Mapping of a Plasmid, Coding for Colonization Factor Antigen I and Heat-Stable Enterotoxin Production, Isolated from an Enterotoxigenic Strain of Escherichia coli

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The non-autotransferring plasmid NTP113 codes for production of colonization factor antigen I and heat-stable enterotoxin. NTP113, which has a molecular weight of $58 \times 10^6$, was digested with BamHI, EcoRI, and HindIII and combinations of these restriction endonucleases, and the products of these digestions were analyzed by agarose gel electrophoresis. The results were used to construct a partial restriction map of NTP113. Transposons coding for resistance to ampicillin, kanamycin, and tetracycline were inserted into NTP113, and we obtained a series of deletion mutants, as determined by the loss of tetracycline or kanamycin resistance from strains carrying the insertion mutants. A number of plasmid mutants obtained by insertion or deletion did not code for colonization factor antigen I, but most of these mutants still coded for heat-stable enterotoxin production. The positions of the inserted transposons and of the deletions were determined on the restriction map. Two regions of NTP113 were required for the expression of colonization factor antigen I, and the two sites were separated by a length of DNA corresponding to a molecular weight of about $25 \times 10^6$.

Some enterotoxigenic strains of Escherichia coli produce specific surface antigens that are responsible for adhesion to intestinal epithelial cells. Colonization factor antigen I (CFA/I) and CFA/II have been identified in enterotoxigenic strains isolated from humans with diarrhea (6, 7, 11, 13, 15, 17, 32). K88 and 987P antigens are found in strains of porcine origin (30, 34), and K99 antigen has been identified in E. coli strains from calves and lambs (33). These antigens are serologically distinct fimbrial structures that can be detected by mannose-resistant hemagglutination (MRHA) of different types of erythrocytes (12, 21, 32, 33). Type 1 fimbriae are also produced by many E. coli strains (10); these fimbriae can be detected by mannose-sensitive hemagglutination of guinea pig erythrocytes and also differ serologically from the other fimbriae described above.

The syntheses of K88, K99, CFA/I, and CFA/II are determined by plasmids (13, 31, 35, 40). The presence of CFA/I in strain H10407, serotype O78:H11, was correlated with a plasmid having a molecular weight of $60 \times 10^6$ (13). Further studies have shown that in several strains belonging to serogroup O78, including strain H10407, a single plasmid codes for production of CFA/I and heat-stable enterotoxin (ST) and that a CFA/I-ST plasmid, NTP113, is mobilized by R-factor R1-19K- to E. coli K-12 from one of these strains (39). Mobilization of CFA/I-ST plasmids to E. coli K-12 has now been demonstrated with several other strains of serogroup O78 (24) and also with a strain belonging to serotype O128:H12 (36). In addition, a plasmid coding for the production of heat-labile enterotoxin (LT), as well as CFA/I and ST, has been mobilized from a strain of O63:H-; the genetic properties of the CFA/I-ST plasmids and the CFA/I-ST-LT factor are very similar (24).

We characterized the CFA/I-ST plasmid NTP113 which was mobilized from strain E7473, serotype O78:H12, in more detail in order to identify the regions that coded for production of CFA/I and ST. We examined NTP113 and mutants obtained by insertion of transposons or deletion by restriction enzyme analysis. In this paper we describe a restriction map of NTP113; it appears that two regions of the plasmid are required for expression of CFA/I.

MATERIALS AND METHODS

Bacterial strains and plasmids. The E. coli K-12 strains used in the conjugation and transposition experiments were strains 14R519 (K-12 F- lac pro trp his Nalr), 21R868 (K-12 F- lac+ Strr), 14R525 (K-12 F- lac+ Nafr), and AB1157. The non-autotransferring CFA/I-ST plasmid NTP113 was mobilized by the R factor R1-19K- from E. coli E7473, serotype O78:H12, which was isolated in South Africa (39). The genetic properties of NTP113 and the other CFA/I-ST plasmids have been described previously (24). The R-
factor R1-16 (28) and the heat-labile enterotoxin plasmid TP236-Tc (25) were also used to mobilize non-autotransferring plasmids. R1-19K codes for resistance to ampicillin (Ap), chloramphenicol (Cm), streptomycin (Sm), and sulfathiazole (Su). R1-16 codes for kanamycin resistance (Km), and TP236-Tc codes for tetracycline resistance (Tc).

Resistance to antibacterial agents. Strains were tested for drug resistance as described previously (1).

Enterotoxin tests. ST was detected by the infant mouse assay (9).

Hemagglutination and immunodiffusion tests. The presence of CFA/I was determined by MRHA of human or bovine erythrocytes and by immunodiffusion with a specific CFA/I antiserum, as described previously (6, 24). Strains tested only by hemagglutination are referred to below as MRHA+ or MRHA-, whereas strains examined for CFA/I by immunodiffusion tests in addition to hemagglutination tests are referred to as CFAI+ or CFAI-.

