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Genetic Transfer of Antimicrobial Resistance and Enterotoxigenicity Among *Escherichia coli* Strains

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To understand the role of enterotoxin (Ent) plasmids in epidemics of enterotoxigenic (ET) *Escherichia coli* diarrhea in the United States, we studied the genetics of Ent plasmids in relation to *E. coli* serotypes and R plasmids. Twenty-nine ET *E. coli* strains, including all epidemic isolates available at the Centers for Disease Control, Atlanta, Ga. (CDC), were assessed for the ability to transfer antimicrobial resistances (if present) by conjugation, to mobilize a nonconjugative R plasmid, and to cotransfer enterotoxigenicity with R determinants. Of the 12 ET *E. coli* strains isolated in the United States, 5 were able to transfer R plasmids; one strain cotransferred detectable enterotoxigenicity. Another four U.S. isolates were able to mobilize plasmid DNA, but no toxin production was detected in transconjugants. Of 17 resistant ET *E. coli* from South Asia, 13 were able to transfer R plasmids; 5 of those 13 cotransferred detectable Ent plasmids. In all, 22 ET *E. coli* strains (76%) were able to initiate conjugation and genetic transfers. Six of these strains (20%) were able to cotransfer enterotoxigenicity with a conjugative R plasmid at a detectable frequency. One of the six strains transferred R and Ent determinants on a single plasmid. These data are addressed in relation to the observed immobility of Ent and R during outbreaks of ET *E. coli*, the efficacy of prophylactic tetracycline, and the worldwide occurrence of a limited number of ET *E. coli* serotypes.

Enterotoxigenic (ET) *Escherichia coli* strains are well-documented pathogens in infants and adults, and their ability to elaborate heat-labile toxin (LT) or heat-stable toxin (ST) or both can be determined readily in the laboratory (K. Wachsmuth, *in* P. D. Ellner, ed., *Recent developments in laboratory diagnosis of diarrheal diseases*, in press). These organisms possess the potential for genetic transfer of toxin production as well as other plasmid-mediated traits such as hemolysin production, resistance to antimicrobial agents, and colonization factors (3, 11). Genes responsible for ST production have been found on a transposable genetic element which can by definition ,incorporate into nonhomologous DNA (25). In view of these data, it is surprising that toxin production is reported in a limited number of serotypes (4, 18). Since 1975, the Centers for Disease Control, Atlanta, Ga. (CDC) has published epidemiological investigations of four large outbreaks of gastroenteritis caused by ET *E. coli* in the United States, and in each outbreak, the toxigenic strains were limited to the epidemic serotype (15, 19, 20, 26). Laboratory investigations to explain these observations were initiated in 1975 at the time of the first reported epidemic of ET *E. coli* diarrhea in the United States. Many of these studies, including attempts to identify the enterotoxin (Ent) plasmid(s) of the first outbreak strain (19), have yielded largely negative data.

One of the four outbreaks occurred in a hospital nursery, and the epidemic strain was found to be resistant to most of the antimicrobial agents tested (20). Further genetic transfer studies showed that ST production was transferred to 30% of the resistant transconjugants (27). Although the hospital environment presented an excellent selective environment for the migration of the large R plasmid, this was not detected when diarrheal stool was plated onto antibiotic selective media (20). Ent plasmids in the remaining outbreaks were generally stable during ET *E. coli* strain storage and were not transmissible at detectable frequencies (28).

The successful use of prophylactic antibiotics in Kenya and Morocco suggested that, within certain serotypes of *E. coli*, toxin plasmids are resident and incompatible with endemic R plasmids and the inverse (21, 22). Unlike ET *E. coli*

