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Transposition of Ampicillin Resistance to an Enterotoxin Plasmid in an *Escherichia coli* Strain of Human Origin

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We examined a strain of *Escherichia coli*, serotype O159.H34, of human origin which produced heat-stable and heat-labile enterotoxins, was resistant to ampicillin, and produced colicin. By conjugation and transformation experiments plasmids coding for enterotoxin production (Ent), enterotoxin production and ampicillin resistance (Ap-Ent), ampicillin resistance (Ap), and colicin production were isolated. Both the Ent and Ap-Ent plasmids were autotransferring and belonged to the F-incompatibility complex. However, the Ap^r Ent^+ transconjugants showed differences in their levels of resistance and in their abilities to propagate F-specific phages and to transfer resistance. The results suggested there was transposition from the small Ap plasmid to the Ent plasmid. The Ap-Ent plasmids were larger than the enterotoxin factor and when treated with restriction endonuclease *Bam*HI showed an additional fragment not present in the enterotoxin plasmid. The insertion of ampicillin resistance probably occurred at different sites on the enterotoxin plasmid, resulting in the observed variation in phenotype.

Enterotoxin production in certain strains of *Escherichia coli* is controlled by plasmids (22, 38). Some of these enterotoxin (Ent) plasmids have been shown to be similar to resistance (R) factors; they are autotransferring (22, 38) and belong to the F-incompatibility complex (34, 39). Enterotoxigenic strains have also been isolated which are multiply resistant to antibiotics (10, 11, 21, 22, 30, 35), and this has been shown to be R-factor controlled in *E. coli* of porcine and human origins (11, 21, 22). Since antibiotic resistance genes are able to move from genome to genome by an illegitimate recombination event called transposition (7, 24), it is possible that in strains carrying both Ent and R factors such a transfer will take place and a single plasmid coding for enterotoxin production and drug resistance will be formed. A plasmid of this type has been constructed in the laboratory by transposition of ampicillin resistance from a non-autotransferring plasmid to the Ent factor P307 (40). A naturally occurring enterotoxin-drug resistance plasmid has also been reported; this was transferred from an enterotoxigenic strain of *E. coli* isolated from a piglet and coded for both heat-labile enterotoxin (LT) and heat-stable enterotoxin (ST) production as well as resistance to streptomycin (Sm), sulfonamides (Su), and tetracycline (Tc) (21). In vivo transfer of this Ent plasmid from the porcine *E. coli* to an *E. coli* K-12 strain in the intestine of newly weaned pigs has been shown to occur (20). Enterotoxin-drug resistance plasmids may be important in increasing the number of wild-type *E. coli* able to produce enterotoxins, since any transfer of antibiotic resistance would result in the cotransfer of enterotoxin production.

During a survey of enterotoxigenic *E. coli* strains for antibiotic resistance and for the cotransfer of enterotoxin production and resistance, one strain, E2985/76, was found from which ST and LT production transferred with and without ampicillin resistance (35). The linkage of the genes coding for antibiotic resistance and enterotoxin production was briefly described in that paper and is described in detail here. The other plasmids present in the strain have also been identified.

MATERIALS AND METHODS

Bacterial strains. Enterotoxigenic *E. coli* E2985/76, serotype O159.H34, was isolated in Canada in 1975 from an adult with a severe cholera-like illness and was sent to us by M. J. Gurwith (19).

The recipient *E. coli* K-12 (K-12) strains used in bacterial crosses were: an F^- *lac*$^+$ *pro met*, nalidixic acid-resistant (Nal^r) strain, J53-1; and an F^- *lac his pro trp*, streptomycin-resistant (Str^r) strain, 1R716. For transformation, the recipient K-12 was AB1157 F^- (9).

Media. Strains for conjugation experiments were

grown in nutrient broth (Difco) or Hedley-Wright broth. For solid media, New Zealand powdered agar was added to the broth. Crosses were plated on MacConkey or Diagnostic Sensitivity Test agar supplemented with the appropriate antibiotics.

Strains for enterotoxin testing were grown in Trypticase soy broth. For the preparation of radioactively labeled DNA, M9 liquid minimal medium supplemented with 0.2% glucose was used.

Drug resistance and colicinogeny. Bacterial strains were tested for resistance by the strip diffusion method or on solid media as described by Anderson and Threlfall (3). Colicinogeny was detected by the agar overlay method of Fredericq, and colicins were identified using standard indicator strains (14).

MICs. The minimal inhibitory concentrations (MICs) of strains resistant to ampicillin were estimated as previously described (3).

Phage testing in *E. coli* K-12. F-specific phages $\mu 2$ and fd were used in surface spot tests to identify derepressed F-like factors and in propagation experiments to detect F fimbriae. Phage If1 was used to detect I fimbriae (15).

Tests for enterotoxin production. Strains were tested for their ability to produce ST and LT by the methods described previously (18). Sterile culture filtrates were tested in infant mice for ST and on Chinese hamster ovary cells and Y1 adrenal cells for LT. When large numbers of recipient colonies not selected for Ap^r were screened for LT production, pools of five colonies were tested in the Y1 tissue miniculture assay. Filtrates of individual colonies from positive pools were subsequently tested for LT. Not all LT^+ or Ap^r transconjugants were tested for ST since it was found that in all cases when transconjugants were examined after transfer from the wild-type strains to K-12, and in subsequent crosses, all the ST^+ colonies were LT^+ and all the ST^- colonies were LT^-.

Test for hemolysin production. The wild-type strain was plated on newly poured nutrient agar (Oxoid) containing 2.5% horse blood.

Transfer of plasmids. (i) Direct. Two methods were used for conjugation experiments. For mating experiments where the wild-type strain was used as donor, 0.1 ml of overnight cultures of the donor and recipient cells was inoculated into 10 ml of Hedley-Wright broth, and the mixture was incubated for 1, 3, or 24 h at 37°C. For subsequent transfer experiments the donor and recipient cultures were grown in nutrient broth to a density of 2×10^8 cells per ml. For 1- or 3-h crosses, 0.2 ml of donor culture was added to 1.8 ml of recipient culture, whereas for overnight crosses 1 ml each of donor and recipient cultures was used. The recipient K-12 were resistant to nalidixic acid or streptomycin. Mating mixtures were plated on MacConkey or Diagnostic Sensitivity Test agar containing the appropriate drugs to select the transconjugants and counterselect against the donor.

