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Plasmids Coding for Drug Resistance and Localized Adherence to HeLa Cells in Enteropathogenic *Escherichia coli* O55:H$^-$ and O55:H6

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Plasmids coding for drug resistance and localized adherence (LA) to HeLa cells were found in two enteropathogenic *Escherichia coli* strains belonging to serotypes O55:H$^-$ and O55:H6. Strain 49-81 HSJ (O55:H$^-$) carries two plasmids, one coding for both ampicillin resistance (Ap^r) and LA (pMS49). Strain 71-82 HSJ (O55:H6) harbors only one plasmid, coding for resistance to sulfadiazine, chloramphenicol, kanamycin, ampicillin, and LA (pMS71). Plasmids pMS49 and pMS71 were transferred to *E. coli* K-12 711 and from this strain to *E. coli* K-12 J53. Curing with acridine orange of an Ap^r LA$^+$ transconjugant showed that both characteristics were lost simultaneously. The plasmids have a molecular weight of approximately 55 × 10^6 and are the first naturally recombinant plasmids coding for adherence and drug resistance described in enteropathogenic *E. coli*.

The mechanisms of virulence of enteropathogenic *Escherichia coli* (EPEC) have so far not been established. Toxin production (8, 9, 13) and adherence to human intestinal epithelium (4, 15, 21) and to cells in culture (4, 5, 10) have been studied. It was shown in our laboratory (16) that *E. coli* organisms adhere to HeLa cells in two different patterns, called localized adherence (LA) and diffuse adherence. The bacteria showing LA adhere to localized areas of the HeLa cells, in which they form very clear-cut microcolonies, whereas those showing diffuse adherence adhere to the whole surface of the cells. LA was observed in strains belonging to serogroups O55, O86, O111ab, O119, O125, O128ab, and O142. Further experiments showed that LA is characteristic of serotypes considered to be EPEC (17). Focal adherence of *E. coli* O111:H$^-$ cells to the small bowel epithelium and packed aggregates on HEp-2 cells were previously described by Clausen and Christie (4).

Baldini et al. (1) demonstrated that strain E2348 (O127:H6), which causes diarrhea in volunteers and is HEp-2 adhesive, has a 55-megadalton plasmid coding for adherence. This plasmid was also shown to correlate with in vivo adhesion to intestine when the colostrum-deprived piglet model was used. Recently it was shown by Nataro et al. (12) that the LA pattern corresponds to that observed by Baldini et al. (1) in HEp-2 cells and described as microcolonies on the cells.

As drug resistance has been frequently observed in adherent EPEC strains (4, 10, 15), the purpose of this work was to find naturally occurring recombinant plasmids coding for LA and drug resistance in this group of *E. coli*.

Thirty-one EPEC strains showing LA and belonging to serotypes O55:H$^-$, O55-H6, O86:H34, O111ab:H$^-$, O111ab:H2, O119:H$^-$, O119:H6, O125:H21, O142:H$^-$, and O142:H6 were studied. All strains were isolated from infants with diarrhea. Five strains of serotype O119:H$^-$ were from the St. Louis Children's Hospital, St. Louis, Mo., and the others were isolated in São Paulo, Brazil. *E. coli* K-12 strains 711 (*phe his pro trp lac* Nal^r) and J53 (*pro met thi*) were used as recipients for the mating experiments. A rifampin (Rif) mutant of strain 711, obtained in our laboratory, was also used.

The plasmid profiles of the EPEC strains and the transconjugants were determined by the technique of Birnboim and Doly (3). The strains were characterized for drug resistance to sulfadiazine (Su), streptomycin (Sm), tetracycline (Tc), chloramphenicol (Cm), kanamycin (Km), ampicillin (Ap), gentamicin (Gm), cephalothin (Cf), co-trimoxazole (Cot), and nalidixic acid (Nal) by the disk method of Bauer et al. (2) with Mueller-Hinton agar (Difco Laboratories, Detroit, Mich.) plates. Resistance to rifampin was determined with nutrient agar (Difco) plates containing 100 μg of this drug per ml.