TABLE 1. ET *E. coli* isolated from humans in the United States

Strain	Source	Yr	Serotype[a]	Diarrheal illness[b]	Antimicrobial resistance[c]	Toxin production	Approx plasmid content (Mdal)
M9682C1	Oregon	1975	06:H16	Epidemic, adult	None	LT ST	70, 55, 33
A2511	Wisconsin	1980	06:H16	Epidemic, adult	Tc	LT ST	68, 52, 37, 26
2513	Massachusetts	1972	06:H16	Sporadic, adult	None	LT ST	85, 37, 3.9, 2.7
E2539C1	Caribbean	1976	025:NM	Epidemic, adult	Tc, Su	LT	57, 27, 4.2, 2.1, 1.5
E8544	Caribbean	1979	027:H7	Epidemic, adult	None	ST	88, 3.3
TX1	Texas	1975	078:H12	Epidemic, infant	Tc, Su, Sm, Km, Cm, Ap, Cb	ST	67, 30, 3
1116	California	1973	078:H11	Sporadic, adult	Su	LT	46, 7, 4.4, 4.1
5203	New Mexico	1970	0128:H21	Sporadic, adult	None	ST	85, 4.2, 2.9
1452	Louisiana	1980	0128:H7	Epidemic, infant	None	ST	80, 50
E4889	Caribbean	1977	0148:H28	Epidemic, adult	Tc	LT ST	100, 50, 36, 29, 2.7
0423	North Carolina	1970	0148:H28	Sporadic, adult	Su, Cm	LT ST	63, 50, 41, 28, 2.6, 2.0
5448	U.S. Army	1972	0148:H28	Sporadic, adult	None	LT ST	55, 35, 2.3

[a] NM, Nonmotile.
[b] Epidemic, Outbreak of diarrheal illness; Sporadic, single case of diarrheal illness.
[c] Tc, Tetracycline; Su, sulfadiazole; Sm, streptomycin; Km, kanamycin; Cm, chloramphenicol; Ap, ampicillin; Cb, carbenicillin.

isolated in Kenya, Morocco, and the United States (5, 21, 22), ET *E. coli* from the Far East are commonly (75%) resistant, perhaps reflecting antibiotic usage (7, 8). To identify Ent plasmids and study their interactions with R plasmids, we compared our U.S. isolates with multiresistant ET *E. coli* isolated from travelers in South Asia. These studies were, in part, reinitiated in response to the rapidly improved methodologies for Ent and plasmid indentification (2; K. Wachsmuth, in press).

MATERIALS AND METHODS

Bacterial strains. Tables 1 and 2 list the human ET *E. coli* strains in this study and some of their relevant characteristics. Table 1 contains all of the available ET *E. coli* strains from CDC epidemiological investigations of diarrheal disease occurring in the United

TABLE 2. ET *E. coli* isolated from humans in South Asia

Strain	Serotype[a]	Diarrheal illness[b]	Antimicrobial resistance[c]	Toxin production	Approx plasmid content (Mdal)
A2	04:NM	Unknown	Tc, Cm	ST	>100, 47
180-72	06:H16	Yes, adult	Tc, Cm	ST	85, 63, 50, 37, 4.9, 4.0
C10	06:H16	Yes, adult	Su, Sm	LT	60, 3.4, 3.1
D2	07:H18	No, adult	Tc, Su, Sm	LT	84, 58
A7	025:NM	Unknown	Tc, Su, Sm	LT	61, 55, 3.4, 2.7
B7	045:H10	No, adult	Tc, Su, Sm	LT	48, 39, 29, 21, 3.3
A6	060:H19	Unknown	Tc, Su, Sm	LT ST	88, 78, 52, 2.5
C3	064:H10	Yes, adult	Su, Sm	LT	50, 43, 3.6, 2.8
A4	076:H12	Unknown	Su	ST	70, 47
C9	077:H45	No, adult	Tc, Su, Sm, Ap, Cb	LT ST	100, 60, 4.0
A3	078:H12	Unknown	Tc, Su	LT ST	61, 50, 2.8, 1.9
B1	078:H12	Unknown	Tc, Su, Sm, Cm	LT	78, 52, 2.7, 2.4
A9	098:NM	Unknown	Tc, Su, Sm, Ap, Cb	LT	100, 78, 46, 2.7
B3	0153:H10	Unknown	Tc, Su, Sm, Cm	ST	78, 49, 28
C4	Rough:H19	Yes, adult	Su, Sm	LT	50, 34, 3.6, 2.9
C5	Ound:H26	No, adult	Tc, Su, Sm	LT	80, 3.6, 2.4
C7	Ound:Hund	No, adult	Tc, Su, Sm, Cm, Ap, Cb	LT ST	100, 60, 3.6, 2.5

[a] NM, Nonmotile; Ound, O group undetermined; Hund, H group undetermined.
[b] Sporadic cases of diarrheal illness.
[c] Tc, Tetracycline; Su, sulfadiazole; Sm, streptomycin; Km, kanamycin; Cm, chloramphenicol; Ap, ampicillin; Cb, carbenicillin.

