

Plasmid-Mediated Properties of a Heat-Stable Enterotoxin-Producing *Escherichia coli* Associated with Infantile Diarrhea

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The plasmid mediation and transmissibility of heat-stable enterotoxin production and multiple antibiotic resistance have been demonstrated for *Escherichia coli* O78:K80:H12 epidemiologically incriminated in a hospital outbreak of infantile diarrhea. The conjugal transfer of a 67×10^6 - and a 30×10^6 -dalton plasmid was associated with the transfer of resistances and enterotoxin production, respectively. Using antibiotics to select *E. coli* K-12 transconjugants from a one-step bacterial cross, all of the monitored resistances were transferred concurrently, and 36% of the resistant transconjugants produced enterotoxin.

The plasmid-mediated nature of important properties of enteropathogenic porcine strains of *Escherichia coli* has been well documented (23). These strains contain plasmids that code for the production of heat-labile (LT) and heat-stable (ST) toxins (Ent), the production of hemolysin, and the possession of a protein fimbria (K88) associated with the ability to colonize the porcine small intestine. More recently, Ent plasmids have been observed in strains of *E. coli* isolated from stool-specimens from ill persons (21), and these, as well as the porcine, have been characterized by deoxyribonucleic acid (DNA) sequence homology and compatibility with other known plasmid groups (10, 22). The human strains of *E. coli* used in these investigations were usually LT-ST-producing organisms because the LT-ST isolates were more clearly defined pathogens. The ST-only-producing strains were known pathogens in piglets and calves, but their role in human illness had been uncertain until 1975, when ST-only-producing *E. coli* were shown to be associated with diarrhea in travelers in Mexico (18).

Earlier studies of the transmissibility of enterotoxigenicity depended on the antibiotic selection of the total recipient population and the assay of this population in a ligated intestinal loop that detected enterotoxin production at a minimum transfer rate of 0.02. This method had the obvious disadvantage of detecting only autotransmissible plasmids, which may or may not describe the Ent (21). Using the resistance determinant mobilization test, Skerman et al. were able to select for only those recipients that

had successfully conjugated (transconjugants) and thereby increase the frequency of Ent transfer to 0.06 (21).

During an outbreak of intestinal illness in the nursery of a large children's hospital, an ST-only-producing *E. coli* was isolated and epidemiologically incriminated (R. W. Ryder, I. K. Wachsmuth, A. Buxton, D. G. Evans, H. L. DuPont, E. Mason, and F. F. Barrett, "Infantile diarrhea produced by heat stable enterotoxigenic *Escherichia coli*," N. Engl. J. Med., in press). The epidemic strain was serotype O78:K80:H12 and possessed a distinctive pattern of antibiotic resistances, including chloramphenicol, streptomycin, sulfathiazole, tetracycline, ampicillin, carbenicillin, and kanamycin, with which enterotoxin production was always associated. This association led to the investigation of a possible "epidemic plasmid" and provided the necessary markers to select resistant transconjugants from a one-step transfer experiment.

There is considerable controversy about the exact nature of an *E. coli* "infant diarrheal" enterotoxin (2, 8, 13, 16; D. J. Evans, Jr., D. G. Evans, R. P. Silver, and S. L. Gorbach, Abstr. Annu. Meet. Am. Soc. Microbiol. 1974, M210, p. 101; M. Gurwith, D. Wiseman, and N. Nelson, Abstr. Annu. Meet. Am. Soc. Microbiol. 1975, B65, p. 22). The epidemic strain may not be representative of this unique infant-associated pathogen. However, the ability of this ST-producing *E. coli* to conjugate and transfer the potentially virulent characteristics reported here provides insight into (i) the nature of an

otherwise ill-defined human pathogen and (ii) its survival and selection in a hospital environment.

MATERIALS AND METHODS

Bacterial strains. During a nursery outbreak of diarrheal illness, *E. coli* strains were isolated from rectal and pharyngeal swabs of infants, rectal swabs and hand washings of nursery personnel, and from the nursery environment. We were unable to isolate other recognized intestinal pathogens, including enteropathogenic serotypes of *E. coli* from infant stools. Strains TX1 and TX85 were isolated from infant stools at the time of well-documented illness, and strain BC was isolated from the blood of an infant with necrotizing enterocolitis. Standard methods for complete serotyping, biochemical characterization (5), and antibiotic susceptibility testing (1) were used. All strains were assayed for LT in the Y1 adrenal cell tissue culture (4, 19), for ST in infant mice (3, 14), for a more generalized, culture-stimulated diarrhea in infant rabbits (9), and for invasiveness in guinea pig eyes (20). Isolation procedures and laboratory methods are described in more detail elsewhere (Ryder et al., in press).