Conjugation experiments. (i) Direct transfer. Nutrient broth cultures of the donor and recipient strains (approximately 2 × 10^8 cells per ml) were mixed in equal volumes, and the mixtures were incubated overnight at 37°C. Mating mixtures were plated onto MacConkey agar containing the appropriate drugs.

(ii) Mobilization of non-autotransferring plasmids. Broth cultures of an E. coli K-12 strain carrying the R factor used for mobilization and a K-12 strain carrying the non-autotransferring plasmid were mixed in equal volumes, and the mixtures were incubated overnight at 37°C; 1 ml of a culture of the final K-12 recipient was added to 2 ml of the mating mixture. After overnight incubation, the mating mixture was plated onto drug-containing MacConkey agar.

Preparation of plasmid DNA. (i) Partially purified DNA. Plasmid-carrying strains of E. coli K-12 were grown in 50-ml volumes of nutrient broth. The bacteria were lysed with a mixture of Brij 58 and sodium deoxycholate (5), and plasmid DNA was partially purified by phenol extraction of cleared lysates (26). Crude plasmid DNA for restriction enzyme digestions was obtained from cleared lysates that were treated with 1% (vol/vol) diethylpyrocarbonate. After 20 min at 65°C, denatured protein was removed by centrifugation, and the plasmid DNA was precipitated with ethanol in the presence of 0.2 M NaCl at -10°C for 16 h.

(ii) Pure plasmid DNA. Bulk preparations of pure plasmid DNA from NTP113 or its derivatives were obtained from 500-ml cultures. Plasmid DNA was precipitated from cleared lysates with polyethylene glycol 6000 and purified by cesium chloride-ethidium bromide dye-buoyant density gradient centrifugation (20).

Treatment with restriction endonucleases. Pure or partially purified plasmid DNA preparations were dialyzed for 36 h against 0.01 M Tris-hydrochloride (pH 8.0) containing 0.001 M EDTA. Digestions with restriction enzymes EcoRI, HindIII, and BamHI (5 U; Boehringer or Bethesda Research Laboratories) were performed in 40-μl volumes at 37°C for 5 to 6 h in the reaction mixtures recommended by the manufacturer.

DNA was digested with two enzymes, it was treated first with the enzyme that required the lower ionic strength. Reactions were halted by incubation at 65°C for 10 min; 5 μl of a solution containing 60% (wt/vol) sucrose and 0.25% (wt/vol) bromophenol blue was added to each mixture before gel electrophoresis.

Agarose gel electrophoresis. The molecular weights of NTP113 derivatives were determined by electrophoresis of partially purified plasmid DNA preparations on vertical slab gels containing 0.6% (wt/vol) agarose (type II; Sigma Chemical Co.) (44). These gels were run for 4 h at 140 V in 89 mM Tris buffer (pH 8.0) containing 89 mM boracic acid and 2.5 mM EDTA. The fragments produced by restriction endonuclease treatments were analyzed as described previously (43) on 0.7% or 1% agarose gels run for 15 to 18 h in pH 7.9 buffer containing 40 mM Tris, 5 mM sodium acetate, and 1 mM EDTA. The molecular weights of these fragments were determined by comparison with the molecular weights of EcoRI- or HindIII-generated fragments of phage λ DNA.

For further analysis of restriction fragments, digested plasmid DNA was subjected to electrophoresis in low-gelling-temperature (LGT) agarose (Marine Colloids), as described above. Partially purified plasmid DNA (1 to 3 μg) was digested at 37°C for 5 h, and the products were separated on vertical tube gels (120 by 6 mm) containing 0.7% LGT agarose. The gels were run for 15 to 18 h at 0.75 mA/gel in the Tris-acetate buffer described above containing 0.5 μg of ethidium bromide per ml. Gel slices containing DNA fragments were melted in 1.5-ml Eppendorf centrifuge tubes, and the DNA was digested with a second restriction enzyme. The mixtures were loaded onto 1 or 1.4% (wt/vol) horizontal agarose gels (type II; Sigma), and these secondary gels were run in Tris-acetate-EDTA buffer containing ethidium bromide at 25 V for 16 h.

Insertion of transposons into the CFA/I-ST plasmid NTP113. (i) Ampicillin resistance transposon Tn4. The F-T plasmid (2) carrying Tn4 was used for transposition of ampicillin resistance. This Tn4 was derived from non-autotransferring plasmid NTP21 (26). F-T-Ap was transferred into K-12(NTP113), and transconjugants were tested for MRHA and drug resistance. MRHA+ApTcTc' strains were tested with acridine orange (75 μg/ml) (16), and cultures were plated onto media containing 100 μg of ampicillin per ml. Colonies were tested by replica plating for the loss of tetracycline resistance. Strains that were Ap Tc' and still MRHA* were first tested for direct transfer of drug resistance, and if the strains were positive, they were discarded since the CFA/I-ST plasmid NTP113 is non-autotransferring. Strains showing no direct transfer of ampicillin resistance were tested for mobilization by using R1-16. If Tn4 had transposed onto CFA/I-ST plasmid NTP113, there should have been 100% linkage of MRHA and Ap markers in further mating experiments when the resulting plasmid (NTP113-Ap) was mobilized by R1-16. We selected strains in which the mobilization tests indicated that Tn4 had been transposed onto NTP113.