States through 1980. Strains from sporadic cases of diarrhea in the United States are described in more detail in a previous publication (4). Strains from Dacca, Bangladesh were isolated at the International Center for Diarrhoeal Disease Research during studies of endemic ET *E. coli* disease and traveler's diarrhea. Strain 180-72 was isolated in India and sent to the CDC by the U.S. Army. Three recipient *E. coli* K-12 strains were used in bacterial conjugation experiments. Initially, either strain 711 (F^- *his lac phe pro trp* Nal^r) or strain Mut2, a derivative of the subline AB1157 (27), was mated with donor ET *E. coli* strains because transconjugants from these recipients have been shown to express both ST and LT production (11, 27). When no conjugal transfer of plasmid DNA was apparent from initial matings, a derivative of C600 (F^- *lacY1 leuB6 supE44 thi-1 thr-1 tonA21* λ^- Nal^r) which is deficient in restriction and modification enzymes was used. All antimicrobial susceptibility testing employed the high-potency disk diffusion method (1).

Toxin assays. Assays for both LT and ST, including growth conditions and preparation of supernatants or filtrates, have been described in detail elsewhere (4). Routinely, the infant mouse assay was used to detect ST, and a modification of the enzyme-linked immunosorbent assay was used to detect LT.

Conjugation experiments. When the donor ET *E. Coli* strain was resistant to antibiotic(s), direct matings were performed as described elsewhere (27). Transconjugants were isolated from MacConkey agar plates containing antibiotic to which the donor was resistant and also containing 20 μg of nalidixic acid per ml for counterselection. All matings were incubated at both 37 and 25°C to achieve the most efficient transfer (37°C) and to control for potentially temperature-sensitive transfer mechanisms in donor strains (25°C). The frequency of transfer was determined by the ratio of the number of transconjugants to the number of recipients. Transconjugants were subcultured to nonselective media before antibiograms and toxin tests were conducted.

When ET *E. coli* were susceptible to antibiotics, indirect matings were used, specifically, the resistance determinant mobilization technique described by Skerman et al. (24). Donor ET *E. coli* strains were mated with an intermediate recipient C600, which is susceptible to antibiotics and contains the nonconjugative streptomycin- and sulfonamide-resistant plasmid RSF1010. After 2-h matings at 37°C, the final recipient 711 was added and the mixture was reincubated for 2 to 4 h. MacConkey agar containing 20 μg of streptomycin and 20 μg of nalidixic acid per ml was used to select transconjugants containing RSF1010 which had been mobilized by a putative conjugative plasmid in the donor ET *E. coli*.

Plasmid analyses. Cleared lysates of cultures were prepared by the sodium dodecyl sulfate high-salt lysis procedure, employing one phenol extraction of protein (11), or by an alkaline-sodium dodecyl sulfate method (2). Plasmid DNA preparations were examined by vertical agarose gel electrophoresis (17).

Plasmid sizes were determined by reference to known molecular weight plasmids included in all gels. These control plasmids consisted of R1 (62 megadaltons [Mdal]), RP4 (36 Mdal), S-a (23 Mdal), and two plasmids of 105 to 140 Mdal from an avirulent derivative of *Shigella flexneri* provided by Dennis Kopecko, Walter Reed Army Institute of Research, Washington, D.C.

