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Linkage of Heat-Stable Enterotoxin Activity and Ampicillin Resistance in a Plasmid Isolated from an *Escherichia coli* Strain of Human Origin

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In an *Escherichia coli* strain of human origin, ampicillin resistance and heat-stable enterotoxin activity were shown by *Eco*RI restriction endonuclease and genetic analysis to be in an 80-megadalton plasmid.

The role of enterotoxigenic strains of *Escherichia coli* in the etiology of diarrhea in humans and livestock has been well documented (2, 7-9, 15, 18, 19, 22). Like antibiotic resistance in clinical enterobacteria (8), heat-stable enterotoxin (ST) and heat-labile enterotoxin (LT) production, separated or together, is plasmid coded (12, 21, 22). Some studies of human diarrheal disease (3, 13; Kupersztoch-Portnoy, unpublished data) have shown that enterotoxigenic strains are also antibiotic resistant and that they can transfer, by conjugation, toxin activities and antibiotic resistance independently (24) or simultaneously (4, 19). We report here the genetic and physical linkage of ampicillin resistance (Ap^r) and ST enterotoxin activity in a plasmid of an *E. coli* strain of human origin.

E. coli 40IEC-4 is one of 890 clinical strains of the same species isolated from an epidemiological study on the etiology of diarrhea (H. Stieglitz, J. Olarte, R. Fonseca, and Y. M. Kupersztoch-Portnoy, manuscript in preparation); it is ST^+ and resistant to ampicillin and tetracycline (Tc^r). *E. coli* K-12 J54 (*pro met* Nal^r) was kindly provided by N. Datta. Susceptibility to antibiotics was established by following the recommendations of the International Collaborative Study (5), substituting Trypticase soy agar for Mueller-Hinton agar. Conjugal transfer experiments were performed in L-broth (16), and selection of transconjugants was performed on L-agar plates with 50 μg of nalidixic acid, 100 μg of ampicillin, or 25 μg of tetracycline per ml, or a combination of these drugs. ST enterotoxin production was assayed in suckling mice as described by Dean et al. (2); however, we used the media reported by Evans et al. (6) for bacterial growth. Plasmid content determination (3 ml of culture) and large-scale plasmid isolation (5 liters of culture) were performed using exponentially growing L-broth cultures lysed by the lysozyme-ethylenediaminetetraacetic acid-Triton X-100 method (14), omitting the clearing spin. The lysates were made 1 M NaCl and incubated for 5 min; they were then diluted to 0.5 M NaCl, incubated with 1 mg of pronase per ml (predigested for 15 min at 37°C) for 15 min at 37°C, and then extracted for 15 min by immersion with a mixture of 1 volume of phenol saturated with TES [0.05 M NaCl-0.005 M ethylenediaminetetraacetic acid-0.05 M tris(hydroxymethyl)aminomethane, pH 8.0] and 0.5 volume of chloroform. The phases were separated by centrifugation; the aqueous phase was extracted free of phenol with ether and then adjusted to pH 12.0 with 5 M NaOH, incubated for 30 min at 37°C, and neutralized with 2 M tris(hydroxymethyl)aminomethane-hydrochloride. One gram of nitrocellulose (1) per ml of solution was added and mixed by inversion for 15 min. The nitrocellulose was removed by centrifugation, and the deoxyribonucleic acid (DNA) was precipitated with ethanol-sodium acetate as described previously (14). The precipitate was suspended in TE [10 mM tris(hydroxymethyl)aminomethane, pH 8-1 mM ethylenediaminetetraacetic acid]. Details of this method have been submitted for publication (J. Atzin, L. Cervantes, C. Cervantes, H. Stieglitz, and Y. M. Kupersztoch-Portnoy). Restriction endonuclease treatment of the DNA and slab gel electrophoresis were carried out as described by Greene et al. (10).

Table 1 shows the linkage of Ap^r, Tc^r, and ST activity; all transconjugants selected as Ap^r were ST^+, whereas only 4% were Tc^r. When selecting for Tc^r, 6% were Ap^r and 50% coded for ST activity. These data suggest the presence of at least two linkage groups, one of Ap^r and the other of Tc^r. ST activity could be linked to Ap or be present on a third linkage group, or both.

