Acquisition and Maintenance of Enterotoxin Plasmids in Wild-type Strains of *Escherichia coli*

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Enterotoxigenic strains of *Escherichia coli* (ETEC) may produce a heat-labile enterotoxin (LT), a heat-stable enterotoxin (ST) or both enterotoxins. Certain serogroups are represented more frequently than others in ETEC isolated from humans. The transfer of three plasmids encoding enterotoxin production (Ent) to 22 non-toxigenic *E. coli* strains of many different O:H serotypes was studied. The Ent plasmids encoded ST (TP276), or LT (TP277), or ST + LT (TP214), and all carried antibiotic-resistance determinants. Twenty-one recipient strains acquired TP214, 18 acquired TP277 and 14 acquired TP276. Strains of those serotypes to which ETEC in diarrhoeal studies commonly belong neither acquired nor maintained Ent plasmids with a higher frequency than strains of those serotypes to which ETEC rarely belong. The recipient strains, with one exception, all expressed ST, or LT, or ST and LT, when they had acquired the appropriate plasmid; a non-motile strain belonging to O serogroup 88 expressed LT but failed to express ST when it acquired TP214 or TP277.

INTRODUCTION

Enterotoxigenic strains of *Escherichia coli* (ETEC) are an important cause of diarrhoeal disease in man and animals. In a number of studies it has been noted that ETEC from humans commonly belong to a restricted range of serogroups (Cravioto *et al.*, 1979; DeBoy *et al.*, 1980; Merson *et al.*, 1979; Ørskov *et al.*, 1976). In the study of ETEC strains isolated in various countries by Cravioto *et al.* (1979), 108 (66%) of 164 strains which belonged to identifiable O groups, belonged to serogroups O6, O8, O63, O78 or O159; an additional 41 strains (25%) belonged to serogroups O15, O25, O27, O114, O115, O128 or O148. In a similar study by Ørskov *et al.* (1976), 101 (98%) of 103 ETEC strains which belonged to identifiable O groups belonged to serogroups O6, O8, O15, O20, O25, O78, O115 or O128. The restriction of ETEC to a small number of O serogroups may be of practical importance in the preliminary screening of strains for enterotoxigenicity (Merson *et al.*, 1980). In some serogroups, for example *E. coli* O128, the enterotoxigenic strains are of many different flagellar types (Reis *et al.*, 1980; Scotland *et al.*, 1981). In other serogroups enterotoxigenic strains usually belong to only one flagellar type; for example O6.H16, O8.H9, O148.H28 (Back *et al.*, 1980; DeBoy *et al.*, 1980; Merson *et al.*, 1979; Ørskov & Ørskov, 1977; Smyth *et al.*, 1979). ETEC of the same O:H serotype have been isolated in widely separated geographical areas.

ETEC isolated from humans may produce a heat-stable enterotoxin (ST), a heat-labile enterotoxin (LT), or both enterotoxins, and it has been noted that certain serotypes are usually correlated with a particular pattern of enterotoxin production. For example enterotoxigenic strains of *E. coli* O6.H16 usually produce ST and LT (Merson *et al.*, 1979; Scotland *et al.*, 1977; Smyth *et al.*, 1979). In contrast, enterotoxigenic strains of *E. coli* O128 of most H types, including H7, H12, H21 and H27, usually produce ST, but so far only strains of *E. coli* O128.H49

Abbreviations: ETEC, enterotoxigenic strains of *Escherichia coli*; LT, heat-labile enterotoxin; MRHA, mannose-resistant haemagglutination; ST, heat-stable enterotoxin.

producing LT have been reported (DeBoy *et al.*, 1980; Merson *et al.*, 1979; Reis *et al.*, 1980; Scotland *et al.*, 1981). The production of ST and LT has been shown to be plasmid mediated; enterotoxin (Ent) plasmids have been identified which code for LT only, ST only, or LT and ST (Evans *et al.*, 1975; Gyles *et al.*, 1974; McConnell *et al.*, 1980, 1981; Scotland *et al.*, 1979).