The recipient *E. coli* K-12 was a lactose-negative derivative of the AB1157 subline, resistant to streptomycin (200 µg/ml), nalidixic acid (NA) (40 µg/ml), and colicine b, and was obtained from Eric Moody of the University of Texas Medical School at San Antonio.

Transfer experiments. An overnight, stationary broth (Mueller-Hinton) culture of donor strains and an overnight, aerated broth culture of the recipient strain were mixed 1:10, respectively, and incubated without agitation for 2 h and 37°C. These mixtures containing approximately 2×10^8 recipient cells per ml as well as appropriate control cultures were plated onto five sets of media: (I) MacConkey agar; (II) MacConkey agar + 20 µg of NA (Sterling Drugs,

Inc.); (III) MacConkey agar + 20 µg of NA + 20 µg of chloramphenicol (Parke-Davis and Co.); (IV) MacConkey agar + 20 µg of NA + 20 µg of kanamycin sulfate (Bristol Laboratories); (V) MacConkey agar + 20 µg of NA + 8 µg of achromycin tetracycline (Lederle Laboratories). Broths were streaked onto medium I, and 0.1 ml of each was spread onto media II through V. From donor-recipient mixtures, only transconjugants should grow on medium III, IV, or V; at least 10 colonies from each medium were subcultured to heart infusion agar slants and were subsequently tested for enterotoxin production and quantitative antibiotic susceptibilities. The same experiments were conducted with only medium II (NA containing), and 100 presumptive transconjugants were examined as above.

Plasmid analysis. Analyses were performed as previously described (10). The number of plasmid species and their molecular weight in ethanol-precipitated lysates of TX1 and *E. coli* K-12 transconjugants were also analyzed by an agarose gel electrophoresis method (J. A. Meyers, D. Sanchez, L. Elwell, and S. Falkow, submitted for publication). Strains were grown overnight in 30 ml of brain heart infusion broth and harvested by centrifugation. Cleared lysates were prepared (10) and precipitated with 95% ethanol in the presence of 0.3 M sodium acetate, pH 8.0. The precipitated DNA was collected by centrifugation, resuspended in 100 µl of TES buffer [50 mM NaCl, 5 mM ethylenediaminetetraacetate, and 30 mM tris(hydroxymethyl)aminomethane, pH 8.0]. The DNA solution was applied to a Lucite vertical slab gel apparatus, and electrophoresis was carried out at 60 mA, 120 V, for 2 h. The gel was placed in a solution of ethidium bromide (0.4 µg/ml) and, after 15 min, the fluorescent DNA bands were photographed under long-wave ultraviolet light. The molecular weight of the plasmid DNA was calculated from the linear relationship between migration rate and the molecular mass of covalently closed circular DNA.

TABLE 1. Resistance transfer results

Strain	Medium ^a									
	I		II		III		IV		V	
	Pos. ^b	Neg.	Pos.	Neg.	Pos.	Neg.	Pos.	Neg.	Pos.	Neg.
Resistant control (unmated)	0 ^c	TNC ^d	0	TNC	0	TNC	0	TNC	0	TNC
Recipient (unmated)	0	TNC	0	TNC	0	0	0	0	0	0
TX1										
Unmated	TNC	0	0	0	0	0	0	0	0	0
Mixture ^e	TNC	TNC	0	TNC	0	15	0	33	0	19
TX85										
Unmated	TNC	0	0	0	0	0	0	0	0	0
Mixture	TNC	TNC	0	TNC	0	31	0	36	0	39
BC										
Unmated	TNC	0	0	0	0	0	0	0	0	0
Mixture	TNC	TNC	0	TNC	0	31	0	54	0	59

^a Described in Materials and Methods.

^b Lactose utilization. Pos., Postive; Neg., negative.

^c Number of colonies.

^d TNC, Too numerous to count (≥ 300).

^e Donor plus recipient.

TABLE 2. Number of resistant transconjugants producing enterotoxin

Donor	Medium ^a			Percent
	III	IV	V	
TX1	5/10	5/10	4/10	47
TX85	3/10	3/10	7/10	43
BC	2/10	2/10	1/10	17

^a Described in text.

RESULTS

Cultures of the epidemic strain (TX1, TX85, BC) were biochemically typical *E. coli* serotype O78:K80:H12. They were negative for LT production and invasiveness but were positive in the infant rabbit and infant mouse tests. All three strains were uniformly resistant to chloramphenicol, streptomycin, sulfathiazole, tetracycline, ampicillin, carbenicillin, and kanamycin. The zones of inhibition were measured, and no quantitative differences were seen between strains with any of the 12 antibiotics tested.