(ii) Mobilization. Two non-autotransferring plasmids, the Sm Su plasmid NTP2 (1, 36) and the kanamycin resistance (Km) plasmid NTP107, were used. NTP107 was previously designated K (2, 37). Broth cultures of the strains to be tested for transfer factor activity and of the strain carrying the non-autotransferring plasmid were mixed in a ratio of 1:1 and incubated overnight. To 2 ml of this suspension was added 1 ml of the final recipient culture, again with overnight mating (1). Transconjugants were selected as described above. Non-autotransferring plasmids were mobilized by the derepressed transfer factors F-T (4) and T-Δ*drp* (15), both of which code for tetracycline resistance.

Surface exclusion and incompatibility tests. Exclusion of one R factor by a second plasmid during conjugation was measured by comparing the transfer frequency of the donor R factor to a recipient strain carrying the second plasmid with that to the same host strain devoid of this plasmid. Progeny were isolated on plates containing antibiotics which selected for recipient strains carrying the incoming R factor. Compatibility was investigated by testing such progeny for the presence of both plasmids: an incompatible resident factor may be displaced by the introduced plasmid. Progeny from this selection carrying both R factors, and additional transconjugants from the original cross isolated on plates containing antibiotics to select only for recipient strains carrying both R factors, were picked, subcultured to nutrient broth, and examined after 24 h for segregation. Suitable dilutions were plated on nutrient agar and replicated onto plates containing antibiotics to detect the presence of each R factor. At least three hybrid clones were plated, and more than 100 colonies of each were replicated. Incompatible R factors showed a rate of segregation higher than that of spontaneous loss of either factor over this time period: usually only 10 to 50% of colonies carried both plasmids. When segregation did not occur, further transfer experiments were carried out to show that the plasmids present in the transconjugants had not recombined but could be transferred independently.

The plasmids of the F groups used in these tests were F-T (F_I) (4), TP160 (F_1me) (4), R100-1 (F_{II}) (12), ColB4-K98 (F_{III}) (23), TP129 (= R124) (F_{IV}) (23), and F_0lac (F_V) (13). The plasmids of the I groups were T-Δ*drp* (I_1) (15) and TP114 (I_2) (16). A non-autotransferring plasmid was tested with NTP16 which codes for ampicillin and kanamycin resistance (Ap Km) (4). This plasmid belongs to incompatibility group 1 of non-autotransferring plasmids.

Fertility inhibition. Cells carrying derepressed F-like factors produce fimbriae which act as receptors for the F-specific phages and cause visible lysis of the cultures. Factors which are fi^+ inhibit the synthesis of F fimbriae. F-T was transferred into K-12 strains carrying plasmids to be tested or the plasmids were introduced into K-12 Hfr strains, and broth cultures were examined with phage $\mu 2$ in surface spot tests (15). fi^+ factors reduce or abolish visible lysis of the culture by this phage.

Preparation and agarose gel electrophoresis of plasmid DNA. Plasmid DNA was partially purified from wild-type *E. coli* or K-12 strains and subjected to electrophoresis as described (G. A. Willshaw, H. R. Smith, and E. S. Anderson, in preparation). The procedures used were based on those of Meyers et al. (31). The molecular weights of plasmids in E2985/76, and in K-12 lines derived from it, were measured by ref-

erence to plasmids of known molecular weight (4.7×10^6 to 78×10^6) run on the same gel.

Transformation of *E. coli* K-12. A preparation of partially purified plasmid DNA from E2985/76 was dialyzed for about 36 h against 75 mM $CaCl_2$. The DNA was used to transform $CaCl_2$-treated cells of AB1157 by the method of Cosloy and Oishi (9), as modified by Humphreys et al. (29). The transformation mixture (0.5 ml in 75 mM $CaCl_2$) contained 0.2 ml of recipient cells and 0.2 ml of dialyzed, partially purified plasmid DNA. Transformants were selected on medium containing 200 μg of ampicillin per ml.

Restriction endonuclease treatment of plasmid DNA. Plasmid DNA, partially purified from K-12 derivatives of E2985/76, was dialyzed for 36 h against 0.01 M Tris-hydrochloride, pH 7.5, containing 0.001 M EDTA. Samples of the preparations were digested for 1 h at 37°C with the restriction endonuclease *Bam*HI or *Eco*RI (Miles Laboratories). Reaction mixtures (30 μl) contained 0.1 M Tris-hydrochloride, pH 7.5, and 0.01 M $MgCl_2$; *Eco*RI digests also contained 0.1 M NaCl and 0.01 M 2-mercaptoethanol. The reactions were terminated by incubation at 65°C for 10 min. Sucrose and bromophenol blue were then added to final concentrations of 10 and 0.04%, respectively. Digested DNA was subjected to electrophoresis on a 0.75% agarose vertical slab gel for 16 h at a voltage gradient of 1.6 V/cm. The electrophoresis buffer, pH 7.9, contained 0.04 M Tris, 0.005 M sodium acetate, and 0.001 M EDTA.

Electron microscopy. Techniques for the examination of plasmid DNA have been described previously. The contour length of plasmid DNA was determined, and molecular weights were calculated on the assumption that 1 μm = 2.07×10^6 (17).

Copy number of the plasmids. The approximate number of copies of a plasmid per chromosome was estimated from the relative amounts of plasmid and chromosomal DNA as measured by uptake of tritiated thymidine over several generations of growth. Cultures of plasmid-carrying strains were grown in M9 minimal medium with 0.2% glucose as carbon source. [*methyl*-^{3}H]thymidine (100 μCi) and deoxyadenosine to a final concentration of 250 μg/ml were added in early exponential growth. The cells were lysed with a mixture of Brij 58 and sodium deoxycholate by the method of Clewell and Helinski (6). The proportion of acid-precipitable ^{3}H label recovered in the cleared lysate, which contained the plasmid DNA, was compared with that in the whole lysate before the clearing spin. The copy number could then be calculated for plasmids of known molecular weight, assuming the molecular weight of the chromosome to be 2.5×10^9 (8).