The prevalence of certain serotypes amongst ETEC, and their isolation from various parts of the world, may reflect the particular ability of such serotypes to acquire or maintain Ent plasmids. In the present investigation, 22 non-enterotoxigenic strains of *E. coli* were tested to see if they differed in their ability to acquire three enterotoxin plasmids coding for LT only, ST only or ST and LT. These Ent plasmids carried antibiotic-resistance determinants so that their transfer to and stability in the recipient strains could be studied more easily. The wild-type recipients included *E. coli* strains of serotypes to which ETEC commonly belong, such as O6.H16 and a number of different H types of O128; they also included strains belonging to serogroups in which ETEC are rarely found, for example, O1 and O154.

METHODS

Donor strains of bacteria. Three strains of *E. coli* K12 carrying autotransferring plasmids coding for enterotoxin production were used; they were auxotrophic, lactose non-fermenting and streptomycin-resistant.

Strain 50R219 carried plasmid TP214 coding for ampicillin resistance and production of both ST and LT; this plasmid had been isolated from *E. coli* strain E2985/76 (O159.H34) (Scotland *et al.*, 1979) and had been characterized as a derepressed F-like plasmid by McConnell *et al.* (1979).

Strain 52R83 carried plasmid TP277 coding for kanamycin resistance and production of LT. The LT plasmid (TP235) had been isolated from *E. coli* strain E5798/76 (O7.H18) (Scotland *et al.*, 1979). It has been 'marked' with a kanamycin resistance gene from pML2 and characterized as an F-like plasmid by McConnell *et al.* (1980).

Strain 52R90 carried plasmid TP276 coding for tetracycline resistance and ST production. The ST plasmid (TP224) had been isolated from *E. coli* strain E7476/77 (O7H27) (Scotland *et al.*, 1979); this strain has now been assigned to a new serogroup O166 (Dr F. Ørskov, personal communication). TP224 had been marked with tetracycline resistance by Dr H. R. Smith using the following procedure. The ST plasmid was transferred into *E. coli* K12 carrying the tetracycline resistance transposon Tn10 (Foster *et al.*, 1975) on the chromosome. Enterotoxigenic transconjugants were mated with *E. coli* K12 selecting for tetracycline resistance and a number of resulting strains including 52R90 were analysed to show that a single plasmid coded for drug resistance and ST production.

Recipient strains of bacteria. Wild-type strains of *E. coli* isolated from human faecal specimens were examined for use as possible recipient strains in conjugation experiments. These strains had been maintained on Dorset egg agar at room temperature in the culture collection of the Division of Enteric Pathogens. They had been identified by biochemical tests and by serotyping techniques using O groups 1-164 and H antigens 1-56. They were originally selected as being representative of serotypes in which ETEC are either commonly found or rarely found. They were then tested for colicinogeny, haemolysis, haemagglutination, drug resistance and the presence of transfer factors. These properties are often plasmid-encoded and might affect the ability of the strain to acquire an Ent plasmid, either by exclusion of the incoming plasmid or by failure of the two plasmids to co-exist in the host bacterium. So when possible, wild-type strains were chosen for further study only if they lacked the aforementioned properties. Occasionally such negative strains were not available and some of the wild-type strains studied further did possess these properties.

A number of non-toxicogenic wild-type strains were of serogroups to which ETEC rarely belong. These included three strains of serogroup O1, with flagellar antigens H5, H34 or H45 from healthy infants; Taylor (1961) reported that strains of serogroup O1 are frequently isolated from the faeces of healthy infants. Strains of serotype O40.H3 and O71.H48, studied by Klipstein *et al.* (1978) and O88.H⁻ from healthy infants were also included. Strain SC449 (*E. coli* O154.H4) was isolated from many healthy adults after their arrival in the Persian Gulf (Rowe, 1973); feeding experiments have shown that this strain is able to colonize the human intestine, but it has not been associated with diarrhoeal disease or toxin production (unpublished results).