HeLa adherence tests were performed as described by Scaletsky et al. (16) by growing the cells on cover slips in Leighton tubes. However, to test large numbers of transconjugants eight-chambered cell culture slides were used (Miles Scientific, Div. Miles Laboratories, Inc., Naperville, Ill.). An amount of 0.3 ml of a suspension with approximately 7.5 × 10^4 cells per ml of Dulbecco modified Eagle medium (GIBCO Laboratories, Grand Island, N.Y.) with 10% fetal calf serum was added to each chamber, and the slides were incubated at 37°C for 48 h. The cells were then washed with phosphate-buffered saline, and 288 μl of the same medium containing 2% fetal calf serum was replaced in the chambers just before use. Bacterial strains were grown in 3 ml of Trypticase soy broth (BBL Microbiology Systems, Cockeysville, Md.) for 16 to 18 h at 37°C. An inoculum of 12 μl of each bacterial culture was added to each chamber. The slides were then incubated for 30 min (infection period), washed, incubated again for 3 h (multiplication period), fixed, and stained as described by Scaletsky et al. (16). The tests were done in the presence of 1% D-mannose added to Dulbecco modified Eagle medium.

Twelve strains belonging to the 10 EPEC serotypes studied and harboring no more than four plasmids each were chosen for mating experiments. The strains were grown in Trypticase soy broth, and the mating was performed at 37°C for 18 h without agitation. Transconjugants resistant to drugs

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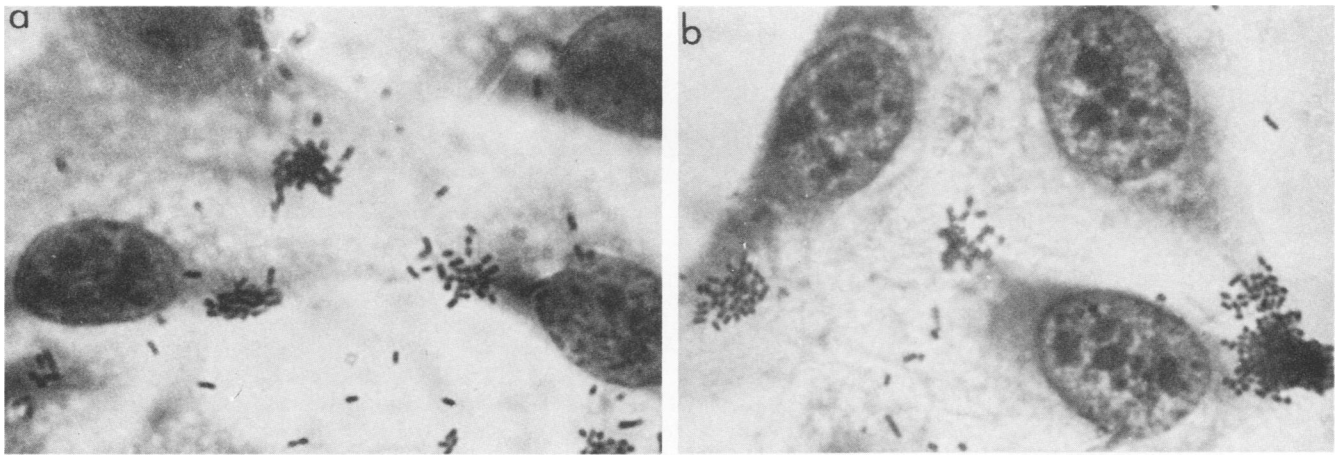

FIG. 1. *E. coli* 71-82 HSJ (a) and transconjugant (b), showing LA pattern of attachment to HeLa cells.

were selected on nutrient agar containing the drugs to which the donor strains were resistant or on Mueller-Hinton agar (Difco) containing sulfadiazine; both kinds of plates had nalidixic acid or nalidixic acid and rifampin according to the recipient strain used (711 or 711 Rif^r). The concentrations of drugs (per milliliter) in the selective plates were 100 μg for sulfadiazine, ampicillin, co-trimoxazole, and rifampin and 20 μg for streptomycin, tetracycline, chloramphenicol, kanamycin, cephalothin, gentamicin, and nalidixic acid. The colonies grown on each selective plate were purified in the same medium and then tested for the other drugs according to the resistance pattern of the donor strain by replica plating.

Six EPEC strains transferred drug resistance genes. Two transconjugants of each pattern were tested for adherence to HeLa cells. It was observed that only transconjugants from strains 49-81 HSJ (O55:H^-) and 71-82 HSJ (O55:H6) showed LA.

Strain 49-81 HSJ carries two plasmids, one (pMS49) of high molecular weight and another one of low molecular weight. Only plasmid pMS49 is conjugative. The phenotype of donor strain and transconjugants was Ap LA.

Strain 71-82 HSJ harbors only one plasmid (pMS71), coding for Su Cm Km Ap LA. Three different phenotypes were found among the transconjugants, i.e., Su Cm Km Ap LA, Cm Km Ap LA, and Ap LA. Fig. 1 shows the LA pattern presented by strain 71-82 HSJ and an Ap LA transconjugant.