RESULTS

Properties of E2985/76. *E. coli* E2985/76 produced both heat-stable and heat-labile enterotoxins. It was resistant to ampicillin at an MIC of 2,000 μg/ml and produced colicin. It did not produce hemolysin. The F-specific phages μ2 and fd were propagated by E2985/76, suggesting that it contained at least one F-like plasmid. Gel electrophoresis of its plasmid DNA showed five bands corresponding to molecular weights of 53 $\times 10^6$, 43 $\times 10^6$, 5.6 $\times 10^6$, 3.6 $\times 10^6$, and 2.7 $\times 10^6$ (Fig. 1, track 2). We have used conjugation and transformation to separate the plasmids and have examined their properties in K-12 strains.

E2985/76 was tested for its ability to transfer ampicillin resistance (Ap^r), enterotoxin production (Ent$^+$), and colicinogeny (Col$^+$) by mating with J53-1 for 3 or 24 h. Transconjugants were selected on Diagnostic Sensitivity Test agar containing nalidixic acid (30 μg/ml) and ampicillin (100, 500, or 1,000 μg/ml) or cephaloridine (10 μg/ml). Unselected recipient colonies were also tested for their ability to produce LT or colicin.

The three properties described above were all transferred to the K-12 strain. The transfer frequencies for each marker are shown in Table 1.

The transfer frequency of Ap^r from the wild-type strain to K-12 in a 24-h cross was compared using different levels of ampicillin for selection. It was found that the frequency was slightly higher with the lower level of ampicillin, i.e., 1.3 $\times 10^{-3}$ transconjugants per recipient cell at 100 μg/ml, 4.7 $\times 10^{-4}$ at 500 μg/ml, and 1.6 $\times 10^{-4}$ at 1,000 μg/ml.

The Ap^r marker was also introduced into K-12 by transformation. Partially purified DNA from the wild-type strain E2985/76 was used to transform AB1157, and transformants were selected on nutrient agar containing ampicillin (200 μg/ml). Ap^r transformants were obtained at a frequency of 2×10^{-6} per recipient cell.

Properties of plasmids isolated from E2985/76. (i) Ampicillin resistance, enterotoxin plasmid (Ap-Ent). Seventy-one Ap^r transconjugants isolated from the crosses between E2985/76 and J53-1 described above, were examined for LT and colicin production. Only one transconjugant, G99, did not produce LT. Twenty-three strains, including G99, were tested for ST. All but G99 were positive. Fifty-two of the 71 transconjugants were colicinogenic.

TABLE 1. *Cross between E2985/76 and J53-1*

Time of cross (h)	Transfer frequency[a]		
	Colonies selected on media containing ampicillin (500 μg/ml) and nalidixic acid (30 μg/ml)	Colonies selected on media containing nalidixic acid (30 μg/ml)[b]	
		LT	Colicin
3	6×10^{-5}	ND[c]	ND
24	3×10^{-3}	2.7×10^{-2}	1.7×10^{-2}

[a] The transfer frequencies are expressed as the proportion of transconjugants per recipient cell.

[b] Thirty to 100 individual colonies from each cross were tested for LT and colicin production as previously described.

[c] ND, Not detected.

One Ap^r Ent^+ transconjugant, G72, which was non-colicinogenic was examined for further transfer of Ap^r and Ent^+ by mating with 1R716 and selecting for Ap^r. The two markers transferred as a single linkage group at a frequency of 1.8×10^{-2} transconjugants per recipient cell in a 1-h cross. Examination of the DNA of G72 showed a single band at a position corresponding to a molecular weight of 55×10^6 (Fig. 1, track 5). Drug resistance and enterotoxin production were therefore coded for by a single plasmid, TP215 (Tables 2 and 3).

G72 was lysed by the F-specific phages $\mu 2$ and fd and therefore contained a derepressed F-like factor. However, TP215 was compatible with plasmids of incompatibility groups F_I through F_V. We have not been able to assign this plasmid to any F incompatibility group. Other F-like plasmids which do not belong to any of these designated groups have also been described (32).

The genetic and molecular properties of other Ap^r Ent^+ transconjugants were examined in a similar fashion. Although all were found to contain an F-like plasmid of molecular weight 53 ×

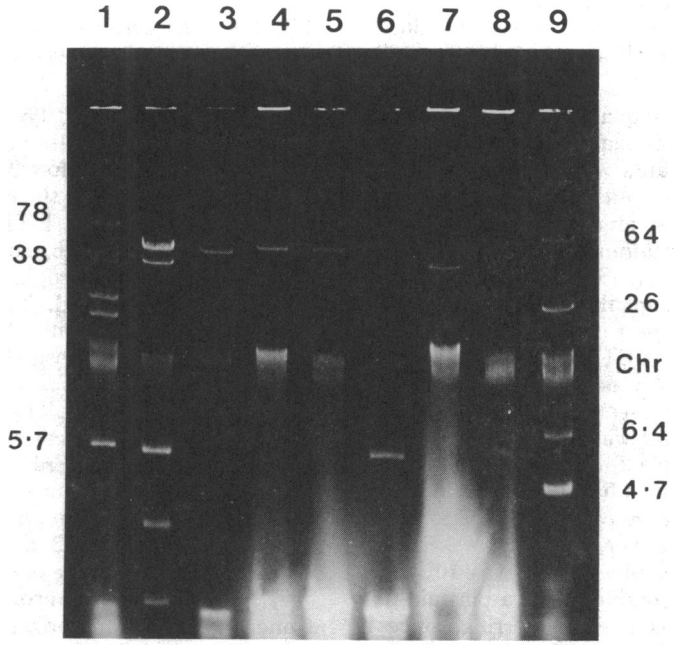

FIG. 1. *Identification of the plasmids in E. coli E2985/76. Analysis by gel electrophoresis of K-12 lines carrying single plasmids. Tracks 1 and 9 contain standard plasmids for determination of molecular weight; the molecular sizes (× 10^6) of some of these plasmids are indicated on the figure. The faint band running ahead of the chromosomal fragments (Chr) in track 9 is the open circular form of the 4.7 × 10^6-molecular-weight plasmid. Track 2, Wild-type strain E2985/76; 3, TP213, 54 × 10^6; 4, TP214, 55 × 10^6; 5, TP215, 55 × 10^6; 6, NTP21, 5.7 × 10^6; 7, TP221, 42 × 10^6; 8, NTP22, 5.7 × 10^6.*