Seven strains of serogroup O6 were studied as recipients. Strains of serotype O6.H16 are usually toxigenic but two non-toxicogenic strains, E3483 and E1437/69, were available in our culture collection. Spontaneous non-toxicogenic variants were also isolated from two ST⁺LT⁺ strains, E219/69 and E1392 to compare their ability to acquire Ent plasmids. E219/69 and E1392 also possessed colonization factor antigen CFA/II as described by Evans & Evans (1978). The variant from strain E219/69 lost the ability to produce ST, LT and CFA/II. Two variants were obtained from E1392; both failed to produce ST or LT, but one had retained the ability to produce CFA/II. Strains of serotype O6.H1 and O6.H⁻ were compared as representatives of serotypes in which ETEC are rarely found; Taylor (1961) reported that strains of *E. coli* O6 are common isolates from the faeces of healthy infants.

Table 1. *Properties and sources of nalidixic acid-resistant mutants of wild-type strains of Escherichia coli*

Reference no. of mutant	Serotype*	Derived from wild-type strain no.†	Country of isolation	Production of colicin or haemolysin	MRHA‡	Antibiotic resistance§	Ability to mobilize NTP107
G259	O1. H5	E16040	Bangladesh	None	None	None	+
G257	O1. H34	E14137	Bangladesh	None	None	None	+
G258	O1. H45	E14247	Bangladesh	None	None	None	—
G153	O6. H16	E3483	Canada	Colicin	None	None	—
G176	O6. H16	E219/69	Sharjah	None	None	None	—
G177	O6. H16	E1437/69	Sharjah	None	None	None	—
G337	O6. H16	E1392	Hong Kong	None	Calf	Sm Su	—
G338	O6. H16	E1392	Hong Kong	None	None	Sm Su	—
G178	O6. H1	E3998	Britain	Haemolysin	None	None	—
G157	O6. H ⁻	E6339	Britain	Haemolysin	Human	None	—
G154	O25. H42	E9031	Mexico	None	None	None	—
G155	O25. H ⁻	E6655	Brazil	None	None	None	—
G182	O40. H3	E2722/1	Britain	None	None	None	—
G181	O71. H48	E2698/1	Britain	None	None	None	—
G158	O88. H ⁻	E8026	Britain	None	None	None	—
G256	O128. H10	E14854	Britain	None	None	None	—
G254	O128. H18	E8124	Britain	None	None	None	—
G255	O128. H27	E9055	Costa Rica	None	None	None	—
G260	O128. H49	E8412	Gambia	None	None	None	—
G334	O154. H4	SC449	Sharjah	None	None	None	—
G159	O159. H34	E8013	Canada	None	None	None	—

* H⁻, non-motile.

† Strains E1392 and E219/69 were ST⁺ and LT⁺ and strain E8412 was LT⁺ when first isolated, and non-toxicogenic variants were selected. All other strains were non-toxicogenic.

‡ MRHA, mannose-resistant haemagglutination of human or calf erythrocytes.

§ Sm, streptomycin; Su, sulphathiazole.

Strains representative of serotypes to which ETEC commonly belong, in addition to *E. coli* O6. H16, included those of serotypes O25. H⁻, O25. H42, O128. H10, O128. H18, O128. H27 and O159. H34. The wild-type strains chosen were all non-toxicogenic when received by our laboratory. All strains of serotype O128. H49 in our culture collection produced LT, so a spontaneous non-toxicogenic variant was selected from strain E8412.

A mutant resistant to nalidixic acid (50 µg ml⁻¹) was selected from each of the 21 wild-type strains for use as a recipient in transfer experiments; the characteristics of these strains are given in Table 1. For comparison, a nalidixic acid-resistant sub-strain of *E. coli* K12 (J53-1) was also tested as a recipient.

Characterization of recipient strains. Bacterial strains were tested for resistance to ampicillin, chloramphenicol, furazolidone, gentamicin, kanamycin, nalidixic acid, streptomycin, sulphathiazole, tetracycline and trimethoprim by the strip diffusion method or on solid media as described by Anderson & Threlfall (1974).

Colicinogeny was detected by the agar overlay method of Fredericq (1957) using the sensitive indicator strain *E. coli* ROW.

Strains were plated on newly poured nutrient agar (Oxoid) containing horse blood (2.5%, v/v) to detect haemolysin production.