(ii) Kanamycin resistance transposon Tn5. We used plasmid F' proAB lac: Tn5 (22) for transposition of Tn5 (3); this plasmid was transferred into E. coli K-12 carrying NTP113 and F-T-Ap. F' proAB lac Tn5 and F-T-Ap are incompatible plasmids that joint selection for Km and Ap markers may result in recombination between the two F' factors or in transposition of either Tn5 or Tn4 onto NTP113. The mating mixture was plated onto a medium containing ampicillin and kana-
mycin, and transconjugants were tested for ampicillin, kanamycin, and tetracycline resistance. Strains that were MRHA Ap Km Tc were mated for 1 h with E. coli K-12 to test for linkage of MRHA and the resistance markers. If Tn5 had transposed onto NTP113, transconjugants carrying this plasmid would be kanamycin resistant and cause MRHA. Strains that were MRHA Ap Km were selected and tested for direct transfer or mobilization. MRHA Km transconjugants in which kanamycin resistance was not transferred directly but could be mobilized by R1-K9 were analyzed further.

(ii) Insertion of a second transposon into derivatives of NTP113. NTP119 is a derivative of NTP113 which carries Tn5 (see Table 3). This plasmid was used to obtain additional derivatives carrying TnA or Tn10, which codes for tetracycline resistance (14).

NTP119 was mobilized by TP236-Tc into an E. coli K-12 strain carrying TnA on the bacterial chromosome. This transposon was derived from non-auto-transferring plasmid NTP21. The resulting strains were mated with K-12, and transconjugants were selected on a medium containing ampicillin and kanamycin; the frequency was usually between 10^{-6} and 10^{-8}. If TnA had transposed onto NTP119, the resulting plasmid should have coded for ampicillin resistance and kanamycin resistance. NTP119-Ap derivatives were identified, and 20 of these derivatives were used in further studies. Similar experiments were performed with a K-12 strain carrying Tn10 on the chromosome; the R factor R1-K9 was used to mobilize NTP119 and the NTP119-Tc derivatives. Transconjugants that were resistant to kanamycin and tetracycline were obtained at frequencies of 10^{-6} to 10^{-8}, and 34 NTP119-Tc derivatives were used in further tests.

Strains carrying derivatives of NTP113 with different transposons were tested for MRHA and for ST production by using the infant mouse assay.

Formation of deletion mutants. Deletions of plasmid DNA result from excision of Tn10 (23). Three strains carrying NTP119 with different Tn10 insertions were examined. Cultures were grown in nutrient broth, and suitable dilutions were plated onto nutrient agar containing 20 μg of kanamycin per ml. Colonies were examined for the loss of tetracycline resistance by replica plating. Strains which were Km Tc were tested for MRHA and ST production. K-12 strains carrying NTP119-Tc derivatives were also examined for the loss of kanamycin resistance and the retention of tetracycline resistance.

Strategy for mapping NTP113 and its derivatives. Initially, NTP113 was digested with EcoRI, BamHI, and HindIII separately and with combinations, and the sizes of the restriction fragments were measured. From double digestions we deduced the presence of sites for a second enzyme within the fragments generated by the first enzyme. The location of a cleavage site(s) within a particular fragment was then obtained by digesting the fragment after excision from LGT agarose. Similar experiments were then performed with NTP113 mutants obtained by inserting drug resistance transposons containing known sites for BamHI, EcoRI, or HindIII. The positions of the transposon restriction sites within the restriction fragments of NTP113 were determined, and these inserted sites provided markers with which neighboring restriction sites of NTP113 were located. Combining all of these results permitted conclusions on the ordering and orientation of the BamHI fragments and most of the EcoRI fragments and on the positions of those HindIII fragments that contained EcoRI or BamHI sites. Finally, we performed a restriction analysis on derivatives obtained after deletion of drug resistance transposons in order to determine the regions of NTP113 DNA that had been lost and to provide further evidence to order the fragments.

Electron microscopy. Strains were grown and prepared for electron microscopy as described previously (24).

RESULTS

Restriction enzyme analysis of NTP113. The genetic properties of CFA/I-ST plasmid NTP113 have been described previously (24). This plasmid (molecular weight, 58 × 10^6) has 3 cleavage sites for BamHI, at least 9 EcoRI sites, and probably more than 17 HindIII sites (Tables 1 and 2). We investigated the relationships of these sites by digesting NTP113 with combinations of these three restriction endonucleases and fractionating the products on 0.7 or 1.0% agarose gels. In addition, fragments larger than 2 × 10^6 generated from primary digestions separated on 0.7% LGT agarose were excised from the gels and subjected to treatment with a second enzyme. These secondary digestions were run on horizontal 1 or 1.4% agarose gels. The results of these experiments are summarized in Tables 1 and 2.