Agarose gel electrophoresis was used to estimate the molecular weight values of the plasmids. The reference plasmids were pMAR2 (adherence to HEp-2 cells [1]; molecular weight, 55×10^6), Ent P307 (heat labile, heat stable; 59.4×10^6), RP4 (Tc Km Ap; 35×10^6), and Sa (Su Sm Cm Km; 23×10^6). Plasmids pMS49 and pMS71 have a slightly different molecular weight (Fig. 2), of approximately 55×10^6.

Transconjugants with phenotype Ap LA from strains 49-81 HSJ and 71-82 HSJ were mated with *E. coli* K-12 J53 for retransfer tests. The selective plates were minimal medium A (6) containing ampicillin. All Ap transconjugants also showed LA.

An Ap LA transconjugant was also treated with 200 μg of acridine orange per ml for curing (11). This concentration was determined in a previous experiment with 50, 100, 150, 200, and 250 μg of acridine orange per ml. Among 500 colonies tested, 6 were Ap^s and no longer adherent to HeLa cells. Agarose gel electrophoresis showed no plasmids in these strains.

Virulence plasmids have been described coding for enterotoxin production plus colonization factors (14, 20) or plus resistance genes (7, 18). We describe here the occurrence of new plasmids, coding for adherence to HeLa cells and drug resistance.

The relationship between adherence and drug resistance in EPEC was stressed by Lacroix et al. (10), who found 15 strains of *E. coli* O111:K58:H2, isolated from cases of severe protracted diarrhea, showing adherence to HeLa cells in

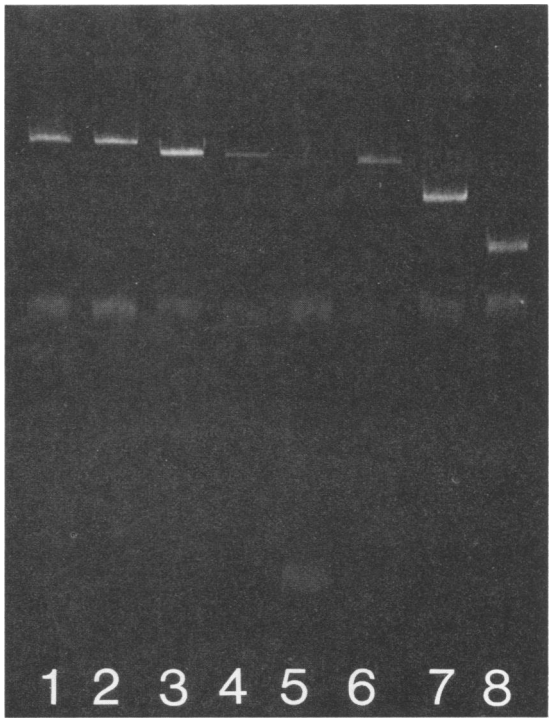

FIG. 2. Agarose gel electrophoresis of strain 49-81 HSJ (lane 1), *E. coli* 711 Ap LA transconjugant (lane 2), strain 71-82 HSJ (lane 3), and *E. coli* 711 Su Cm Km Ap LA transconjugant (lane 4). Reference plasmids (lanes 5 to 8, respectively): pMAR2 (55 megadaltons, in strain E2348), Ent P307 (59.4 megadaltons), RP4 (35 megadaltons), and Sa (23 megadaltons).

large bacterial clumps and resistance to chloramphenicol, kanamycin, ampicillin, cephalothin, and ticarcillin. Rothbaum et al. (15), studying 15 infants with protracted diarrhea, found EPEC strains of serotype O119:B14 adherent to enterocytes and resistant to ampicillin and carbenicillin. Clausen and Christie (4) described the isolation of EPEC strains of serotype O111:K58:H$^-$, which showed focal adherence to the small bowel and to HEp-2 cells and resistance to sulfadiazine, kanamycin, ampicillin, cephalothin, carbenicillin, and neomycin. Multiple resistance was also observed among the EPEC strains isolated in São Paulo (19). Among 90 strains studied, 48 were resistant to seven or eight drugs, with most of these strains belonging to serogroups O55, O111, O119, and O142. The majority of the strains were resistant to sulfadiazine (78 strains) and ampicillin (71 strains).

Further experiments are under way to search for other naturally recombinant plasmids in strains belonging to the remaining EPEC serotypes.

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