TABLE 2. *Genetic and molecular properties of the plasmids isolated from the enterotoxigenic strain of E. coli, E2985/76*

Transconjugant or transformant no.	DEP plasmid no.[a]	Ampicillin resistance	Toxin production (LT and ST)	Colicinogeny	Phage propagation	Mol wt[b] (×10^6)
G72	TP215	+	+	−	F specific	55
G118	TP213	−	+	−	F specific	54
50R174	NTP21	+	−	−		5.7
G102	TP221	−	−	−	I specific	42
G99	TP222	+	−	−	I specific	45
G120	NTP22	−	−	+		5.7

[a] DEP, Division of Enteric Pathogens; TP, autotransferring plasmid; NTP, non-autotransferring plasmid.
[b] Molecular weights were determined by agarose gel electrophoresis of plasmid DNA.

TABLE 3. *Genetic and molecular properties of transconjugants carrying Ap-Ent plasmids isolated from E. coli E2985/76*

Transconjugant no.	MIC of ampicillin (μg/ml)	Lysis by phages μ2 and fd[a]	Phage propagation[b]		Transfer frequency of Ap^r to K-12 in 1-h crosses (transconjugants/recipient cell)	Ap-Ent plasmid	
			μ2	fd		No.	Mol wt (×10^6)
G72	300	OL	10^6	10^6	1.8×10^{-2}	TP215	55
G79	300	OL	10^6	10^6	5.7×10^{-2}	TP216	53
G91	500	OL	10^6	10^6	1.8×10^{-1}	TP214	55
50R526	300	OL	10^6	10^6	2.0×10^{-2}	TP220	55
G80	750	OL	10^5	10^6	2.2×10^{-3}	TP217	53
G102	400	—	10^4	10^3	5.8×10^{-4}	TP238	55
G100	1,500	—	10^3	10^4	4.0×10^{-4}	TP218	55
G101	1,500	—	10^2	10^3	1.2×10^{-4}	TP219	55

[a] OL, Opaque lysis; —, no lysis.

[b] Expressed as the difference between the titer when the phage was incubated with a plasmid-free control and the titer when the phage was incubated with the strain containing the plasmid being tested.

10^6 to 55×10^6, some quantitative differences in Ap^r and phage propagation were observed. These transconjugants will be described later.

(ii) Enterotoxin plasmid. Six transconjugants which were isolated on Diagnostic Sensitivity Test agar containing nalidixic acid (30 μg/ml) produced LT and ST. These six colonies were sensitive to ampicillin, and five were colicinogenic. The properties of the non-colicinogenic transconjugant G118 were examined further. When its DNA was run on an agarose gel, only one band was seen, corresponding to a plasmid of molecular weight 54×10^6 (TP213) (Fig. 1, track 3; Table 2). A culture of G118 was not lysed by F-specific phage μ2 or fd in a surface spot test. There was no phage propagation of μ2 by this strain. However, fd phage was propagated by G118; the phage titer was 10^4 times greater than that obtained with a plasmid-free control. TP213 was autotransferring, since it transferred from G118 to 1R716 at a frequency of 6×10^{-2} transconjugants per recipient cell in a 24-h cross and was able to mobilize two standard non-autotransferring plasmids, NTP2 and NTP107.

(iii) Ampicillin resistance plasmid. Ten Ap^r colonies isolated after transformation of AB1157 by DNA from the wild-type strain E2985/76 did not produce enterotoxin. Examination of their DNA showed that they contained one plasmid of molecular weight 5.7×10^6. A representative plasmid, NTP21, is shown on the gel (Fig. 1, track 6), and its properties are given in Table 2. The plasmid was non-autotransferring but could be mobilized from the transformant by the transfer factors F-T and T-Δ*drp*. The transformant containing NTP21 had an ampicillin MIC of 2,000 μg/ml. It was tested for compatibility with a variant of NTP16 which coded only for Km resistance (NTP16 Ap$^-$) and which belonged to group 1 (4). Three clones carrying the two plasmids NTP21 and NTP16 Ap$^-$ were tested for segregation after 24 h of incubation, when 55% (214 of 393) of the colonies lost one or the other of the plasmids. NTP21, therefore, was incompatible with NTP16 and belonged to group 1. It existed in about 19 copies per chromosome.

(iv) Colicin plasmid. The transconjugant G120 produced colicin only. Examination of its DNA by gel electrophoresis showed that it contained one plasmid of size 5.7×10^6 (NTP22) (Fig. 1, track 8; Table 2). The plasmid was non-autotransferring, but it cotransferred at a high rate with Ent$^+$ or Ap^r Ent$^+$ from E2985/76 to J53-1. The colicin has not been precisely identified. Colicin Ib indicator strains were resistant to it, but *E. coli* K-12 derivatives carrying NTP22 were not immune to colicin Ib.

(v) I-like transfer factor. When the DNA of one Ap^r Ent$^+$ transconjugant, G102, from a 24-h cross between E2985/76 and J53-1 was examined by gel electrophoresis, two large plasmid bands corresponding to molecular weights of 55×10^6 and 42×10^6 were seen. Ap^r and Ent$^+$ were transferred together from G102 into 1R716 in a 1-h cross when a single large plasmid of molecular weight 55×10^6 was isolated (TP238) (Table 2). When Ap^r and Ent$^+$ were lost together from G102, the 42×10^6 plasmid remained (TP221) (Fig. 1, track 7; Table 2). This plasmid was a transfer factor since the strain was able to mobilize the standard non-autotransferring plasmids NTP2 and NTP107 and could propagate phage If1 when there was a 10-fold increase in phage titer.