BamHI sites were located in EcoRI fragments E1, E3, and E8 (Table 1). Among the products of double digestions with these enzymes, five fragments (designated EB2, EB4, EB5, EB6, and EB8) corresponded to the primary EcoRI fragments E2, E4, E5, E6, and E7; these contained no BamHI sites and therefore arose as internal fragments when primary BamHI fragments were redigested with EcoRI (Table 1). LGT agarose analysis showed that fragment EB9 (molecular weight, 0.9 × 10^6) comprised two comigrating EcoRI-BamHI fragments (EB9a, derived from EcoRI fragment E3, and EB9b, derived from EcoRI fragment E8).

Treatment of NTP113 DNA with HindIII and BamHI together showed that BamHI sites were present in HindIII fragments H4 and H8. After excision from LGT agarose, HindIII fragment H4 was cleaved with BamHI to give fragments having molecular weights of 2.0 × 10^6 and 2.2 × 10^6, and HindIII fragment H8 yielded fragments having molecular weights of 2.6 × 10^6 and 0.3 × 10^6. The HindIII fragment containing the third BamHI site was not identified. It may have been smaller than HindIII fragment H17 and thus not recovered on gels; alternatively, the third BamHI site was located so close to a HindIII...
<table>
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<tr>
<th>Primary EcoRI fragment</th>
<th>Products formed by BamHI digestion</th>
<th>Primary BamHI fragment</th>
<th>Products formed by EcoRI digestion</th>
<th>Double digestion fragment</th>
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<td>B1</td>
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<td>EB9b, EB11$^e$</td>
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<td>0.5</td>
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<tr>
<td>E11</td>
<td>—$^e$</td>
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$^a$ Data from secondary digestions of primary fragments excised from LGT agarose gels and from double digests of NTP113 DNA.

$^b$ Fragments are numbered in decreasing order of size.

$^c$ Molecular weights were measured relative to fragments of lambda phage digested with HindIII or HindIII plus EcoRI.

$^d$ An equivalent fragment was present in the primary EcoRI digest, as it lacked a BamHI site.

$^e$ EB11 was the postulated smaller fragment which resulted from digestion of EcoRI fragment E8 with BamHI. Because of its small size this fragment was never located or measured on our gels.

$^f$ EB9 was composed of two nonidentical but comigrating fragments, EB9a and EB9b.

$^g$ ND, Not determined.
TABLE 2. Relative locations of EcoRI and HindIII fragments of NTP113a

<table>
<thead>
<tr>
<th>Designation</th>
<th>Mol wt (10^6)</th>
<th>Products of EcoRI treatment</th>
<th>Primary EcoRI fragment</th>
<th>Designation</th>
<th>Mol wt (10^6)</th>
<th>Mol wt of products of HindIII treatment (10^6)</th>
<th>HindIII fragments containing EcoRI fragment termini</th>
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<tr>
<td>H1</td>
<td>15</td>
<td>6.2, 5.5, 1.4, 1.7</td>
<td>E2, E6, E7, E3</td>
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a Data from secondary digestions of primary restriction fragments excised from 0.7% LGT agarose gels and from double digestions of NTP113 and NTP128 (Fig. 1 and 4) with HindIII and EcoRI.

b Fragments were numbered in decreasing order of size. HindIII fragments H4 and H8 contained cleavage sites for BamH1.

c Molecular weights were measured relative to fragments of phage λ DNA digested with HindIII or HindIII plus EcoRI.

d NS, No cleavage site detected.

e Intense bands on agarose gels probably indicated the presence of at least two fragments of this size.

f The presence of an EcoRI or HindIII site was deduced from double digestion of NTP113. The sizes of the products were not determined.
site that the mobility of the HindIII fragment which contained it was not altered detectably after digestion with both restriction enzymes. It is also possible that the site was in a small HindIII fragment which comigrated with another fragment; in this case it would have been difficult to detect a difference between HindIII digests and HindIII-BamHI digests.

Because of the relatively large number of HindIII sites, we made no attempt to order these sites completely. However, we examined the fragments for the presence of EcoRI sites to determine the positions of the HindIII cleavage sites around the EcoRI sites (Table 2). The largest HindIII fragment (fragment H1) contained three EcoRI sites and yielded two internal fragments that were indistinguishable in size from EcoRI fragments E6 and E7. These EcoRI fragments contained no HindIII sites (Table 2), a finding consistent with location within HindIII fragment H1.