RESULTS

The results of serotyping, antimicrobial susceptibility testing, toxin assays, and plasmid analysis are given in Tables 1 and 2. Table 1 consists of all the available ET *E. coli* strains isolated in the United States from outbreaks of diarrheal disease before 1981 (15, 19, 20, 26) and ET *E. coli* strains of the same or similar serotype isolated from sporadic disease in the United States (4). Table 2 consists of strains from South Asia which were initially selected by toxigenicity and chosen for this study because they were resistant to several antimicrobial agents. Unlike the South Asian isolates, 9 of the 12 U.S. strains of ET *E. coli* were resistant to one or no antibiotics. The seven known U.S. epidemic strains of ET *E. coli* were initially recognized and selected by their toxigenicity, and all belong to the traditional ET *E. coli* serotypes. Five of the 17 South Asian strains (29%), selected for this study by resistances, belong to the traditional types.

Results of conjugation experiments are shown in Table 3. Five of the six U.S. ET *E. coli* strains with antimicrobial resistances were able to transfer these resistances. R plasmids varied in molecular mass from 46 to 100 Mdal. Only one of the U.S. ET *E. coli* strains (TX1) that transferred antimicrobial resistance was able to transfer its ability to produce toxin as detected in our assay systems. Of the 17 foreign ET *E. coli* strains, 13 conjugally transferred antimicrobial resistance, and 5 of these 13 concomitantly transferred the ability to produce toxin as detected in this study. The number of transconjugants which were assayed for toxin production varied greatly, depending on public health importance (high in U.S. outbreak situations) and on the total number of transconjugants (e.g., one transconjugant from parent D2). Four of the seven U.S. strains with no apparent R plasmid (including strain A2511) were able to initiate conjugation and genetic transfer. Strains 2513, 5203, and 1452 possessed plasmid DNA but were unable to mediate transfer of genetic material as detected in this study.

In the six transconjugant strains expressing toxigenicity, transfer was detected in the initial mating experiments with either 711 or Mut2 recipients. C600 was used as an alternative recipient with strains E2539C1, 0423, C10, D2, and A3 when matings with 711 or Mut2 or both did not result in transfer of R determinants. In no incidence did resistant C600 transconjugants

TABLE 3. Properties and plasmids of *E. coli* K-12 transconjugants

Parent strain	Antibiotic resistance[a]	Resistance plasmid (Mdal)	Toxin production (No. tested)	Toxin plasmid
M9682C1	RMD[b]		0 (100) LT or ST	
A2511	Tc, Su, RMD		0 (100) LT or ST	
E2539C1	Tc, Su	57	0 (430) LT	
E8544	RMD		0 (20) ST	
TX1	Tc, Su, Sm, Km, Cm, Ap, Cb	67	30 (100) ST	30
1116	Su	46	0 (80) LT	
E4889	Tc	100	0 (323) ST or LT	
0423	Su, Cm	63	0 (292) ST or LT	
5448	RMD		0 (20) ST or LT	
A2	Tc, Cm	47	0 (20) ST	
180-72	Tc, Cm	80	0 (80) ST	
C10	Su, Sm	60	0 (20) LT	
D2	Tc, Sm	58	0 (1) LT	
A7	Tc, Su, Sm	55	0 (20) LT	
B7	Tc	48	0 (20) LT	
A6	Tc	52	1 (20) ST	78
			0 (40) LT	
C9	Tc, Su, Sm, Ap, Cb	60	8 (20) ST and LT	100
A3	Tc, Su	50	0 (20) ST or LT	
B1	Tc, Su, Sm, Cm	78	2 (25) ST and LT	52
A9	Tc, Su, Sm	46	0 (20) LT	
B3	Tc, Sm	49	5 (20) ST	78
C7	Tc, Su, Sm, Cm, Ap, Cb	60 and 70	4 (20) ST	60
			2 (20) LT	100

[a] Tc, Tetracycline; Su, sulfadiazole; Sm, streptomycin; Km, kanamycin; Cm, chloramphenicol; Ap, ampicillin; Cb, carbenicillin.

[b] RMD, Resistance mobilization determinant (24).

express toxin as detected in this study. The methods and dilutions of mating mixtures employed should detect transfer frequencies of $\geqq 10^{-7}$.

One of the most unusual mating experiments involved the parent strain C7, which possessed two R plasmids, one of which contains ST genes, and a 100-Mdal plasmid containing genes for LT production. Initial antibiotic testing and assays yielded two distinct patterns of resistance and three patterns of toxigenicity (negative, ST producing, and LT and ST producing) among transconjugants from Mut2. Plasmid profiles corresponding to the toxin and resistance phenotypes provided the associations shown in Table 3.