TP221 was tested for incompatibility with two I-like plasmids: T-Δ (incompatibility group I_1), which codes for Tc, and TP114 (incompatibility group I_2), which codes for Km. No exclusion or segregation of plasmids was observed when T-Δ

was transferred into a K-12 strain carrying TP221. However, when TP114 was transferred into the K-12 strain carrying TP221 there was 100-fold exclusion as compared to its transfer into plasmid-free K-12. Three clones were tested for loss of the Km marker. From one, 23% (64 of 278) of the replicated colonies had lost Km after 24 h of incubation. There was no loss of the Km marker from the other two clones. However, since TP221 has no resistance marker its loss could not be followed in these clones. The surface exclusion and instability of Km in one clone suggested that TP221 belonged to incompatibility group I_2. Examination of transconjugant G99 supported this suggestion.

Transconjugant G99 was isolated from a 3-h cross between E2985/76 and J53-1. It was ampicillin resistant and colicinogenic, but it did not produce enterotoxin or propagate the F-specific phages. G99 contained a plasmid of molecular weight 45×10^6, TP222, which transferred at a frequency of 6.0×10^{-2} transconjugants per recipient cell in a 24-h cross. Surface exclusion, 100-fold, was observed when the I_2 plasmid TP114 was transferred into G99. Ap^r was lost from 10 of 10 clones selected on plates containing kanamycin. When three colonies, selected on plates containing ampicillin and kanamycin, were tested for segregation, 53% (316 of 598) of the replicated colonies had lost one or the other of the markers. TP222 therefore belonged to incompatibility group I_2. Its similarity to TP221 suggested that it was probably derived from that plasmid by transposition of Ap^r.

(vi) Cryptic plasmids. Two small plasmids of molecular weights 3.6×10^6 and 2.7×10^6 were also seen in the wild-type strain E2985/76. They were not detected in any of the transconjugants examined. No properties have been assigned to these plasmids.

Variation in properties coded for by Ap-Ent plasmids isolated from E2985/76. Seventy Ap^r Ent^+ transconjugants derived from the 3- and 24-h crosses between E2985/76 and J53-1 were examined for colicinogeny, lysis by the F-specific phages $\mu 2$ and fd, and cephaloridine resistance.

From the 3-h cross, 20 of 25 recipient clones were obtained which were lysed by phages $\mu 2$ and fd in surface spot tests, indicating the presence of a derepressed F-like factor. From 24-h crosses the number with derepressed factors was 12 of 45.

By using the strip diffusion method to detect resistance, it was possible to compare roughly the degree of resistance of one culture with that of others on the plate (3). All the Ap^r transconjugants were also resistant to cephaloridine, but there was a continuous variation in degree from a low to a high level. Colonies were selected for further study on the basis of these differences in their levels of cephaloridine resistance and the differences in lysis by the phages. The properties of seven transconjugants isolated from these crosses from E2985/76 to J53-1 are shown in Table 3. Transconjugant 50R526 was obtained after further transfer to 1R716.

The DNA from all of these transconjugants was examined by gel electrophoresis, and in every case a plasmid band was seen at a position corresponding to a molecular weight of 53×10^6 to 55×10^6. In transconjugants G79, G101, and 50R526, as in G72, only a single band was seen. Again, Ent^+ and Ap^r were transferred together when these strains were mated with K-12 and selection was made for Ap^r, showing that these properties were coded for by single plasmids numbered TP216, TP219, and TP220, respectively. The DNA of the other transconjugants was shown to contain other plasmid bands such as the colicin plasmid NTP22 and the transfer factor TP221, but in every case it was possible to separate a single Ap-Ent plasmid by transfer to 1R716.

Four types of Ap-Ent, F-like plasmids having the following properties were found in the transconjugants: (i) derepressed and coding for an ampicillin MIC of 300 to 500 μg/ml, TP214, TP215, TP216, and TP220; (ii) derepressed and coding for an ampicillin MIC of 750 μg/ml, TP217; (iii) repressed and coding for an ampicillin MIC of 400 μg/ml, TP238; and (iv) repressed and coding for an ampicillin MIC of 1,500 μg/ml, TP218 and TP219.

TP218 and TP219 coded for ampicillin MICs of 1,500 μg/ml. This suggested that these plasmids might be present in more copies than, for example, TP214, which coded for an ampicillin MIC of 500 μg/ml. The copy numbers of three plasmids, TP213, TP214, and TP219, were compared. Both TP213 and TP214 were present as single copies per chromosome, whereas TP219 was present in three to four copies.

These variations in the properties of the Ap-Ent plasmids together with the presence in the wild-type strain of the Ent plasmid (TP213) and an Ap plasmid (NTP21) suggested that they may have been formed as a result of transposition of ampicillin resistance from NTP21 to TP213.

Genetic and molecular evidence for transposition. (i) Transposition in a K-12 transconjugant. Ap^r transconjugants from a 24-h cross between E2985/76 and J53-1 were selected on Diagnostic Sensitivity Test agar containing ampicillin (1,000 μg/ml) and nalidixic

acid (30 μg/ml). Three of 10 recipient clones had an ampicillin MIC of 2,000 μg/ml. The properties of one of these transconjugants, 50R469, were examined. Agarose gel electrophoresis of its DNA showed three bands at positions corresponding to molecular weights of 52×10^6, 42×10^6, and 5.8×10^6. 50R469 was enterotoxigenic and colicinogenic. It was not lysed by the F-specific phages μ2 and fd but could propagate fd, but not μ2. In this respect it was similar to transconjugant G118, which carried the Ent factor TP213. When NTP16 Ap^- was introduced into the strain, segregation occurred; Ap^r was lost from a number of colonies, but they still produced LT. 50R469, therefore, contained TP213 and the Ap plasmid NTP21. The transfer factor TP221 was also present.

In a 24-h cross to IR716, Ap^r was transferred from 50R469 at a low frequency, 3.0×10^{-6} transconjugants per recipient cell. Transfer of Ap^r from the Ap^r transconjugants described in Table 3 occurred at frequencies of 1.0 to 1.0×10^{-2} in 24-h crosses. Two of the Ap^r Ent^+ clones from the cross between 50R469 and 1R716 were compared. One was like the original strain except that TP221 was not present. The second 1R716 transconjugant had a low ampicillin MIC (300 μg/ml), was lysed by phages μ2 and fd, and transferred Ap^r at a frequency of 2.7×10^{-1} transconjugants per recipient cell in a 24-h cross. These properties were similar to those coded for by the Ap-Ent plasmid TP215. Like that plasmid, its formation had probably been the result of transposition of the ampicillin resistance region (Tn*A*) from NTP21 to TP213.