Digestion of NTP113 with HindIII and EcoRI indicated that HindIII fragments H4, H6, H7, H8, and H12 also contained EcoRI sites, and the positions of these sites within HindIII fragments H4, H6, H7, and H8 were determined by secondary digestion of excised fragments (Table 2). HindIII sites were present in EcoRI fragments E1, E2, E3, E4, E5, and E8; Table 2 shows the molecular weights of the products of HindIII digestion of these fragments. An examination of all of these data resulted in the conclusions shown in Table 2 concerning the relative locations of the EcoRI and HindIII sites of NTP113. EcoRI fragment E8 and HindIII fragment H12 were not examined by the LGT agarose method, and the derivation of these fragments was inferred from treatment of NTP113 with combinations of HindIII, EcoRI, and BamHI. This was confirmed by a similar examination of NTP128, a deletion mutant which had a molecular weight of $32 \times 10^6$ and was derived from NTP113 (see below). This plasmid contained HindIII fragments H8 and H12 and EcoRI fragment E8 but was simpler to analyze because of its smaller size. From BamHI digestion of HindIII fragments of NTP128, we concluded that HindIII fragment H8 was derived from BamHI fragments B1 and B2 of NTP113. Further analysis of HindIII fragment H4, EcoRI fragments E3 and E4, and EcoRI-BamHI fragment EB3 of NTP113 with LGT agarose showed that HindIII fragment H4 was derived from BamHI fragments B1 and B3.

We studied the structure of plasmid NTP113 further by using derivatives obtained by insertion of transposons TnA, Tn5, and Tn10.

**Mutants obtained by transposition. (i) Derivatives of NTP113 carrying Tn5 or TnA.** NTP119 (Table 3) was formed by transposition of Tn5 onto NTP113, as described above; this plasmid coded for MRHA and ST production and had a molecular weight of $62 \times 10^6$. NTP119 has been used previously in genetic studies of CFA/I-ST plasmids (24).

NTP120 and NTP121 were obtained by transposition of TnA onto NTP113 (Table 3). Both of these plasmids coded for ST production, but strains carrying NTP121 were MRHA negative. Immunodiffusion tests confirmed that CFA/I was not produced by K-12(NTP121). NTP119, NTP120, and NTP121 were analyzed further to locate the positions of insertion of Tn5 and TnA.

Both Tn5 and TnA contain single cleavage sites for BamHI (22, 37). In both NTP119 and NTP120, insertion of the transposon occurred into EcoRI fragment E3 of NTP113 (Table 3 and Fig. 1). The plasmid derivatives produced four BamHI fragments, two of which were equivalent in size to BamHI fragments B2 and B3 of NTP113. NTP119 also gave a fragment having a molecular weight of $11 \times 10^6$ and one which comigrated with BamHI fragment B2, whereas NTP120 yielded fragments having molecular weights of $7 \times 10^6$ and $>14 \times 10^6$. NTP121, which did not code for MRHA, was derived from NTP113 by insertion of TnA into EcoRI fragment E5 (Table 3 and Fig. 1), an event which generated a new BamHI fragment that had a molecular weight of $2.4 \times 10^6$ and as shown by LGT studies, had an EcoRI site very close to one end. The other BamHI fragments of NTP121 could not be distinguished on the basis of size from the BamHI fragments of NTP113. A comparison of HindIII digests of mutant and parent plasmids showed that in NTP121 the site of insertion was within HindIII fragment H8, a fragment which had a molecular weight of $2.7 \times 10^6$ and contained BamHI and EcoRI sites of NTP113 (see above). We concluded that the EcoRI site in HindIII fragment H8 was the site at one end of EcoRI fragment E5 and that the $2.4 \times 10^6$ BamHI fragment of NTP121 was formed from the BamHI site in TnA and the site in HindIII fragment H8. The EcoRI fragment containing the BamHI site of HindIII fragment H8 was EcoRI fragment E8, a fragment that had a molecular weight of approximately $10^6$ and a BamHI site within $0.2 \times 10^6$ of one end. The latter requirement was necessary to explain the internal structure of the $2.4 \times 10^6$ BamHI fragment of NTP121. Results from digestions with combinations of EcoRI, BamHI, and HindIII and from LGT agarose studies were consistent with a structure in which EcoRI fragments E5 and E8 were contiguous.

**Derivatives of NTP119 carrying TnA.** TnA was inserted into NTP119, and strains carrying the derivatives from this procedure were tested for MRHA and ST production. Of the 20 strains...
examined, 19 were ST⁺ MRHA⁺, and 1 was ST⁻ MRHA⁻. Two of the plasmids from these experiments were examined in more detail (Table 3).

Both NTP135, which coded for ST and MRHA, and NTP132, which coded for only ST, carried Tn5 as it was located in NTP119. Insertion of TnA into both NTP135 and NTP132 occurred within EcoRI fragment E5 (Table 3 and Fig. 1); for NTP132 this insertion site was in HindIII fragment H8, but in NTP135 transposition probably occurred into a small HindIII fragment of <1 × 10^5 in EcoRI fragment E5. Thus, in two independent mutants (NTP121 and NTP132) transposition of TnA resulting in the loss of CFA/I occurred in HindIII fragment H8, suggesting that this was a region which controlled CFA/I production (designated region 1).