Transfer studies. Table 1 presents the results of one experiment and shows that transfer was detected with each antibiotic in the selective media. Antibiotic susceptibilities of the transconjugants showed the uniform pattern described in the donor, epidemic strain with the exception of NA resistance already chromosomally determined in the recipient *E. coli* K-12. The resistances appeared to be determined by one R-factor. The production of enterotoxin (ST) was observed in 36% of the R-factor transconjugants (Table 2), whereas the R-factor and Ent plasmid transferred at respective frequencies of 3.5×10^{-5} and 1.3×10^{-5} .

Transconjugants. Culture supernatants used in the infant mouse assay were heated, and six twofold dilutions were prepared to compare the quality and quantity of donor and transconjugant enterotoxin (Table 3). The data show that ST production in the transconjugant was equal to or greater than that in the donor strain and that both toxins were indeed stable at 60°C for 15 min. No transfer of antibiotic resistance or enterotoxin production was detected in experiments using only medium II.

Plasmids. The clinical isolate TX1 possessed 67×10^6 -, 30×10^6 -, and 3×10^6 -dalton plasmid species. The R⁺ transconjugants contained only the 67×10^6 -dalton plasmid, and the R⁺ Ent⁺ transconjugants contained both a 67×10^6 -dalton and a 30×10^6 -dalton plasmid, indicating that the 30×10^6 -dalton plasmid carries the gene governing the production of the ST enterotoxin. Figure 1 illustrates the electrophoretic migration of DNA isolated from the *E. coli* K-12 recipient and transconjugants.

DISCUSSION

Although rectal isolates of enterotoxigenic *E. coli* are uncommon in the United States in a healthy or hospitalized population without gastrointestinal illness (14, 24), there is well-documented evidence of increased occurrence of multiply resistant, R-factor-containing enterobacteriaceae with the increased use of antibiotics in hospitals (7). This study demonstrates the transmissible nature of an R-factor for multiple drug resistance and of a plasmid mediating ST production in *E. coli* O78:K80:H12 epidemiologically incriminated as the cause of infantile diarrhea in a hospital nursery. There have been at least two other nursery outbreaks associated with a specific serotype of *E. coli* in which similar resistance to multiple antibiotics was noted (2, 15); in one the resistances emerged after oral neomycin treatment of a strain initially resistant only to sulfonamides (15).

The significant number (36%) of R⁺ transconjugants that received Ent and the relative ease of this one-step transfer caution against the indiscriminate use of antibiotics that might select for this organism and lead to widespread distribution of enterotoxigenic strains in areas where the antibiotics are used. We hasten to emphasize that most transfer experiments with and without the selective pressures of antibiotics have been less successful and may be more representative of the rate of transfer of other Ent-containing *E. coli* (21). Resistance to ampicillin, carbenicillin, kanamycin, streptomycin, sulfathiazole, chloramphenicol, and tetracycline was transferred from TX1, TX85, and BC regardless of the selective antibiotic medium used and was found to be mediated by one 67×10^6 -dalton plasmid. When the same bacterial matings were performed with selection of the total recipient population, the frequency of transfer of Ent⁺ or R⁺ was too low to be detected.

TABLE 3. Enterotoxin production by donor and transconjugant strains of *Escherichia coli*

Strain	Titer ^a	
	Unheated	Heated (60°C, 15 min)
TX1	16	8
TX1 T ^b	32	16
TX85	16	8
TX85 T	16	16
BC	16	16
BC T	32	16

^a Titer is the reciprocal of the highest dilution giving a positive mouse test (intestinal to remaining body weight ≥ 0.083)

^b T, Transconjugant.