(ii) Determination of molecular weights. The contour lengths of TP213 and the Ap-Ent plasmid TP214 were determined by electron microscopy, which is a more accurate technique than gel electrophoresis. The molecular weights were calculated as described in Materials and Methods. TP213 had a molecular weight of $50.4 \pm 0.6 \times 10^6$, and TP214 was $54.6 \pm 1.1 \times 10^6$ in size. This finding supported the view that Tn*A* of molecular weight 3.2×10^6 (25) had been inserted into TP213.

(iii) Treatment of plasmid DNA with restriction endonucleases. The Ent plasmid TP213 and Ap-Ent derivatives were compared by analysis of the plasmid DNA fragments produced by treatment with restriction endonucleases. Digestion with *Eco*RI was performed to show if insertion of Tn*A* resulted in an increase in size of the *Eco*RI fragments of TP213. The plasmids were also treated with *Bam*HI, which has a cleavage site within Tn*A* (33), to determine whether the insertion resulted in an extra DNA fragment. Plasmid NTP21, which presumably contained Tn*A*, gave a single linear fragment with *Bam*HI.

The Ent plasmid TP213 and the Ap-Ent plasmids TP214, TP215, TP219, and TP220 were examined. Partially purified plasmid DNA, prepared from K-12 lines carrying these Ap-Ent plasmids or TP213, was dialyzed and treated with restriction enzymes as described earlier. With *Eco*RI, all the plasmids showed four bands of size $< 13 \times 10^6$ and combined molecular weight of about 24×10^6 (Fig. 2a, tracks 1–5). A slow-moving band was also present, and this could represent one or more large DNA fragments not resolved from each other. Small fragments, $< 1 \times 10^6$, were not visible on the gel. These results were obtained reproducibly with both partially purified and pure plasmid DNA preparations. The patterns for TP213, TP214, TP215, TP219, and TP220 were very similar and confirmed the close relationship of the Ent and Ap-Ent plasmids. For each Ap-Ent plasmid, insertion of Tn*A* had probably occurred at a site which did not result in a measurable increase in size of the four *Eco*RI fragments smaller than 13×10^6, but presumably into the large fragment(s).

When treated with *Bam*HI, TP213 gave a single slow-moving band, which may indicate the presence of one *Bam*HI cleavage site (Fig. 2b, track 1). Each Ap-Ent plasmid also gave a slow-moving band; the migration of these bands and that in TP213 were very similar. However, digestion of each of the Ap-Ent plasmids also generated a small fragment (tracks 2–5) which suggested that they possessed a *Bam*HI site not present in the Ent plasmid. The size of this extra fragment was different for each of the four Ap-Ent plasmids examined: TP214, 5.8×10^6; TP215, 4.5×10^6; TP219, 2.4×10^6; and TP220, 6.0×10^6. The distinct sizes of these fragments indicated that insertion of Tn*A* had probably occurred at different sites on the TP213 plasmid. These insertions may have occurred on different sides or on the same side of the *Bam*HI site present in TP213. Examination of a larger number of Ap-Ent plasmids with *Bam*HI should show whether insertion of Tn*A* occurs randomly on the Ent plasmid.

DISCUSSION

The wild-type enterotoxigenic *E. coli* strain E2985/76 carried the following types of plasmids: (i) a repressed F-like Ent plasmid, TP213; (ii) Ap-Ent factors; (iii) an I_2 transfer factor, TP221; (iv) an Ap I_2 factor, TP222; (v) an Ap plasmid, NTP21; (vi) a colicin plasmid, NTP22; and (vii) two cryptic plasmids. The Ap-Ent plasmids varied in their ability to code for ampicillin

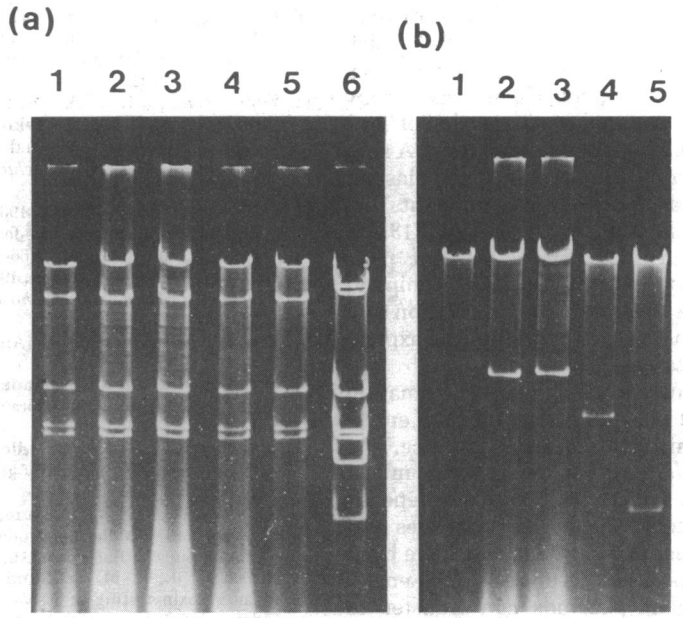

FIG. 2. *Treatment of Ent and Ap-Ent plasmids with restriction endonucleases: (a) EcoRI, (b) BamHI. In each case, tracks 1 through 5 contain the following plasmids: 1, Ent, TP213; 2, Ap-Ent, TP214; 3, Ap-Ent, TP220; 4, Ap-Ent, TP215; 5, Ap-Ent, TP219; 5, phage λ DNA.*

resistance, phage propagation, and transfer. This suggested that transposition of Ap^r from NTP21 to TP213 had occurred, leading to the formation of these Ap-Ent plasmids. The enterotoxin plasmid TP213 has been marked with tetracycline resistance and tested for incompatibility with the eight Ap-Ent factors described in Table 3. In all cases these plasmids were found to be incompatible with TP213 (unpublished data).

The I_2 factor TP222 could also have been formed by transposition of ampicillin resistance, in this case to the transfer factor TP221. A similar transposition of Ap^r from a non-autotransferring plasmid to a transfer factor has been shown to occur in a natural isolate of *Salmonella panama* (41).