DISCUSSION

In the first three outbreaks of ET *E. coli* diarrhea reported by the CDC (15, 19, 20), we could directly identify the Ent plasmid in only one laboratory investigation, in which it was associated with a conjugative R plasmid (27). The epidemic strain M9682C1 from the first outbreak was antibiotic susceptible and did not contain a recognizable or mobilizing Ent plasmid (24). The epidemic strain, E2539C1, from the third outbreak (15) did possess a conjugative R plasmid, but we were unable to transfer Ent at a detectable frequency (28).

This study expands those initial observations of Ent and R plasmid interactions to additional U.S. isolates of ET *E. coli* and to ET *E. coli* with multiple antimicrobial resistances. The latter were not available among the U.S. strains stored at the CDC and were collected from travelers in South Asia. Our resistant South Asian strains may not be typical of ET *E. coli* from that area, because only 5 of 17 belong to the traditional ET *E. coli* serotypes as described by Orskov et al. and based largely on studies of ET *E. coli* from South Asia (18). Conversely, all U.S. ET *E. coli* strains belong to those traditional serotypes (18). We can speculate that by selecting for resistant ET *E. coli*, we may have selected against the more commonly occurring strains.

Studies by Hartley et al. (12, 13) have shown that certain *E. coli* serotypes in human feces are more likely than others to carry R plasmids. Additionally, antibiotic use is not the only selective influence that operates on these plasmids (14). A study of ET *E. coli* in the Far East demonstrated that antibiotics did not increase the point prevalence of LT^+ ST^+ strains (6). Serotype may play an important role in the ability of a strain to accept and maintain plasmids and plasmid-mediated virulence factors

responsible for enterotoxigenicity, alpha-hemolytic activity, and colonization (4, 9, 10). Our data, including the variety of serotypes among the resistant ET *E. coli* strains, may reflect these strain specificities and plasmid incompatibilities.

A study by Franklin et al. (10) suggested that a ColB plasmid interfered with the transfer of an LT-producing Ent plasmid. In the present study, LT alone was detected in one transconjugant clone (parent strain C7). R plasmids, like ColB plasmids, may have interfered with the transfer of the LT-producing Ent plasmid; the transmissibility of ST was more easily demonstrated. This is consistent with at least two earlier studies (5, 16) which showed that LT^- ST^+ strains were more frequently resistant to tetracycline than were LT^+ ST^+ or LT^+ ST^- ET *E. coli* strains.

In reports of the successful use of prophylactic doxycycline against *E. coli* traveler's diarrhea in Kenya and Morocco, ET *E. coli* strains were predominantly susceptible to tetracycline (21, 22). In Morocco, the strains that were tetracycline resistant were LT^- ST^+ isolates (22). In the present study, 8 of 13 ET *E. coli* from South Asia and 3 of 4 ET *E. coli* from the United States were able to transfer resistance to tetracycline, and 6 of these transconjugants also received genes for Ent production (Table 3). It would be interesting to test the efficacy of doxycycline in areas harboring these tetracycline-resistant ET *E. coli* strains.

In support of the epidemiological and genotypic evidence for the spread of single ET *E. coli* clones within and beyond (18, 23) defined geographic areas, our data suggest that certain serotypes of ET *E. coli*, regardless of R plasmids, do not readily transfer Ent plasmids by conjugation. This was observed for the five strains of serotype O6:H16. All but one of these strains (2513) could initiate conjugation and genetic transfers; two possessed conjugative R plasmids. This was also true for strains of serotype O148:H28. This genetic stability does not appear to be prerequisite, because another of the globally defined serotypes, O78:H12, contains strains which are able to transfer Ent and R plasmids at a detectable frequency. Plasmid profiles among ET *E. coli* of the same (traditional) serotypes (O6:H16, O25:NM, O78:H12) are not identical and do not support this clonal concept. Plasmid DNA is subject to alteration with time through accretion, deletion, exchange, or transposition, so that descendents of the original parent strain are no longer traceable by plasmid analysis.

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