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Plasmid Coding for Drug Resistance and Production of Heat-Labile and Heat-Stable Toxins Harbored by an *Escherichia coli* Strain of Human Origin

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In a study of enterotoxigenic strains of *Escherichia coli* isolated from children with diarrhea in São Paulo, Brazil, a new enterotoxin and antibiotic resistance plasmid that carries heat-labile toxin, heat-stable toxin, and drug resistance genes was found. This is the first such plasmid to be found in a human strain of *E. coli*. The plasmid is nonconjugative, has a molecular weight of at least 54×10^6, and is mobilized by the R plasmid present in the host strain.

Enterotoxigenic *Escherichia coli* strains have been associated with diarrhea in both children and adults (10, 12, 14), as well as in animals (8). These strains produce the heat-labile enterotoxin (LT) or the heat-stable enterotoxin (ST) or both. The production of LT and ST is coded by plasmids termed Ent (17). Several LT, ST, or LT ST plasmids from human and animal strains have been described (7, 14). However, genetic transfer of such plasmids is difficult to study because of the absence of a direct selection procedure or a convenient method for screening very large numbers of colonies for toxin production. Some Ent plasmids have been described which also carry genes for drug resistance and can therefore be detected by direct selection. These plasmids include pCG86, coding for sulfadiazine (Su), streptomycin (Sm), and tetracycline (Tc) resistance and LT ST production (9); a plasmid coding for ampicillin (Ap) resistance and ST synthesis (18); and a plasmid coding for Sm and Tc resistance and LT production (5). The first plasmid was isolated from a porcine strain, and the other two were isolated from human strains. The objective of this work was to find naturally occurring recombinant Ent plasmids containing some other marker in human strains of *E. coli*. The plasmid characteristics we studied were drug resistance and production of colicin, hemolysin, and H_2S.

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Five *E. coli* strains producing both LT and ST toxins, isolated in São Paulo, Brazil (13), from feces of children with diarrhea were studied. *E. coli* K-12 strains MA335 (*pro met his trp* Nal^r), C600 (*thr leu thy thi lac* Nal^r), 711 (*phe his pro trp lac* Nal^r), and J53 (*pro met thi*) were used as recipients for the mating experiments. Strain MA335 was used as an indicator strain for colicin production. The technique of Azevedo and Costa (1) for detection of colicin was used. For the test of production of hemolysin, the strains were grown on Columbia agar base (Oxoid Ltd.) plates containing 5% sheep erythrocytes previously washed with phosphate-buffered saline. Hemolysis was detected after incubation at 37°C for 16 to 20 h. The production of H_2S was tested by inoculating the strains in EPM medium (M. R. F. Toledo, C. F. Fontes, and L. R. Trabulsi, Rev. Microbiol., in press). The positive strains develop a black color in the medium. The strains were characterized for drug resistance to Su, Sm, Tc, chloramphenicol (Cm), kanamycin (Km), Ap, and nalidixic acid (Nal) by the plate dilution method (19). Mueller-Hinton agar (Difco Laboratories) was used for the plates containing Su, and nutrient agar (Difco) was used for the other drugs. The strains were considered resistant when they grew at 100 μg of Su per ml, 10 μg of Sm, Cm, Km, and Ap per ml, and 5 μg of Tc and Nal per ml. For the detection of LT enterotoxin, the system used was the Y1 mouse adrenal tumor cell assay (4), as modified for microtiter plates by Sack and Sack (15). For the test of ST enterotoxin, the infant mouse assay described by Dean et al. (3) was used.

TABLE 1. Transfer of the genetic markers of strain TR432/6 by using *E. coli* MA335 and *E. coli* C600 as recipient strains

Recipient strain	Selective plate	Transfer frequency	No. of colonies tested	No. and pattern of transconjugants
MA335	Su	1.33×10^{-7}	38	5 Su Sm Tc LT ST 18 Su Sm Tc 15 Su Sm LT ST
	Sm	5.84×10^{-6}	40	2 Su Sm Tc LT ST 14 Su Sm Tc 24 Su Sm LT ST
	Tc	4.06×10^{-6}	40	3 Su Sm Tc LT ST 37 Su Sm Tc
C600	Su	1.53×10^{-7}	10	10 Su Sm LT ST
	Sm	2.30×10^{-7}	10	10 Su Sm LT ST
	Tc	3.38×10^{-6}	10	4 Su Sm Tc LT ST 6 Su Sm Tc

The five strains showed the following phenotypes: LT ST (two strains), Sm LT ST, Sm Col LT ST, and Su Sm Tc LT ST. H_2S and hemolysin production were not detected in any of the strains.

The three strains that showed characteristics that could be associated with plasmids were mated with *E. coli* MA335, and transconjugants were selected according to the resistance pattern of the donor strain. The strains were grown in brain heart infusion broth (Difco), and the mating was performed at 37°C for 2 h without agitation. Transconjugants resistant to drugs were selected on nutrient agar containing the drugs to which the donor strains were resistant or on minimal medium A of Davis and Mingioli (2) containing Su; both kinds of plates had Nal. The transfer of colicin production was tested in 50 colonies grown on the selective plates containing nutrient agar plus Nal. The transfer studies were also performed with *E. coli* C600 as the recipient strain. The selective plates used were minimal medium A containing Su, Sm, or Tc. Ten colonies of each selective plate were used for cotransfer studies and LT and ST production tests.