TABLE 1. *Cotransfer frequency of antibiotic resistance and ST*[a]

Donor strain	Resistance	Selected marker	Unselected marker (%)		
			Ap	Tc	ST
40IEC-4	Ap Tc	Ap	100	4	100
		Tc	6	100	50

[a] Conjugal transfer experiments were done from separately grown (37°C, overnight) L-broth cultures of donor (40IEC-4) and recipient (J54 Nal^r) strains. The cultures were diluted 1:10 and mixed 1:1, and the conjugation mixture was further diluted 1:40 with the same medium. Conjugation was at 37°C without agitation for 18 h. The transmission was interrupted by vigorous shaking in a Vortex mixer; samples of the conjugation mixture were diluted and plated on L-agar plates supplemented with ampicillin plus nalidixic acid or with tetracycline plus nalidixic acid; after incubation of the plates at 37°C, 100 colonies of each medium were purified two times by streaking on L-agar plates without antibiotics and tested for resistance to ampicillin and tetracycline and for the phenotypic properties of the recipient strain. Ten J54 isolates of each pattern of resistance were tested for ST activity (2).

To explore whether these linkage groups correspond to different extrachromosomal genetic elements, plasmid content analyses of the clinical strains and of transconjugants of the various segregation groups were performed. The results of representative strains of each group are shown in Fig. 1A. The clinical strain (slots 2 and 8) showed four bands. The Ap^r ST$^+$ transconjugants (slots 3 and 4) harbored a single plasmid (pYK007) of 80 megadaltons (Md); those that code for Tc^r (slots 5, 6 and 7) contained a plasmid of 21 Md (pYK009). We were therefore able to conclude that Ap^r and ST$^+$ are transferred together on pYK007 and that Tc^r is coded for by pYK009. We have detected, with high frequency, changes in the electrophoretical mobility of plasmids when clinical *E. coli* strains are mated with laboratory strains of the same species (Stieglitz and Kupersztoch-Portnoy, unpublished data). Transconjugant 4T-2 (Tc^r ST$^+$) (slot 6) has two plasmids, pYK009 and pYK008. pYK008 (78 Md) is slightly smaller than pYK007 (as determined by electrophoresis for 6 h, under the same

