was observed, but TP268 displaced kanamycin resistance from 10 of 10 transconjugants. There was no displacement in the experiment involving TP267, but the rate of segregation was very high (82.8% of the hybrid colonies lost either tetracycline resistance or kanamycin resistance). Therefore, TP267 and TP268 were recombinants between Rts1 and CFA/I-ST plasmids. TP267 was the larger plasmid and retained more of the properties of Rts1.

Other properties of NTP113-Km. NTP113-Km was examined for incompatibility with F-like plasmids. It was compatible with IncFII plasmids and with LT plasmids TP236-Tc and TP237-Tc, which were isolated originally from O78 strains (Table 2). We observed a low degree of incompatibility with the IncFI plasmids F-T and TP160. In both cases, when F-T or TP160 was transferred into a strain carrying NTP113-Km, no displacement occurred. However, segregation of the IncFI plasmid and NTP113-Km occurred at a low rate; 10% of the transconjugants lost one or the other plasmid after overnight incubation.

The transfer of NTP113 from the wild-type strain *E. coli* E7473 was mediated originally by R1-19K$^-$, and cotransfer occurred at a very high rate (Table 3). The ability of the marked plasmid NTP113-Km to be mobilized by other F-like factors was examined. Four standard F-like factors were used, and three plasmids were isolated from the O78 strains (Table 4). These factors were introduced into *E. coli* K-12 (NTP113-Km), and the transfer frequencies of the R factor and of NTP113-Km were measured. The crosses were 2 h long when the derepressed factors F-T, R1-19K$^-$, R100-1, and 240 *drp* were used, but overnight with the plasmids which were isolated originally from O78 strains (TP236-Tc, TP237-Tc, and TP273). A total of 10 colonies from each selection were examined for drug resistance. R1-19K$^-$ was able to mobilize NTP113-Km about 6×10^2 times as well as the two other IncFII factors and F-T. The other plasmids which were particularly efficient at transferring NTP113-Km were the LT plasmids TP236-Tc and TP237-Tc. Selection for NTP113-Km usually resulted in transconjugants which also possessed an R factor. However, particularly when F-T was used as a transfer factor, transconjugants containing only NTP113-Km could be obtained. The difference with F-T was probably due to the incompatibility described above.

Electron microscopy. An *E. coli* K-12, strain carrying NTP113-Km and the original *E.*

TABLE 4. *Rate of cotransfer of NTP113-Km with F-like plasmids*

Plasmid	Incompatibility group	Transfer frequencies[a]		Ratio of frequencies (NTP113-Km/R factor)	Time of cross (h)	No. of colonies selected for:	
		R factor	NTP113-Km			NTP113-Km which carry R factor	R factor which carry NTP113-Km
F-T	FI	2.5×10^{-2}	5.2×10^{-6}	2×10^{-4}	2	5/10	0/10
R1-19K$^-$	FII	6.2×10^{-1}	5.5×10^{-2}	9×10^{-2}	2	9/10	2/10
R100-1	FII	2.1×10^{-3}	6.6×10^{-7}	3×10^{-4}	2	10/10	0/10
240 *drp*	FII	3.1×10^{-3}	4.4×10^{-7}	1×10^{-4}	2	10/10	0/10
TP236-Tc	Enterotoxin plasmid group 1	4.6×10^{-1}	2.0×10^{-2}	4×10^{-2}	ON[b]	10/10	10/10
TP237-Tc	Enterotoxin plasmid group 3	8.2×10^{-2}	1.1×10^{-4}	1×10^{-3}	ON	10/10	3/10
TP273	ND[c]	6.4×10^{-1}	3.5×10^{-6}	5×10^{-6}	ON	10/10	0/10

[a] For 2-h crosses transfer frequencies were estimated as fractions of transconjugants per donor cell, and for overnight crosses transfer frequencies were estimated as fractions of transconjugants per recipient cell.
[b] ON, Overnight.
[c] ND, Not determined.

coli K-12 strain (as a control) were grown on CFA agar for two successive 18-h periods. These cultures were negative for type 1 fimbriae when tested for the ability to agglutinate guinea pig erythrocytes (8). The bacteria were examined after negative staining. The majority (93%) of the 225 Formalin-fixed *E. coli* K-12 cells carrying NTP113-Km which we examined were fimbriate (Fig. 1A). The fimbriae were approximately 6.5 nm in diameter. The majority (91%) of the 173 *E. coli* K-12 control cells examined were nude (Fig. 1B), but a few cells had fimbriae (Fig. 1D) that were morphologically indistinguishable from those observed on *E. coli* K-12 cells carrying NTP113-Km. To distinguish between CFA/I and other fimbriae, fresh preparations of strains were made and examined by electron microscopy with specific anti-CFA/I serum. The fimbriae of the *E. coli* K-12 cells carrying NTP113-Km were coated with antibody (Fig. 1C), whereas those on the control *E. coli* K-12 cells were not (Fig. 1D). The fimbriae of the *E. coli* K-12 cells carrying NTP113-Km were not coated with antibody when specific anti-CFA/II serum was used.