Strains were tested for their ability to produce ST and LT by methods described previously (Gross *et al.*, 1976) using the Chinese hamster ovary cell test for LT and the infant mouse test for ST. A gut/body weight ratio in the infant mouse test of greater than 0.10 was considered positive, and a ratio of less than 0.08 negative. In some experiments, filtrates were concentrated tenfold using an Amicon UM2 filter before testing for the presence of ST in the usual manner.

Strains were passaged twice on CFA agar (Evans *et al.*, 1977a) and tested for haemagglutination of human (group A) or calf erythrocytes in the presence and absence of D-mannose (0.5%, w/v). Strain G337 gave mannose-resistant haemagglutination (MRHA) of calf erythrocytes; this strain was derived from strain E1392 (*E. coli* O6. H16), which gave a similar haemagglutination pattern and had been shown by immunodiffusion tests to possess CFA/II. Strain G157 gave MRHA of human erythrocytes only.

Mobilization tests as described by Scotland *et al.* (1979) were used to detect the presence of transfer factors in the nalidixic acid-resistant recipient strains. These were carried out using the *E. coli* K12 substrains 38R960, carrying the non-autotransferring kanamycin-resistance plasmid NTP107 (McConnell *et al.*, 1979) and 10R151 Str^r as the final recipient.

Selection and maintenance of toxigenic transconjugants. For transfer experiments, 0.1 ml of overnight cultures of donor and recipient strains were inoculated into 10 ml Hedley-Wright broth and incubated 24 h at 37 °C without shaking. The mating mixtures were washed twice and concentrated tenfold in 0.25 Ringers solution. Dilutions of mating mixtures were plated on DST agar (Oxoid) containing nalidixic acid (50 µg ml⁻¹) for a count of total recipient bacteria, and on DST agar containing nalidixic acid and an appropriate antibiotic (ampicillin, 50 µg ml⁻¹; kanamycin, 30 µg ml⁻¹; tetracycline, 10 µg ml⁻¹) to select transconjugants which had acquired the toxin plasmid carrying antibiotic resistance.

A single transconjugant from each selection was checked for drug resistance, toxin production and O serogroup, and stored on a Dorset egg slope at room temperature. In a few experiments a number of transconjugants were examined. At 6 and 12 months at least 100 colonies were recovered from the slope culture and examined for loss of drug resistance.

The ability of some transconjugants to transfer the enterotoxin plasmid which they had acquired was tested in overnight mating experiments (see above) using the streptomycin-resistant *E. coli* K12 substrains 712 or 10R151 as recipients.

RESULTS

Transfer of plasmids coding for drug resistance and toxin production

Transfer frequencies of TP214 (ST-LT-Ap), TP277 (LT-Km) and TP276 (ST-Tc), as measured by the proportion of recipient bacteria which acquired the drug-resistance marker after 24 h, are shown in Tables 2, 3 and 4.

Table 2 shows the proportion of recipient bacteria of strains belonging to serogroup O6 which acquired drug resistance. All strains accepted TP214, with the proportion of ampicillin-resistant recipient bacteria varying from 1×10^{-1} to 2×10^{-5} . For all strains fewer bacteria acquired either TP277 or TP276 and in some cases no transfer was detected. G337 and G338 were derived from the same wild-type strain but they differed in their ability to acquire TP214.

Transfer of the Ent plasmids to strains of other serotypes to which toxigenic strains commonly belong is shown in Table 3. One recipient strain, G154 (O25.H42), failed to acquire any of the three plasmids. Strain G254 (O128.H18) failed to acquire TP276 and acquired TP214 and TP277 at a low frequency ($<10^{-6}$). For the other five strains a greater proportion of bacteria acquired TP214 than acquired TP277 or TP276.

Transfer of the Ent plasmids to strains belonging to serogroups to which ETEC rarely belong is shown in Table 4. Two strains, G259 (O1.H5) and G257 (O1.H34) failed to acquire TP276. In all other experiments, transfer of the Ent plasmids was detected, although in most cases a higher proportion of recipient bacteria acquired TP214 than acquired TP276 or TP277.