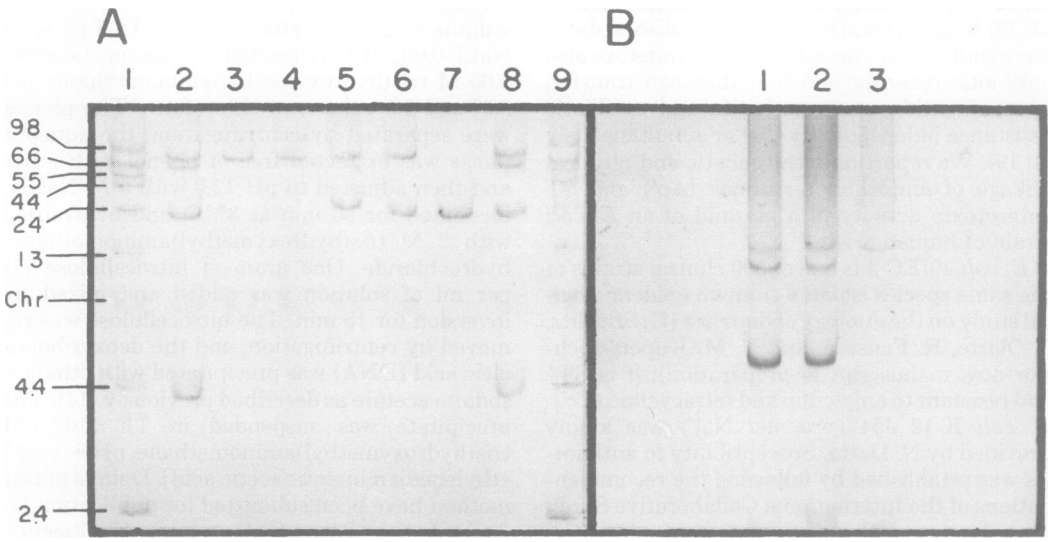

FIG. 1. *Slab agarose (0.7%) gel electrophoresis (125 V) of nitrocellulose-treated lysates of clinical and transconjugant strains. (A) Three milliliters of culture treated as described in the text and electrophoresed for 3 h. Slots 1 and 9: Strain IEC-78 used as a size marker (J. Atzin, L. Cervantes, C. Cervantes, H. Stieglitz, and Y. M. Kupersztoch-Portnoy, submitted for publication), showing eight bands whose molecular weights are indicated at the left of the figure; Chr indicates the position of contaminating chromosomal DNA. Slots 2 and 8: Clinical strain 40IEC-4 (Ap^r ST$^+$ Tc^r) with four plasmid bands: pYK007 (80 Md), pYK015 (59 Md), pYK009 (21 Md), and pYK016 (4.5 Md). Slots 3 and 4: Transconjugants 4A-104 and 4A-35, respectively, both Ap^r ST$^+$, showing pYK007. Slot 5: Transconjugant 4A-3 (Ap^r ST$^+$ Tc^r), where pYK007 and pYK009 are seen. Slot 6: Transconjugant 4T-2 (ST$^+$ Tc^r), showing pYK008 (78 Md) and pYK009. Slot 7: Transconjugant 4T-6 (Tc^r), where pYK009 is the only plasmid present. (B) Samples electrophoresed for 6 h. Slot 1: Treated lysate from 1.5 ml of 40IEC-4. Slot 2: Treated lysate from 1.5 ml of 40IEC-4 and 1.5 ml of 4A-104, mixed before lysis. Slot 3: Treated lysate of 1.5 ml of strain 4A-104. The first letter in the transconjugant designation represents the antibiotic used in the selection procedure (A, ampicillin; T, tetracycline).*

conditions; data not shown) and most likely arose by the excision of Ap^r (Tn*1*, *2*, or *3*) from pYK007. Although these results suggest that pYK007 is the plasmid responsible for Ap^r and ST activity, pYK007 could have arisen from molecular rearrangement in strain 40IEC-4 or during the conjugal transfer. Figure 1B shows the coelectrophoresis (slot 2) of lysates of strain 40IEC-4 (slot 1) and strain 4A-104 (slot 3); under these conditions the largest plasmid of strain 40IEC-4 comigrated with pYK007 of strain 4A-104, suggesting that both plasmids have the same molecular weight.

Even though Ap^r and ST^+ show tight linkage during conjugal transfer (Table 1), the possibility of two plasmids with the same electrophoretical mobility had to be discarded. Figure 2 shows the *Eco*RI digestion products of pYK007 DNA isolated from strain 4A-104. When electrophoresis was run for 1 h (Fig. 2A, slot 2), 10 fragments could be distinguished. However, a densitometer scanning (data not shown) indicated that the largest band had a higher fluorescent intensity than that expected for a single fragment. Further analysis using lower DNA concentrations and electrophoresis for 3 h (Fig. 2B, slots 2 and 3) resolved two fragments for that band. With the addition of the molecular weights of the 11 *Eco*RI fragments, the total is 81.48 Md and is in good agreement with the 80 Md calculated for pYK007. Furthermore, the same fragments resulted when the plasmid DNAs of four Ap^r ST^+ transconjugants, independently isolated in different experiments, were treated with *Eco*RI (data not shown). Thus the likelihood of random molecular rearrangements to account for the generation of pYK007 is greatly diminished.

In two of the four plasmid species present in strain 40IEC-4, phenotypic properties have been identified: pYK007 codes for Ap^r, ST^+, and *tra* (data not shown), and pYK009 codes for Tc^r; pYK015 and pYK016 so far are cryptic plasmids. The four functions identified account for a small portion of the genome of these plasmids. Experiments are in progress to localize the colonization factor (2) present in strain 40IEC-4.