A wild-type strain of serotype O78:H$^-$ which produced CFA/I and its CFA/I$^-$ derivative were examined in a similar fashion. Again, the majority (94%) of the 172 cells from the CFA/I$^+$ strain were fimbriate, whereas the majority (87%) of the 114 cells from the CFA/I$^-$ strain were not. Again, only the fimbriae on the CFA/I$^+$ cells were coated with CFA/I antiserum.

DISCUSSION

Previous work suggested that the genes for CFA/I production and ST production are carried on a single plasmid in enterotoxigenic *E. coli* strains of different serotypes (5, 9, 11, 28, 32). CFA/I-ST plasmids have now been transferred from six O78 strains (32; see above) and two O128 strains (28; unpublished data). If the O78 strains originally produced LT, then CFA/I$^-$ ST$^-$ derivatives retained the ability to produce LT; this property was encoded by a separate plasmid (20, 32). However, CFA/I$^-$ ST$^-$ derivatives from an O63:H− strain were also LT$^-$; in this strain the CFA/I-ST plasmid also coded for LT. A similar type of plasmid has been isolated from an O6:H16 strain; the colonization factor antigen coded for in this strain was CFA/II. ST, LT, and CFA/II were lost together from two O6:H16 strains and one O85:H7 strain (27). However, an examination of the CFA/II$^-$ derivatives of other O6:H16 and O8:H9 strains showed a variety of other patterns (9). Plasmid loss associated with the loss of these properties has been observed, suggesting that CFA/II production and enterotoxin production are not always linked (A. Cravioto, personal communication).

When an *E. coli* K-12 strain carrying one of the CFA/I-ST plasmids (NTP113-Km) was examined by electron microscopy, numerous fimbriae having a mean diameter of 6.5 nm were observed on most cells. Fimbriae of this size were also observed on CFA/I$^+$ wild-type *E. coli*, as previously described (12, 13, 38). This size appears to be the most common size, although Wadstrom et al. (38) found that the fimbriae of some strains had a mean diameter of 9.4 nm. Like Freer et al. (13), we also observed some nonfimbriate cells in our preparation. Although Evans and Evans claimed that strains grown on CFA agar do not produce type I fimbriae (9), we found that 9 to 13% of the CFA/I$^-$ cells were fimbriate in the two cultures examined. "Light" piliation in CFA/I$^-$ cells was also observed by