Transposons coding for ampicillin resistance (Tn*A*) are present in autotransferring and non-autotransferring plasmids (24, 26, 27). Insertion of Tn*A* can occur at different sites on the bacterial chromosome or on other plasmids and can generate mutations. Integration may result in inactivation of a structural gene and, depending on the orientation of insertion, have a polar effect on the expression of distal genes (24, 33).

The molecular weights of TP213 and the Ap-Ent plasmid TP214 were 50.4×10^6 and 54.6×10^6, respectively, as determined by electron microscopy. This difference is consistent with the insertion of Tn*A*, of molecular size 3.2×10^6 (25), into TP213. Integration of a transposon is generally found to increase the size of a plasmid (24, 25). The molecular weight difference between the Ent and Ap-Ent plasmids was correlated with the presence in TP214 of an extra cleavage site for the restriction endonuclease *Bam*HI, an enzyme which has a single cut site in Tn*A* (33). NTP21 (Ap), which had one cleavage site for *Bam*HI, was probably the donor of Tn*A*. Analysis of four Ap-Ent plasmids showed that the size of the extra *Bam*HI fragment was different in each case. This indicated that insertion of Tn*A* had probably occurred at different sites in TP213.

The derepressed Ap-Ent plasmids carried by 32 of 70 Ap^r enterotoxigenic transconjugants probably resulted from insertion of Tn*A* within the transfer region of TP213. Strains carrying these recombinants showed an increase in transfer ability and in propagation of F-specific phages. Such clones would be preferentially selected in conjugation experiments, resulting in the high proportion of transconjugants with this phenotype. Insertion of Tn*A* has been shown to result in recombinant plasmids defective in transfer (28, 40).

Expression of other plasmid functions may also be affected by insertion of Tn*A*. The plasmid TP219 was present in three to four copies per chromosome, and host strains showed high ampicillin resistance (MIC, 1,500 μg/ml), which presumably reflected the increased number of

copies of the β-lactamase gene. This contrasted with an Ap-Ent plasmid such as TP214 which was present as a single copy and conferred an ampicillin MIC of 500 μg/ml. The increased copy number probably resulted from insertion of Tn*A* in a gene controlling plasmid replication. A similar increase in ampicillin resistance and plasmid copy number was found in recombinants between Tn*A* and RSF1010 (26) or CloDF13 (5). Other transconjugants carrying Ap-Ent plasmids showed variation in resistance to ampicillin. This may be the result of the position and orientation of Tn*A* within TP213 on the expression of the β-lactamase gene.

The results demonstrate the in vivo formation of a recombinant plasmid coding for both enterotoxin production and ampicillin resistance. The wild-type *E. coli* strain is probably a mixed population, with some lines carrying independent plasmids determining these properties and other cells in which Ap-Ent plasmids have been formed by transposition. Drug resistance-enterotoxin recombinant plasmids can transfer to other strains. These strains will probably be at a selective advantage in the presence of the appropriate drugs. The use of antibiotics may therefore have the effect of increasing the number of strains carrying plasmids which code for enterotoxin production or other pathogenic properties.