Escherichia coli K12, substrain J53-1, acquired TP214 and TP277 at a high frequency and acquired TP276 at a frequency of 9×10^{-3} , which was higher than the frequencies with which any of the wild-type strains acquired this plasmid.

Acquisition of an Ent plasmid did not result in changes in the following properties of the wild-type strains: transconjugants of G153 and G334 were still colicinogenic; transconjugants of G157 and G178 were still haemolytic; transconjugants of G337 and G338 remained resistant to streptomycin and sulfathiazole; transconjugants of G157 and G337 gave MRHA.

Expression of enterotoxin genes in wild-type E. coli recipient strains

Recipient bacteria which had acquired Ent plasmids were initially recognized by the drug-resistance character with which the plasmid was marked. Such resistant transconjugants were then checked for their ability to produce enterotoxins. All resistant transconjugants from the wild-type recipients which acquired TP277 (LT-Km) were kanamycin-resistant and LT⁺. Similarly, with the exception of only one wild-type recipient strain, all wild-type recipients which acquired TP214 (ST-LT-Ap) were ampicillin-resistant, ST⁺ and LT⁺, and those which acquired TP276 (ST-Tc) were tetracycline-resistant and ST⁺. However, the recipient strain G158 (O88.H⁻) differed from all the others in expression of toxin production. When this strain acquired TP214 or TP276, the drug-resistant transconjugants failed to produce ST. Five ampicillin-resistant transconjugants of G158 from each of two separate transfer experiments with TP214 were negative for ST in the infant mouse test, giving an average gut/body weight

Table 2. *Transfer of enterotoxin plasmids to E. coli strains belonging to serogroup O6*

Recipient strain		Transfer of plasmid:					
		TP214 (ST-LT-Ap)		TP277 (LT-Km)		TP276 (ST-Tc)	
Reference no.	Serotype	Transfer frequency*	Stability in recipient†	Transfer frequency*	Stability in recipient†	Transfer frequency*	Stability in recipient†
G153	O6.H16	1×10^{-1}	80	8×10^{-6}	100	3×10^{-6}	72
G176	O6.H16	3×10^{-2}	93	5×10^{-6}	66	2×10^{-7}	1
G177	O6.H16	3×10^{-3}	100	4×10^{-7}	56	2×10^{-8}	100
G337	O6.H16	2×10^{-2}	86	0	.	0	.
G338	O6.H16	2×10^{-5}	100	0	.	0	.
G178	O6.H1	2×10^{-1}	100	0	.	0	.
G157	O6.H-	8×10^{-4}	91	6×10^{-6}	93	0	.

* Values are the proportion of recipient bacteria which had acquired drug resistance after 24 h. Where no transfer was detected the proportion of recipient bacteria acquiring a plasmid would have been less than 2×10^{-9} in each of two experiments.

† Values are the percentage of colonies derived from a transconjugant culture which were drug-resistant when tested 12 months after transfer.

Table 3. *Transfer of enterotoxin plasmids to E. coli strains belonging to serotypes which are commonly enterotoxigenic*

Recipient strain		Transfer of plasmid:					
		TP214 (ST-LT-Ap)		TP277 (LT-Km)		TP276 (ST-Tc)	
Reference no.	Serotype	Transfer frequency*	Stability in recipient†	Transfer frequency*	Stability in recipient†	Transfer frequency*	Stability in recipient†
G154	O25.H42	0	.	0	.	0	.
G155	O25.H-	4×10^{-1}	83	1×10^{-2}	97	2×10^{-5}	68
G256	O128.H10	1×10^{-1}	98	3×10^{-5}	97	7×10^{-6}	95
G254	O128.H18	9×10^{-7}	100	2×10^{-7}	13	0	.
G255	O128.H27	2×10^{-3}	62	2×10^{-8}	85	4×10^{-8}	100
G260	O128.H49	2×10^{-4}	52	3×10^{-9}	90	2×10^{-7}	77
G159	O159.H34	2×10^{-1}	100	2×10^{-2}	100	5×10^{-5}	100

*, † Footnotes as in Table 2.