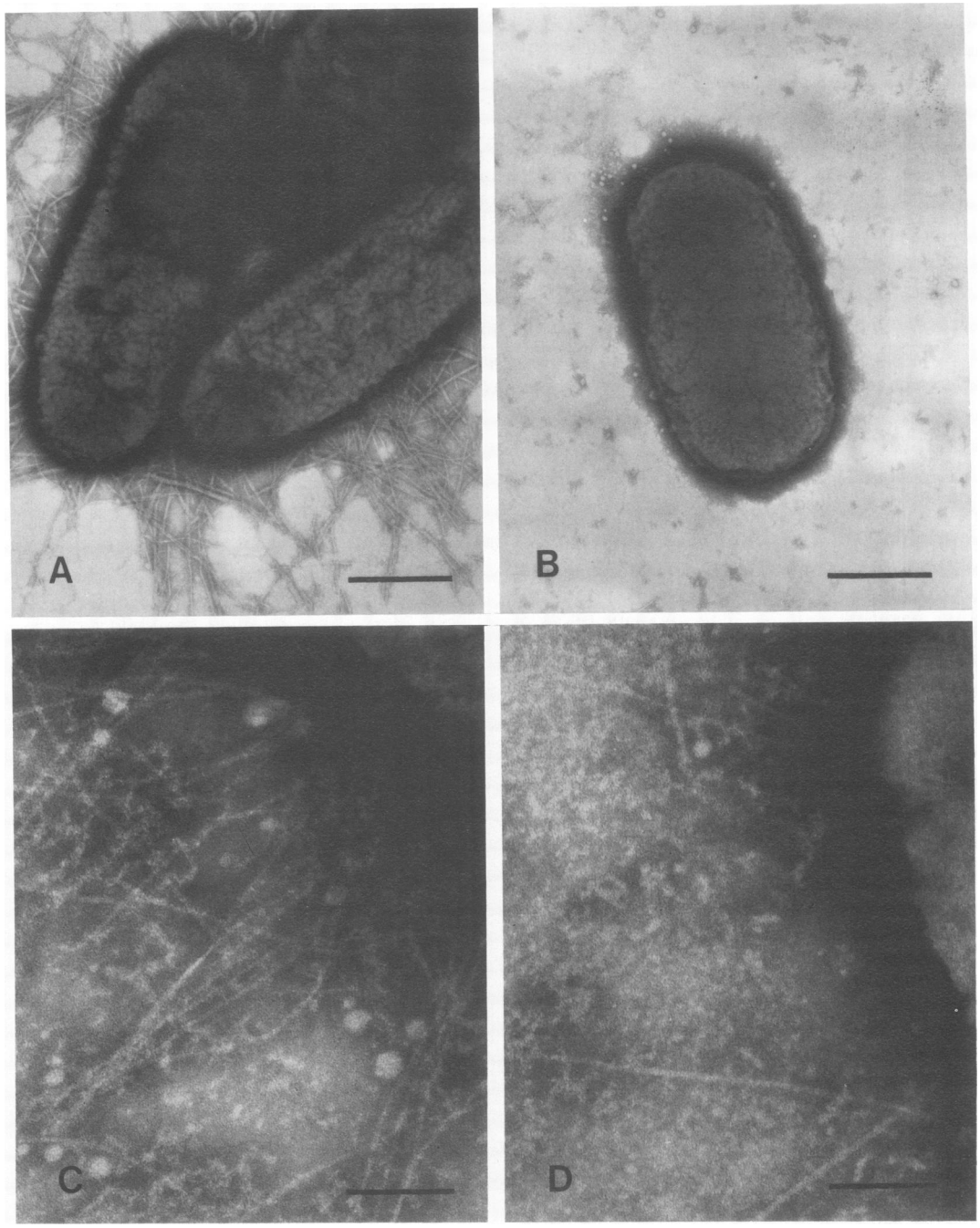

FIG. 1. *Electron micrographs of negatively stained preparations of an E. coli K-12 strain containing a CFA/I-ST plasmid (strain 54R118) (A) and an E. coli K-12 control strain (strain 21R868) (B). Bars = 500 nm. ×30,000. Also shown are micrographs of negatively stained preparations treated with specific anti-CFA/I sera, strain 54R118 (C) (showing fimbriae coated with antibody) and strain 21R868 (D) (showing a few fimbriae not coated with antibody). Magnification, ×157,500. Bars = 100 nm.*

Wadstrom et al. (38). Since these fimbriae did not react with anti-CFA/I serum, they were presumably type 1 fimbriae, and hemagglutination tests for them were negative because these tests are relatively insensitive.

Smith and Huggins (34) have shown that to

convert a nonpathogenic *E. coli* O9:K36:H19 strain to pathogenicity, it is necessary to implant both a plasmid coding for an adhesion factor and a plasmid coding for enterotoxin production. The combination of genes for colonization factor antigen and enterotoxin production on a single plasmid provides an efficient means by which a suitable nonpathogenic *E. coli* strain might be converted to pathogenicity.

The six plasmids that we examined are all non-autotransferring, so that a transfer factor is required to mobilize them between *E. coli* strains. These plasmids are transferred efficiently with R1-19K$^-$ but not with R100 and 240 *drp* (which are also IncFII plasmids) or with the IncFI plasmid F-T. R1-19K$^-$ differs from R100 and 240 *drp* in that the plasmid-specific proteins necessary for transfer are different (14); the F-like pili coded for by R100, 240 *drp*, and F differ serologically from the pili of R1 (22). The two LT plasmids are also able to mobilize NTP113-Km efficiently, suggesting that they code for transfer systems similar to that of R1-19K$^-$. The LT plasmids are related by incompatibility to R1-19K$^-$ in that TP236 is incompatible with IncFII plasmids and TP237 is incompatible with TP236, although TP237 is itself compatible with IncFII plasmids (20). The ability of the two LT plasmids isolated from O78 strains and the ability of a transfer factor in an O128 strain to mobilize tbe CFA/I-ST plasmid (28) suggest a means by which the plasmids could be transferred in vivo. Five of the seven enterotoxigenic strains examined here carry an autotransferring factor.

During our original examination of the O78 strains, we were able to transfer plasmids which coded for LT production but not ST production, as tested in infant mice (20) and in pig gut loops (Bywater, personal communication). The LT plasmids were divided into three groups; those isolated from strains of the same H type and from the same geographical region had the same properties. The CFA/I-ST plasmids that we compared above were almost identical in properties regardless of the country of isolation of the wild-type strain or its H type. The mobilization experiments and the incompatibility of NTP113-Km with F-T suggest that these plasmids are all F-like plasmids. The CFA/I-ST-LT plasmid isolated from the O63:H$^-$ strain also had properties similar to those of the CFA/I-ST plasmids and was incompatible with NTP113-Km. A more detailed examination of the relationships among these plasmids is being made by molecular techniques (Willshaw et al., unpublished data).

The isolation of plasmids coding for CFA/I-ST will enable us to study the effect of chromosomal genes on the stability of these plasmids and on the expression of the CFA/I phenotype. Transposition into a CFA/I-ST plasmid is being used to map the genes for CFA/I production and ST production (Smith et al., unpublished data) and to obtain derivatives which code only for CFA/I production or ST production. Such derivatives could be used to test the pathogenic effects of these factors separately.

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