(iii) Derivatives of NTP119 carrying Tn10. We also used transposition of Tn10 onto NTP119 in an attempt to obtain mutants which coded for production of ST alone or MRHA. K-12 strains carrying NTP119-Tn10 derivatives were tested for MRHA and ST production. Of the 34 strains examined, 33 were ST⁺ MRHA⁺, and 1 was ST⁻ MRHA⁻. Three of the plasmids which carried both Tn5 and Tn10 were examined in more detail (Table 3; see below).

Plasmids NTP122, NTP123, and NTP124 (Table 3) each contained a copy of Tn5 in the same position as on NTP119. Transposition of Tn10 into NTP119 to yield NTP122 and NTP124 occurred at different sites within EcoRI fragment E4. In NTP122 Tn10 inserted into HindIII fragment H4, a fragment having a molecular weight of 4.2 × 10^6; from double digestions and treatment of the excised fragment with EcoRI or BamHI, we determined that HindIII fragment H4 spanned the junction of EcoRI fragment E3 and E4 and contained the BamHI site of EcoRI fragment E3 (Tables 1 and 2). Tn10 was not present in HindIII fragment H4 from NTP124 but probably was present in a small HindIII fragment contained in EcoRI fragment E4. NTP124 coded for MRHA, whereas NTP122 did not. The third plasmid examined, NTP123, was derived by insertion of Tn10 into EcoRI fragment E3 and encoded both ST production and MRHA.

However, unlike NTP123 and NTP124,
NTP122 appeared to carry a second copy of the Tn5 transposon within EcoRI fragment E2. Therefore, this plasmid gave six BamHI fragments, rather than the five fragments of NTP123 and NTP124, which could be attributed to the presence of single copies of Tn5 and Tn10. The extra BamHI site in EcoRI fragment E2 resulted in a BamHI fragment that had a molecular weight of $9 \times 10^6$ and an EcoRI site approximately $0.9 \times 10^6$ from one end. An examination of the relative positions of the BamHI and EcoRI sites in NTP113 (Table 1) suggested that one end of the $9 \times 10^6$ BamHI fragment of NTP122 was in EcoRI fragment E8, the EcoRI fragment that gave rise to an EcoRI-BamHI fragment of $0.9 \times 10^6$. We concluded that EcoRI fragments E8 and E2 were contiguous. Since NTP122 was derived from NTP119 by two transposition events, it was not possible at this stage to decide which insertion resulted in the MRHA\(^+\) mutant phenotype conferred by NTP122.

**Restriction map of NTP113.** From the detailed analysis of the digestion products of the insertion mutants shown in Table 3 we constructed a partially complete restriction map of NTP113 (Fig. 2). The positions of EcoRI fragments E6, E7, and E9 could not be deduced with certainty because no insertions into these fragments were obtained. EcoRI fragments E6 and E7 are shown between EcoRI fragments E2 and E3 since this location is consistent with the sizes of the BamHI fragments of NTP113 and NTP122. EcoRI treatment of HindIII fragment H1 excised from LGT agarose yielded internal fragments that were indistinguishable in size from EcoRI fragments E6 and E7, and it is likely that these fragments may be contiguous, although their orientations are unknown. Further evidence for the locations of these fragments was obtained from an analysis of deletion mutants (see below) which were obtained by deletion of transposons Tn10 and Tn5 from NTP113 derivatives.

**Mutants obtained by deletion.** (i) Loss of Tn10. Strains carrying NTP122, NTP123, and NTP124 were tested for loss of tetracycline resistance; this occurred at a frequency of about 0.2%. Tc\(^+\) lines which were still kanamycin resistant were tested for MRHA and ST production. A number of the plasmid derivatives were examined in detail (Table 4); some of the Tc\(^+\) strains were MRHA\(^-\), but all of these derivatives were still ST\(^+\).

We obtained three derivatives of NTP123 which had lost all or part of Tn10, and two of these (NTP125 and NTP126) did not code for MRHA. These plasmids had molecular weights of $40 \times 10^6$ and $48 \times 10^6$, respectively, and the amounts of DNA deleted in the formation of these plasmids are shown in Fig. 3. In both cases, excision of Tn10 from EcoRI fragment E3 removed DNA extending through EcoRI fragments E4 and EB7. However, in the formation of NTP130, which still coded for MRHA, the loss of the Tc marker from NTP123 was accompanied by excision of DNA from EcoRI fragment E3 only to yield a plasmid having a molecular weight of $64 \times 10^6$.