Table 4. *Transfer of enterotoxin plasmids to E. coli strains belonging to serogroups which are rarely enterotoxigenic*

Recipient strain		Transfer of plasmid:					
		TP214 (ST-LT-Ap)		TP277 (LT-Km)		TP276 (ST-Tc)	
Reference no.	Serotype	Transfer frequency*	Stability in recipient†	Transfer frequency*	Stability in recipient†	Transfer frequency*	Stability in recipient†
G259	O1.H15	3×10^{-5}	100	2×10^{-8}	100	0	.
G257	O1.H34	5×10^{-4}	100	2×10^{-6}	98	0	.
G258	O1.H45	2×10^{-4}	88	1×10^{-6}	84	2×10^{-6}	3
G182	O40.H3	2×10^{-1}	100	2×10^{-4}	99	3×10^{-7}	98
G181	O71.H48	4×10^{-1}	100	4×10^{-5}	18	3×10^{-6}	94
G158	O88.H-	5×10^{-3}	1	6×10^{-5}	95	2×10^{-6}	88
G334	O154.H4	8×10^{-2}	100	8×10^{-1}	81	4×10^{-8}	84
J53-1	O rough.H48	8×10^{-1}	100	6×10^{-1}	98	9×10^{-3}	87

*, † Footnotes as in Table 2.

ratio of 0.068; all ten transconjugants gave a positive result for LT. Five tetracycline-resistant transconjugants of G158 from each of two separate transfer experiments with TP276 were negative for ST and gave an average gut/body weight ratio in the infant mouse test of 0.073.

An ampicillin-resistant, LT⁺ transconjugant of G158 (G206) transferred ampicillin resistance to *E. coli* K12 substrain 10R151; the proportion of strain 10R151 acquiring ampicillin resistance after 24 h was 3×10^{-1} and all three transconjugants of 10R151 tested produced LT and ST, giving a gut/body weight ratio greater than 0.120. A tetracycline-resistant transconjugant of G158 (G345) transferred tetracycline resistance to strain 10R151 with a frequency of 2.3×10^{-4} per recipient after 24 h; all three 10R151 transconjugants tested produced ST, giving a gut/body weight ratio greater than 0.110. So in transfers of both TP214 and TP276, the transconjugants of G158 had acquired a plasmid coding for ST production which they had been unable to express, but which functioned normally after transfer to a strain of *E. coli* K12.

It was noted that loss of TP214 occurred from G206 (see next section) and, as the test for ST is less sensitive than that for LT, it was possible that a culture containing a low proportion of bacteria carrying TP214 might result in a positive LT test and a negative ST test. However, when G206 was grown in trypticase soy broth with ampicillin ($50 \mu\text{g ml}^{-1}$) to allow the growth only of bacteria carrying TP214, ST was still not detected in the culture filtrate (gut/body weight ratio 0.068).

To test whether strain G206 was producing ST at a low rate, two separate culture filtrates of G206 grown in trypticase soy broth with ampicillin ($50 \mu\text{g ml}^{-1}$) were concentrated tenfold using an Amicon UM2 filter. The filtrates gave gut/body weight ratios of 0.092 and 0.061. When culture filtrates of G345 were similarly concentrated tenfold they gave ratios of 0.087 and 0.093 in the infant mouse test. Concentrated preparations of uninoculated trypticase soy broth gave gut/body weight ratios of 0.069 and 0.073. So ST production by derivatives of G158 was not demonstrated at a level characteristic of ST⁺ strains by concentrating the filtrates.

Stability of enterotoxin plasmids after transfer

Transconjugants of the wild-type strains of *E. coli* which had acquired Ent plasmids in the preceding transfer experiments were examined at intervals for the loss of these plasmids as recognized by the loss of resistance to the appropriate drug. At 6 months, loss of an Ent plasmid was only detected from 27 of the 53 transconjugants, and so they were re-examined at 12 months. The proportion of bacteria which still possessed drug resistance 12 months after the acquisition of a plasmid is shown in Tables 2-4. At 12 months, loss of a plasmid was detected in 36 of the 53 transconjugants: in 10 of 21 strains carrying TP214, in 15 of 18 strains carrying TP277 and in 11 of 14 strains carrying TP276. All three plasmids exhibited high, intermediate or low stabilities dependent on the strain in which they were resident. There was no correlation between the ability of a strain to accept the plasmid and its ability to maintain the plasmid.