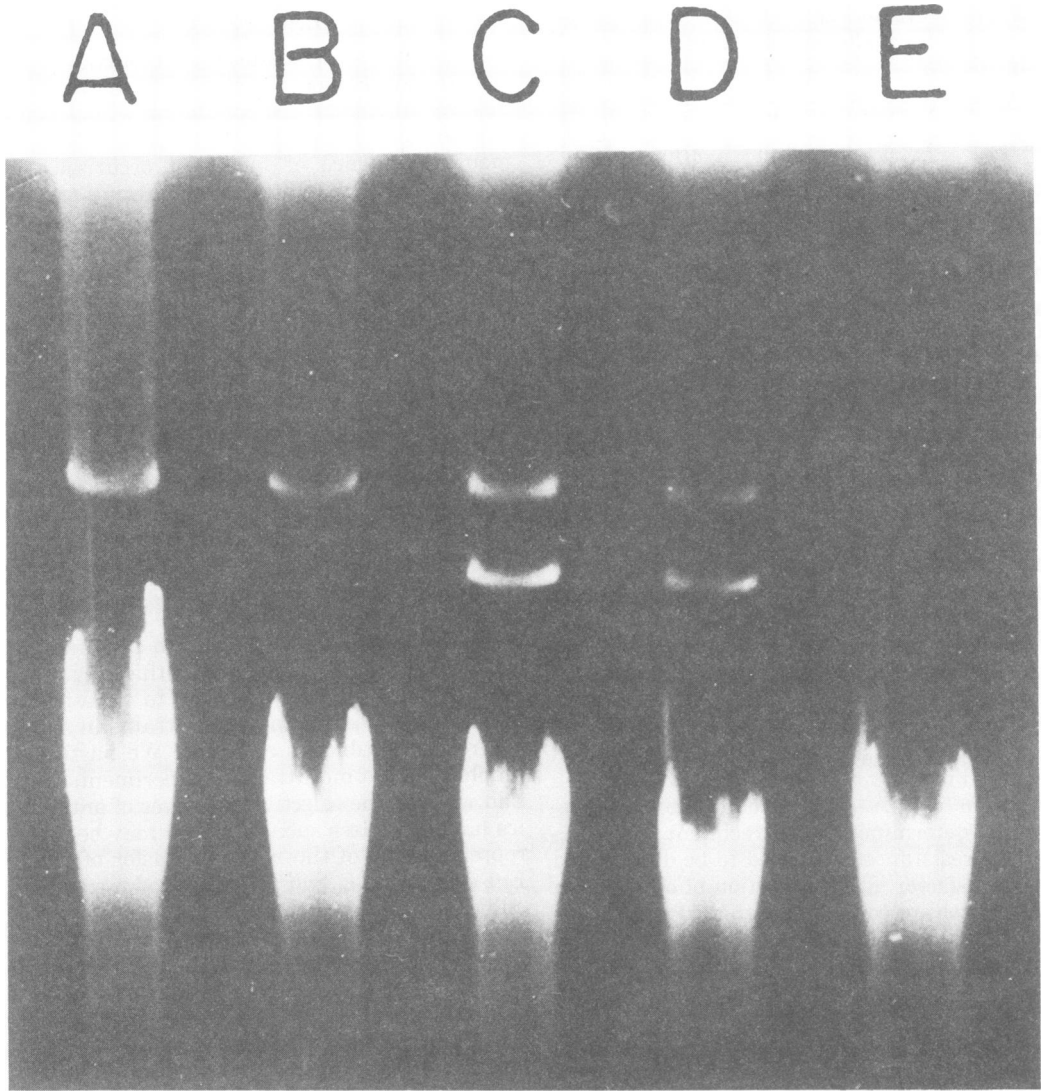


FIG. 1. Gel electrophoresis of *E. coli* K-12 DNA. (A and B) $R^+ Ent^-$ transconjugants; (C and D) $R^+ Ent^+$ transconjugants; (E) $R^- Ent^-$ recipient.

During the initial investigation, a multiply resistant, enterotoxigenic, nontypable *E. coli* and a multiply resistant, non-enterotoxigenic *E. coli* O78:H12 were isolated from infants TX440 and TX206, respectively. Both infants were concomitantly harboring the epidemic organism. These isolates indicate (i) that other strains of *E. coli* may carry the R and Ent plasmids, (ii) that *E. coli* O78 may have been Ent^+ in vivo or have spontaneously lost its Ent plasmid, and (iii) that conjugal transfer of plasmids probably occurred in vivo. No strains were found—either during the epidemic or a subsequent study—that produced enterotoxin without displaying the characteristic antibiogram.

These laboratory data suggest that ST production is not mediated by an autotransmissible plasmid.

Previous studies of LT-ST-producing *E. coli* of human and porcine origin have shown the Ent plasmids to be an homologous population of approximately 60×10^6 daltons with similar polynucleotide sequences and guanine + cytosine mole fraction. The ST-only-producing *E. coli* were porcine strains and contained a heterogeneous population of plasmids (10, 22). The reported heterogeneity may not reflect the type of Ent plasmids associated with ST-only *E. coli* causing human illness.

All but two of the 29 epidemic strains exam-

ined possessed pili that may aid in colonization of the small intestine (Ryder et al., in press); antigenically similar pili have been shown to be mediated by a 60×10^6 -dalton plasmid (6). The finding of pili without the apparent "colonization plasmid" raises many new questions, including the possibility of gene translocation. Its role in pathogenesis is further complicated by the discovery in pigs of phenotypically specific host susceptibility to the K88 fimbriae (17) and the discovery of an alternative pathway for colonization when K88 is not present (11). The pili may or may not contribute, along with antibiotic resistances and ST production, to the overall survival and virulence of this epidemic strain of *E. coli*.

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