Strains carrying NTP124 were MRHA\(^+\), but after the loss of Tn10 from EcoRI fragment E4, both MRHA\(^+\) and MRHA\(^-\) clones were obtained; these clones carried NTP129 and
TABLE 4. Properties and molecular weights of the plasmid mutants obtained by the loss of tetracycline resistance or kanamycin resistance

<table>
<thead>
<tr>
<th>Parent plasmid</th>
<th>Mutation</th>
<th>Plasmid derivative</th>
<th>Properties</th>
<th>Mol wt (10^6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NTP123</td>
<td>None</td>
<td>NTP125</td>
<td>CFA/I+ ST+ Km Tc</td>
<td>68</td>
</tr>
<tr>
<td></td>
<td>Loss of Tc</td>
<td>NTP126</td>
<td>CFA/I+ ST+ Km</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td>Loss of Tc</td>
<td>NTP130</td>
<td>CFA/I+ ST+ Km</td>
<td>64</td>
</tr>
<tr>
<td>NTP124</td>
<td>None</td>
<td>NTP127</td>
<td>CFA/I+ ST+ Km Tc</td>
<td>68</td>
</tr>
<tr>
<td></td>
<td>Loss of Tc</td>
<td>NTP129</td>
<td>CFA/I+ ST+ Km</td>
<td>52</td>
</tr>
<tr>
<td></td>
<td>Loss of Tc</td>
<td>NTP137</td>
<td>CFA/I+ ST+ Km</td>
<td>48</td>
</tr>
<tr>
<td>NTP122</td>
<td>None</td>
<td>NTP128</td>
<td>CFA/I+ ST+ Km</td>
<td>32</td>
</tr>
<tr>
<td>NTP135</td>
<td>None</td>
<td>NTP138</td>
<td>CFA/I+ ST+ Ap</td>
<td>66</td>
</tr>
</tbody>
</table>

NTP127, respectively. In the formation of NTP129, deletion of DNA occurred toward the “right” within EcoRI fragment E4 and extended into EcoRI fragment E1, removing the BamHI site of that fragment; in NTP127, deletion of Tn10 abolished the region around the junction of EcoRI fragments E3 and E4 (Fig. 1 and 3). A comparison of the extents of the deletions from NTP123 and NTP124 suggested that the capacity to determine MRHA was associated with the region of EcoRI-BamHI fragment EB9a around the junction of EcoRI fragments E3 and E4 (designated region 2). This region contained HindIII fragment H4, which was implicated as a possible site controlling CFA/I production based on an analysis of NTP122.

The loss of the tetracycline resistance marker from NTP122 resulted in a large deletion of DNA and yielded NTP128, a plasmid having a molecular weight of 32 × 10^6. This plasmid contained parts of EcoRI fragments E1 and E2 (the latter carrying a copy of Tn5 derived from NTP122) and also complete EcoRI fragments E5 and E8 (Fig. 1, 2, and 4). Therefore, this derivative, although MRHA-, still possessed one of the regions involved in CFA/I production (region 1) and also coded for ST.

(ii) Loss of Tn5. The loss of kanamycin resistance by strains carrying NTP124 or NTP135 occurred at a very low frequency (<0.1%). NTP124 yielded the derivative NTP137, which coded for tetracycline resistance but not for MRHA or ST production; this derivative had a molecular weight of 30 × 10^6. Restriction en-

FIG. 3. Approximate extents of the deletions resulting from the loss of the tetracycline resistance marker of NTP123 or NTP124. This figure shows the regions of these plasmids containing EcoRI fragments E3, E4, and E1 (see Fig. 2). Both plasmids carried a copy of Tn5 in EcoRI fragment E3; in NTP123, Tn10 was also inserted into EcoRI fragment E3, whereas Tn10 was present in NTP124 within EcoRI fragment E4. The internal EcoRI and BamHI sites contained in these transposons are designated Er or Br to distinguish them from NTP113 sites. Only one HindIII fragment (fragment H4), is shown. For each derivative, the DNA region that was deleted is indicated by a horizontal bar beneath the parental structure. E, EcoRI site; B, BamHI site; H, HindIII site.
zyme digestion showed that NTP124 had suffered a deletion extending anticlockwise from the TnI0 insertion in EcoRI fragment E4 (Fig. 2 and 4) to a point somewhere within EcoRI fragment E5; EcoRI-BamHI fragments EB1 and EB7 were present intact. This plasmid contained neither of the putative CFA/I regions. Plasmid NTP138 resulted from a loss of kanamycin resistance by NTP135 (Table 4); this derivative coded for ampicillin resistance but not for MRHA or ST production. NTP138 was the smallest spontaneously occurring deletion derivative (molecular weight, $11 \times 10^6$) and contained TnA, which was inserted into EcoRI fragment E5 of the parent plasmid (NTP135) (Table 3). HindIII treatment of NTP138 yielded a fragment that was indistinguishable in size from HindIII fragment H7 of NTP113 and contained an EcoRI site corresponding to one end of EcoRI fragment E5 (Fig. 4).