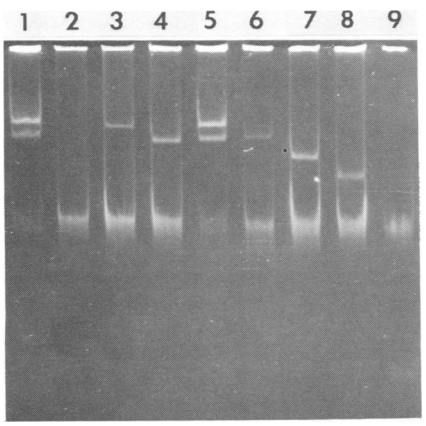

FIG. 1. Agarose gel electrophoresis of strain TR432/6 (lane 1), *E. coli* C600 (lane 2), and C600 transconjugants Su Sm LT ST (lane 3), Su Sm Tc (lane 4), and Su Sm Tc LT ST (lane 5). Reference plasmids (lanes 6 to 9): Ent P307 (54 megadaltons), RP4 (35 megadaltons), Sa (23 megadaltons), and pBR322 (2.6 megadaltons).

The results obtained showed that only strain TR432/6 transferred the drug resistance genes. Colicin production was not found to be transferred on nutrient agar selective plates. Transconjugants (38 to 40) from each selective plate from the mating with MA335 were purified in the same medium and then tested for the other characteristics of the donor. Table 1 shows that two patterns of drug resistance were found: Su Sm and Su Sm Tc. The transfer of the Ent plasmid was studied by testing the drug-resistant transconjugants for LT and ST production. It was observed that all transconjugants with the Su Sm pattern and some with Su Sm Tc pattern were enterotoxigenic. When *E. coli* C600 was used as the recipient strain, the transconjugants studied had the phenotype Su Sm LT ST or Su Sm Tc (Table 1). These transconjugants were later used for retransfer studies and agarose gel electrophoresis.

The agarose gel electrophoresis technique described by Eckhardt (6) was used. The molecular weight values of the plasmids were estimated by using as references the plasmids Ent P307 (LT ST; molecular weight, 54×10^6), RP4 (Tc Km Ap; 35×10^6), Sa (Su Sm Cm Km; 23×10^6), and pBR322 (Tc Ap; 2.6×10^6). Figure 1 shows the analysis made by electrophoresis with the TR432/6 strain and some C600 transconjugants. Strain TR432/6 possesses two bands of plasmid DNA (lane 1). The upper band (A) and

TABLE 2. Transfer of Su Sm Tc and Su Sm LT ST plasmids to *E. coli* 711

Donor strain	Pattern	Transfer frequency	No. of colonies tested	No. and pattern of transconjugants
C600/1	Su Sm Tc	1.30×10^{-6}	6	6 Su Sm Tc
C600/2	Su Sm Tc	5.71×10^{-6}	11	11 Su Sm Tc
C600/11	Su Sm LT ST	—[a]	—	—
C600/12	Su Sm LT ST	—	—	—
C600/13	Su Sm Tc LT ST	6.57×10^{-6}	10	1 Su Sm Tc LT ST 9 Su Sm Tc

[a] —, no transconjugants.

the lower band (B) are also found in the Su Sm Tc LT ST transconjugant (C600/13) (lane 5). Transconjugant Su Sm LT ST (lane 3) possesses only band A, and transconjugant Su Sm Tc (lane 4) possesses only band B. Strain C600 is shown in lane 2. Reference plasmids Ent P307, RP4, Sa, and pBR322 are shown in lanes 6, 7, 8, and 9, respectively. It can be observed that the plasmid related to band A has a molecular weight at least as high as that of Ent P307 (54×10^6). The results obtained with the mating experiments and agarose gel electrophoresis showed that strain TR432/6 carries two plasmids, one coding for Su Sm Tc resistance and the other coding for Su Sm resistance and LT ST production.

Transconjugants with one or the other plasmid and both plasmids were mated with *E. coli* 711 to test whether they were conjugative or not. The selective plates were minimal medium A contianing Sm. Table 2 shows that only the Su Sm Tc plasmid is conjugative. The Su Sm LT ST plasmid is nonconjugative but is mobilized by the Su Sm Tc plasmid, as observed in the mating experiment with C600/13 as the donor strain. Su Sm LT ST transconjugants C600/11 and C600/12 were also mated with another recipient strain, *E. coli* K-12 J53, and no transfer of the plasmid was observed, even after a 24-h mating.

So far, only one plasmid coding for LT and ST toxins that has additional genes has been described (9). This plasmid, pCG86, also codes for Su, Sm, and Tc resistance. Because of the drug resistance genes in such a plasmid, it was possible to obtain LT^- and ST^- mutants by employing a comutagenesis technique (16). Plasmid pCG86 was described in an *E. coli* strain of animal origin. Ent plasmids containing drug resistance genes resulting from recombination events (R. P. Silver, W. Aaronson, and C. F. Garon, Abstr. Annu. Meet. Am. Soc. Microbiol. 1979, B19, p. 18) or generated by transpositional events (11) have also been described.

This note describes the occurrence of a new Ent-R plasmid, designated pMS432. It is the second naturally occurring plasmid described that carries LT, ST, and drug resistance genes, but it is the first such plasmid found in a human strain. The plasmid is nonconjugative, has a molecular weight of at least 54×10^6, and is mobilized by an R plasmid present in the host strain, *E. coli* TR432/6.

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