LITERATURE CITED

1. **Anderson, E. S., and M. J. Lewis.** 1965. Characterisation of a transfer factor associated with drug resistance in *Salmonella typhimurium*. Nature (London) **208**:843–849.
2. **Anderson, E. S., J.-S. Pitton, and J. N. Mayhew.** 1968. Restriction of bacteriophage multiplication by resistance determinants in salmonellae. Nature (London) **219**:640–641.
3. **Anderson, E. S., and E. J. Threlfall.** 1974. The characterisation of plasmids in the enterobacteria. J. Hyg. **72**:471–487.
4. **Anderson, E. S., E. J. Threlfall, J. M. Carr, M. M. McConnell, and H. R. Smith.** 1977. Clonal distribution of resistance plasmid-carrying *Salmonella typhimurium*, mainly in the Middle East. J. Hyg. **79**:425–448.
5. **Andreoli, P. M., N. Overbeeke, E. Veltkamp, J. D. A. van Embden, and H. J. J. Nijkamp.** 1978. Genetic map of the bacteriocinogenic plasmid CloDF13 derived by insertion of the transposon Tn901. Mol. Gen. Genet. **160**:1–11.
6. **Clewell, D. B., and D. R. Helinski.** 1969. Supercoiled circular DNA-protein complex in *Escherichia coli*: purification and induced conversion to an open circular DNA form. Proc. Natl. Acad. Sci. U.S.A. **62**:1159–1166.
7. **Cohen, S. N.** 1976. Transposable genetic elements and plasmid evolution. Nature (London) **263**:731–738.
8. **Cooper, S., and C. E. Helmstetter.** 1968. Chromosomal replication and the division cycle of *Escherichia coli* B/r. J. Mol. Biol. **31**:519–540.
9. **Cosloy, S. D., and M. Oishi.** 1973. The nature of the transformation process in *Escherichia coli* K12. Mol. Gen. Genet. **124**:1–10.
10. **Dupont, H. L., H. West, D. G. Evans, J. Olarte, and D. J. Evans.** 1978. Antimicrobial susceptibility of enterotoxigenic *Escherichia coli*. J. Antimicrob. Chemother. **4**:100–102.
11. **Echeverria, P., L. Verhaert, C. V. Ulyangco, S. Komalarini, M. T. Ho, F. Ørskov, and I. Ørskov.** 1978. Antimicrobial resistance and enterotoxin production among isolates of *Escherichia coli* in the Far East. Lancet **ii**:589–592.
12. **Egawa, R., and Y. Hirota.** 1962. Inhibition of fertility by multiple drug resistance-factor (R) in *Escherichia coli* K12. Jpn. J. Genet. **37**:66–69.
13. **Falkow, S., and L. S. Baron.** 1962. Episomic element in a strain of *Salmonella typhosa*. J. Bacteriol. **84**:581–589.
14. **Fredericq, P.** 1957. Colicins. Annu. Rev. Microbiol. **11**: 7–22.
15. **Grindley, J. N., and E. S. Anderson.** 1971. I-like resistance factors with the *fi*$^+$ character. Genet. Res. **17**:267–271.
16. **Grindley, N. D. F., J. N. Grindley, and E. S. Anderson.** 1972. R factor compatibility groups. Mol. Gen. Genet. **119**:287–297.
17. **Grindley, N. D. F., G. O. Humphreys, and E. S. Anderson.** 1973. Molecular studies of R factor compatibility groups. J. Bacteriol. **115**:387–398.
18. **Gross, R. J., S. M. Scotland, and B. Rowe.** 1976. Enterotoxin testing of *Escherichia coli* causing epidemic infantile enteritis in the U.K. Lancet **i**:629–631.
19. **Gurwith, M. J., C. Bourque, E. Cameron, G. Forrest, and M. Greene.** 1977. Cholera-like diarrhoea in Canada. Report of a case associated with enterotoxigenic *Escherichia coli* and a toxin producing *Aeromonas hydrophila*. Arch. Intern. Med. **137**:1461–1464.
20. **Gyles, C., S. Falkow, and L. Rollins.** 1978. In vivo transfer of an *Escherichia coli* enterotoxin plasmid possessing genes for drug resistance. Am. J. Vet. Res. **39**:1438–1441.
21. **Gyles, C. L., S. Palchaudhuri, and W. K. Maas.** 1977. Naturally occurring plasmid carrying genes for enterotoxin production and drug resistance. Science **198**:198–199.
22. **Gyles, C. L., M. So, and S. Falkow.** 1974. The enterotoxin plasmids of *Escherichia coli*. J. Infect. Dis. **130**: 40–49.
23. **Hedges, R. W., and N. Datta.** 1972. R124, an *fi*$^+$ R factor of a new compatibility class. J. Gen. Microbiol. **71**:403–405.
24. **Hedges, R. W., and A. E. Jacob.** 1974. Transposition of ampicillin resistance from RP4 to other replicons. Mol. Gen. Genet. **132**:31–40.
25. **Heffron, F., C. Rubens, and S. Falkow.** 1975. Translocation of a plasmid DNA sequence which mediates ampicillin resistance: molecular nature and specificity of insertion. Proc. Natl. Acad. Sci. U.S.A. **72**:3623–3627.
26. **Heffron, F., C. Rubens, and S. Falkow.** 1977. Transposition of a plasmid deoxyribonucleic acid sequence that mediates ampicillin resistance: identity of laboratory-constructed plasmids and clinical isolates. J. Bacteriol. **129**:530–533.
27. **Heffron, F., R. Sublett, R. Hedges, A. Jacob, and S. Falkow.** 1975. Origin of the TEM beta-lactamase gene found on plasmids. J. Bacteriol. **122**:250–256.
28. **Hernalsteens, J. P., R. Villarroel-Mandiola, M. Van Montagu, and J. Schell.** 1977. Transposition of Tn*1* to a broad-host-range drug resistance plasmid, p. 179–183. *In* A. I. Bukhari, J. A. Shapiro, and S. Adhya (ed.), DNA insertion elements, plasmids and episomes. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
29. **Humphreys, G. O., A. Weston, M. G. M. Brown, and J. R. Saunders.** 1979. Plasmid transformation of *Escherichia coli*, p. 254–279. *In* S. W. Glover and L. O.

Butler (ed.), Transformation 1979. Cotswold Press, Oxford.

30. **Merson, M. H., G. K. Morris, D. A. Sack, J. G. Wells, J. C. Feeley, R. B. Sack, W. B. Creech, A. Z. Kapikian, and E. J. Gangarosa.** 1976. Travelers diarrhoea in Mexico. A prospective study of physicians and family members attending a congress. N. Engl. J. Med. **294:** 1299–1305.
31. **Meyers, J. A., D. Sanchez, L. P. Elwell, and S. Falkow.** 1976. Simple agarose gel electrophoretic method for the identification and characterization of plasmid deoxyribonucleic acid. J. Bacteriol. **127:**1529–1537.
32. **Monti-Bragadin, C., L. Samer, G. D. Rottini, and B. Pani.** 1975. The compatibility of Hly factor, a transmissible element which controls α-haemolysin production in *Escherichia coli.* J. Gen. Microbiol. **86:**367–369.
33. **Rubens, C., F. Heffron, and S. Falkow.** 1976. Transposition of a plasmid deoxyribonucleic acid sequence that mediates ampicillin resistance: independence from host *rec* functions and orientation of insertion. J. Bacteriol. **128:**425–434.
34. **Santos, D. S., S. Palchaudhuri, and W. K. Maas.** 1975. Genetic and physical characteristics of an enterotoxin plasmid. J. Bacteriol. **124:**1240–1247.
35. **Scotland, S. M., R. J. Gross, T. Cheasty, and B. Rowe.** 1978. Incidence of plasmids carrying genes for both enterotoxin production and drug resistance in *Escherichia coli* strains of human origin. Soc. Gen. Microbiol. **6:**45.
36. **Smith, H. R., G. O. Humphreys, and E. S. Anderson.** 1974. Genetic and molecular characterization of some non-transferring plasmids. Mol. Gen. Genet. **129:**229–242.
37. **Smith, H. R., G. O. Humphreys, N. D. F. Grindley, J. N. Grindley, and E. S. Anderson.** 1973. Molecular studies of an fi^+ plasmid from strains of *Salmonella typhimurium.* Mol. Gen. Genet. **126:**143–151.
38. **Smith, H. W., and S. Halls.** 1968. The transmissible nature of the genetic factor in *Escherichia coli* that controls enterotoxin production. J. Gen. Microbiol. **52:** 319–334.
39. **So, M., J. H. Crosa, and S. Falkow.** 1975. Polynucleotide sequence relationships among Ent plasmids and the relationship between Ent and other plasmids. J. Bacteriol. **121:**234–237.
40. **So, M., F. Heffron, and S. Falkow.** 1978. Method for the genetic labeling of cryptic plasmids. J. Bacteriol. **133:**1520–1523.
41. **van Embden, J. D. A.** 1978. Translocation of an ampicillin resistance determinant within an R-factor aggregate in *Salmonella panama* Antonie van Leeuwenhoek. J. Microbiol. Serol. **44:**203–218.