When tested at six months, the Ent plasmid had been lost from at least 95 of 100 colonies tested from three strains: TP214 from G158 (*E. coli* O88.H⁻), TP276 from G176 (*E. coli* O6.H16) and TP276 from G258 (*E. coli* O1.H45). The stability of the Ent plasmids in these strains was therefore examined after a shorter time. A single antibiotic-resistant colony was isolated from each transconjugant culture and inoculated in nutrient broth. After growth for 24 h, the culture was plated out. All of 100 colonies tested from each culture were still antibiotic resistant, so plasmid loss was not rapid from these three strains.

It was possible that after acquisition of the plasmid the properties of drug resistance and the ability to produce enterotoxins might be lost independently of each other. It was not feasible to test all colonies examined in the stability studies for enterotoxin production. However, colonies obtained from strain G159 (O159.H34) which stably maintained all three Ent plasmids for 12 months showed that ten ampicillin-resistant colonies carrying TP214 were ST⁺LT⁺, ten kanamycin-resistant colonies carrying TP277 were LT⁺, and ten tetracycline-resistant colonies carrying TP276 were ST⁺.

When no loss of drug resistance was detected from a transconjugant after 12 months, transfer experiments were performed and it was confirmed that these strains were able to transmit the resident plasmid to the *E. coli* K12 substrain 10R151. This indicated that stability of the drug-resistance character was not due to chromosomal integration.

DISCUSSION

Only one strain, *E. coli* O25.H42, failed to acquire any of the Ent plasmids used in this study; however, ETEC belonging to this serotype have been reported previously by Cravioto *et al.* (1979), Merson *et al.* (1979) and Ørskov *et al.* (1976). The other 21 recipient strains all acquired TP214 (ST-LT-Ap). The plasmid was stably maintained in a number of recipient strains including many belonging to serotypes to which ETEC rarely belong, such as O40.H3 and O71.H48. In our collection of ETEC from human sources which have been isolated in many countries, strains producing ST and LT are more common than strains producing LT or ST only (Cravioto *et al.*, 1979). Other plasmids encoding ST and LT production should be studied to confirm that this class of Ent plasmid is acquired readily by recipient strains. However, ST⁺LT⁺ strains may be more common because they can arise by more than one mechanism. In some, including the strains from which TP214 originated, the ST and LT genes are encoded on a single plasmid (Gyles *et al.*, 1974; Scotland *et al.*, 1979); in other ST⁺LT⁺ strains, for example *E. coli* O78.H12, the genes for ST and LT production are carried on separate plasmids (McConnell *et al.*, 1980, 1981). The proportion of recipient bacteria acquiring TP214 ranged from 8×10^{-1} (for *E. coli* K12) to 9×10^{-7} (for *E. coli* O128.H18). The stability of TP214 in a recipient was not correlated with the ability of the recipient to acquire it; for example, the stability was high in both *E. coli* K12 and *E. coli* O128.H18, but lower in *E. coli* O25.H⁻ (transfer frequency 4×10^{-1}) and *E. coli* O128.H49 (transfer frequency 2×10^{-4}).

TP277 (LT-Km) was transferred to 18 of the 22 recipient strains and as for TP214 these included strains belonging to serogroups in which enterotoxigenic strains are rarely found. The proportion of recipient bacteria acquiring the plasmid ranged widely from 8×10^{-1} (*E. coli* O154.H4) to 3×10^{-9} (*E. coli* O128.H49) and the stability of TP277 differed in the transconjugants obtained.

There was transfer of TP276 (ST-Tc) to only 14 of the 22 recipients and in all but one experiment (with *E. coli* K12) the proportion of bacteria acquiring the plasmid was less than 5×10^{-5} . Plasmid loss was shown in 11 of the 14 transconjugants. So this particular ST plasmid was neither transferred efficiently nor maintained well by the strains of *E. coli* tested.