Gyles et al. (11) have previously shown that in a plasmid of porcine origin, ST is linked to tetracycline, streptomycin, and sulfonamides resistance and to LT activity. We now report the linkage of ST and ampicillin resistance. To our knowledge, this is the first report of the linkage of ST and drug resistance in a natural plasmid of *E. coli* of human origin. We believe that such plasmids coding for both drug resistance factors

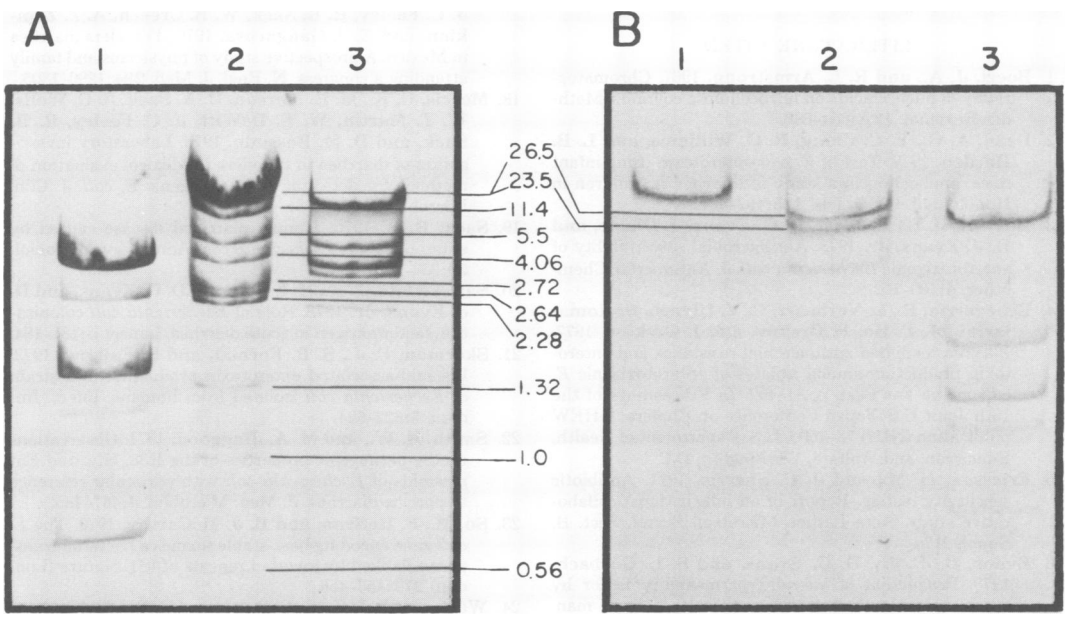

FIG. 2. *Agarose (0.8%) gel electrophoresis of EcoRI restriction endonuclease-cleaved pYK007 DNA. (A) Electrophoresis for 1 h at 125 V. Slot 1: PM2 DNA digested with HindIII. Slot 2: pYK007 DNA digested with EcoRI. The molecular masses of the fragments in megadaltons are indicated to the right of (A). Slot 3: EcoRI digest of λ DNA. Slots 1 and 3 are used as size markers. (B) Electrophoresis for 3 h at 125 V. Slots 1 and 3 are untreated and EcoRI-digested λ DNA, respectively, and are used as molecular weight standards. Slot 2: EcoRI-cleaved pYK007 DNA showing the two bands (26.5 and 23.5 Md) unresolved in (A).*

and enterotoxin are becoming more frequent as the use of antibiotics becomes more widespread. The fact that strain 4OIEC-4 is resistant to ampicillin and tetracycline should not be surprising since these antibiotics are widely consumed in Mexico. During the year of the epidemiological study, 101 and 134 tonnes of ampicillin and tetracycline were consumed in Mexico, respectively, and 63.7% and 86.5%, respectively, of the enterobacteria analyzed were found to be resistant to them (Fonseca and Kupersztoch-Portnoy, unpublished data).

It is known that many R factors are part of transposons. Ap^r has been shown in a variety of plasmids to be in Tn*1*, Tn*2*, and Tn*3* (13). Likewise, ST, in a plasmid isolated from *E. coli* of bovine origin, has been localized in Tn*1681* (23). We can account for the appearance of Tc^r ST^+ transconjugants (Table 1) and for the 3-Md difference between pYK007 (Ap^r ST^+) and pYK008 (ST^+) by proposing the excission of a transposon that codes for Ap^r, since Tn*1*, Tn*2*, and Tn*3* are all 3 Md in molecular size (13).

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