Complementation between CFA/I mutants. We tested a plasmid with a mutation that mapped in CFA/I region 1 for complementation with another plasmid derivative with a mutation which mapped in CFA/I region 2. NTP128 (CFA/I-ST + Km) was mobilized by TP236-Tc into K-12 carrying NTP121 (CFA/I-ST + Ap). The mating mixture was plated onto media containing ampicillin and kanamycin, and colonies were tested for MRHA. All 10 colonies tested showed MRHA with human erythrocytes, although the reaction was weak in most cases. Four strains from this cross were grown in drug-free nutrient broth and were tested for the stability of their ampicillin resistance and kanamycin resistance. Since NTP128 and NTP121 were both derivatives of NTP113, they should have been incompatible, and segregation tests showed losses of ampicillin resistance (20%) and kanamycin resistance (45%), as expected.

Electron microscopy of strains carrying colonization factor antigen mutants. We examined E. coli K-12 strain 1R519 carrying NTP121 or NTP127 for the presence of fimbriae by electron microscopy after the strain was grown on CFA agar (12). Of 211 K-12(NTP121) organisms, 208 (98.5%) were nonfimbriate; in the case of K-12(NTP127), 96.5% of the organisms (171 of 177) were nude. Cultures of the two strains were negative in hemagglutination tests for type 1 fimbriae when guinea pig erythrocytes were used. However, the few cells of K-12(NTP121) and K-12(NTP127) which were fimbriate probably possessed type 1 fimbriae, as observed previously with CFA/I-negative strains (24, 42).

**DISCUSSION**

Previous studies have shown that in several enterotoxigenic E. coli strains a single plasmid codes for production of CFA/I and ST (24, 36, 39). In this investigation we constructed a restriction map of the CFA/I-ST plasmid NTP113 and showed that two regions of the plasmid are required for expression of CFA/I. Electron microscopy confirmed that strains carrying plasmids with mutations in either region do not produce fimbriae. We do not know whether both regions include structural genes for CFA/I fimbriae or whether one of them possesses a control gene(s) which is necessary for expression.

The role of CFA/I in producing disease has been demonstrated in volunteer experiments with strain H10407. The derivative H10407-P, which did not produce CFA/I or ST but still produced heat-labile enterotoxin, did not cause diarrhea in these experiments (38). It would be useful to obtain a derivative of a CFA/I-ST plasmid which does not code for ST production so that the effect of CFA/I alone could be examined. This type of mutant was not obtained in this study, although we did isolate mutants of NTP113 which did not code for CFA/I or ST production. The ST gene may map very closely to CFA/I region 1, so that deletion mutants that affect ST also result in the loss of CFA/I production.

It may be possible to obtain derivatives that
code for only CFA/I by cloning techniques. Genes coding for enterotoxin production or adhesive factors on a number of different plasmids have been cloned onto multicopy vector plasmids [8, 29, 41]. In the case of CFA/I this is complicated by the presence of two regions which are required for the expression of CFA/I, and these have to be cloned separately.

Strains carrying NTP128 were ST+; whereas strains carrying NTP137 were ST−; both of these plasmids were about one-half the size of NTP113 (Fig. 2). The extent of overlap of the deleted DNA indicated that the region coding for ST was located in a segment of the molecule extending clockwise from within EcoRI fragment E5, through EcoRI fragment E8, and into EcoRI fragment E2. This part of the plasmid, corresponding to a molecular weight of \(12 \times 10^6\) to \(15 \times 10^6\), includes CFA/I region 1 and its neighboring DNA. The ST gene from an E. coli strain of bovine origin has been cloned and is on a transposable element (Tn681) that is flanked by IS1 (41). This ST is positive in the infant mouse assay and therefore is the STa or ST1 type (4, 18). The ST encoded by NTP113 is also positive in the mouse test (24), but it is not known whether this plasmid carries a transposon like Tn681. Because of the presence of PstI sites in the inverted repeats of IS1, the majority of Tn681 is carried on a 1.7-kilobase PstI fragment (41). A PstI digest of NTP128, the smallest derivative of NTP113 which coded for ST, was compared with a similar digest of a plasmid containing Tn681 (41). Although the 1.7-kilobase fragment was identified clearly in the control, there was no precisely equivalent comigrating fragment in NTP128 (unpublished data). Further studies with specific hybridization probes will be required to identify and locate the ST gene in CFA/I-ST plasmid NTP113.

The deletion mutants NTP128 (molecular weight, 32 \(\times 10^6\)) and NTP137 (30 \(\times 10^6\)) both contain a region of NTP113 of about 15 \(\times 10^6\) which extends anticlockwise from within EcoRI fragment E5 and includes a large portion of EcoRI fragment E1 (Fig. 2). The smallest naturally occurring NTP113 derivative, NTP138 (molecular weight, 11 \(\times 10^6\)), also contains DNA from around the junction of EcoRI fragments E5 and E1. Therefore, the replication genes of NTP113 may be located in this region of the molecule.

Further mapping studies of NTP113 and some of the derivatives obtained in this study should result in a more precise identification of the regions associated with CFA/I, ST, replication, and other properties encoded by the plasmid. In particular, we plan to use cloning techniques to obtain derivatives which only code for CFA/I production so that the expression of the adhesion system can be examined in more detail.

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LITERATURE CITED