Six of seven strains of serogroup O6 accepted TP214 with transfer frequencies greater than 8×10^{-4} , whereas none acquired the LT or ST plasmids readily. Most ETEC of serotype O6.H16 have similarly been shown to produce both ST and LT, and the genes encoding these enterotoxins and CFA/II were carried on a single plasmid (Evans & Evans, 1978; Peneranda *et al.*, 1980; Smith *et al.*, 1983). The stability of Ent plasmids was not the same in all strains of *E. coli* O6.H16. Differences in the stability of toxin production in three strains of *E. coli* O25.H42 and also in two strains of *E. coli* O25.H⁻ have been reported indicating, as in the present work, that Ent plasmid stability is not related to the serotype of the host strain (Evans *et al.*, 1977b).

Acquisition of Ent plasmids by strains of *E. coli* O128 resulted in transconjugants unlike ETEC of this serogroup isolated from patients. In several clinical studies, *E. coli* O128 of different flagellar types have been shown to produce ST only, with the exception of strains of *E. coli* O128.H49 which produced LT only. G254 (O128.H18) was a poor recipient of TP214 (ST-LT-Ap) or TP277 (LT-Km) and failed to acquire TP276 (ST-Tc). The other strains of serogroup O128 with flagellar antigens 10, 27 and 49 all accepted TP214 with a higher frequency than the other two plasmids. Once acquired, TP277 was not highly stable in G260 (O128.H49) nor was TP276 highly stable in G256 (O128.H10). However, the high stability of certain combinations of plasmid and host bacterium, for example TP276 in G255 (O128.H27), might account for the persistence of certain enterotoxigenic strains *in vivo*.

The failure to acquire a plasmid could be due to a number of factors. The recipient may already carry plasmids which exclude or are incompatible with the plasmid carried by the donor. Such plasmids would confer individual characteristics on a recipient, which would not be associated with serotype. An attempt had been made to select recipients lacking properties which are known to be plasmid-mediated, but even where these properties were absent, the recipients could have carried plasmids coding for other properties. Effects of such plasmids on the three Ent plasmids would differ. Studies with mutants of *E. coli* K12 by Hoekstra & Havekes (1979) have demonstrated that cell envelope components, including lipopolysaccharide, are

important for the functioning of bacteria as recipients in mating experiments, and affect mating pair formation only with certain donors. Antigenic differences in the wild-type strains could therefore directly affect the ability of a strain to act as a recipient.

This survey of recipient strains shows that many serotypes can express ST and LT. However, although the strain of serotype O88.H⁻ expressed LT, it failed to express ST genes carried on two different plasmids. The only enterotoxigenic *E. coli* O88 strain in our culture collection produces LT only, but a strain of *E. coli* O88.H⁻ producing ST and LT has been reported by Evans *et al.* (1977*b*). It is not known whether the production of ST and LT was quantitatively similar in the remaining toxigenic transconjugants. There may be wild-type *E. coli* strains which are apparently non-enterotoxigenic but which carry enterotoxin plasmids which can be transmitted to other strains. The use of DNA probes (Moseley *et al.*, 1980) which reveal the presence of the enterotoxin genes, without the necessity of their expression, would be of value in detecting the occurrence of such strains.

In this study, many serotypes of *E. coli* were able to accept and maintain enterotoxin plasmids, and were able to produce enterotoxins *in vitro*. These included both serotypes to which ETEC commonly belong in diarrhoeal disease studies and serotypes which are frequently isolated from healthy persons but to which ETEC rarely belong. The ability to produce enterotoxins *in vitro* is however probably insufficient to allow a strain to cause diarrhoeal disease, and other properties, such as the ability to colonize the intestinal mucosa, may be necessary. Thus it is not known whether the enterotoxigenic strains produced in this study would be pathogenic.

The studies of the transfer of pathogenic properties in the laboratory may not resemble the transfer of these properties *in vivo* but, with this limitation, we conclude that the prevalence of certain serotypes of ETEC is not due to the particular ability of these strains to acquire or maintain Ent plasmids.

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