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Localization of a Determinant for HEp-2 Adherence by Enteropathogenic *Escherichia coli*

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pMAR2, a 60-megadalton plasmid encoding localized HEp-2 adherence in enteropathogenic *Escherichia coli*, was mapped with *Bam*HI, *Hin*dIII, and *Sal*I. Deletion and insertion mutants were constructed and used to define a potential DNA probe. Preliminary results indicate that this probe is sensitive and specific for the genes encoding the enteropathogenic *E. coli* adherence factor.

Enteropathogenic *Escherichia coli* (EPEC), the first *E. coli* to be described as a causative agent of diarrhea, is a major bacterial cause of infantile gastroenteritis in developing countries (8, 17). Virulence properties of other diarrheagenic *E. coli*, such as the toxins (6) and colonization factor antigens (7, 15) of enterotoxigenic *E. coli* or invasiveness (6) of enteroinvasive *E. coli*, have been well documented. The pathogenic mechanisms of EPEC, however, are only beginning to be elucidated. Electron microscopy of intestinal biopsies reveals a characteristic histopathology consisting of EPEC attached to and effacing the microvilli (14). In the HEp-2 tissue culture assay, we have found that 30 to 40% of EPEC attach and form microcolonies, whereas enterotoxigenic and normal flora *E. coli* are almost always negative (1, 12). E2348, an O127:H6 EPEC which adheres to HEp-2 cells in culture, contains a 60-megadalton (MDa) plasmid, pMAR2. This plasmid has been shown to encode the ability to adhere to HEp-2 cells and is associated with the ability of E2348 to cause the characteristic histopathologic lesion in the colostrum-deprived piglet model (1). The integral involvement of pMAR2 in the pathogenicity of E2348 has also been demonstrated in adult volunteers (9). Diarrhea was seen in 9 of 10 volunteers who received E2348 containing pMAR2, in contrast to only 2 of 9 volunteers who received E2348 cured of pMAR2. Furthermore, the diarrhea seen in these two volunteers was much milder. The gene product responsible for the ability of E2348 to adhere to cultured HEp-2 cells and piglet intestinal mucosa has been designated the EPEC adherence factor (EAF) (9, 11). The volunteer trial suggests that the EAF is an important virulence factor in EPEC. We detail here the mapping of pMAR2 as well as the generation of deletion and insertion mutations and their use to define a potential DNA probe. The preliminary evaluation of the sensitivity and specificity of this probe for the EAF is also reported.

Mapping of pMAR2. pMAR2 was extracted by the method of Birnboim and Doly (2) and isolated by CsCl-ethidium bromide density gradient ultracentrifugation. The purified plasmid was digested with *Bam*HI, *Hin*dIII, and *Sal*I (Bethesda Research Laboratories, Inc., Gaithersburg, Md.), singly or in combination. Fragments were separated by agarose or polyacrylamide gel electrophoresis and visualized by UV transillumination after ethidium bromide staining. Selected fragments were isolated by electroelution and digested with a second enzyme to confirm the position of restriction sites. Digestion patterns were analyzed to determine fragment size and relative position of restriction sites in pMAR2 (Fig. 1).

Localization of EAF genes. We first attempted to isolate the genes encoding HEp-2 adherence by cloning individual fragments from pMAR2. *Bam*HI, *Hin*dIII, and *Sal*I fragments were cloned into a high-copy-number vector, pACYC184 (4), or a low-copy-number vector, pMAR12, derived from R388 (M. M. Baldini and J. B. Kaper, unpublished data). *E. coli* HB101 (3) was transformed with these recombinants by the method of Dagert and Erlich (5) and tested for adherence to HEp-2 cells as described previously (12). Although the fragments cloned encompassed the entire plasmid, none of these recombinants retained the ability to adhere to HEp-2 cells.

The inability to isolate the genes encoding HEp-2 adherence during these preliminary cloning attempts suggests that more than one region of pMAR2 contains gene(s) required for this adherence, as is the case with colonization factor antigen I (16). Alternatively, the recognition sites of these enzymes may interrupt the genes that encode HEp-2 adherence. An alternate cloning strategy, designed to circumvent these problems, was used to delete regions of pMAR2 not essential for HEp-2 adherence. pMAR7, a Tn*801*-marked derivative of pMAR2 (1), was digested with *Sau*3A to generate essentially random fragments. We varied the incubation time and temperature and *Sau*3A concentration to determine digestion conditions which would generate 30- to 90-kilobase-sized fragments. These fragments were ligated overnight at 15°C with T4 DNA ligase (Bethesda Research Laboratories), transformed into *E. coli* HB101, and plated on L agar containing 200 μg of ampicillin per ml to select for the Ap^r of Tn*801*. Plasmids extracted from the resulting transformants were examined by agarose gel electrophoresis. Transformants containing the six plasmids that showed significant deletion were tested for adherence to HEp-2 cells in culture. One plasmid, pMAR15, which retained the ability to encode HEp-2 adherence, was deleted for 60% of pMAR7. The portion of pMAR2 encompassed by pMAR15 is indicated in Fig. 1.

To localize the genes encoding the EAF, the 18-MDa *Sal*I fragment of pMAR7 (corresponding to the large *Sal*I fragment of pMAR15) was isolated and ligated, forming pMAR16. *E. coli* HB101 containing pMAR16 did not adhere to HEp-2 cells. Therefore, the possibility that the adherence genes span one or both of the *Sal*I sites of pMAR15 was examined by cloning a gene encoding kanamycin resistance

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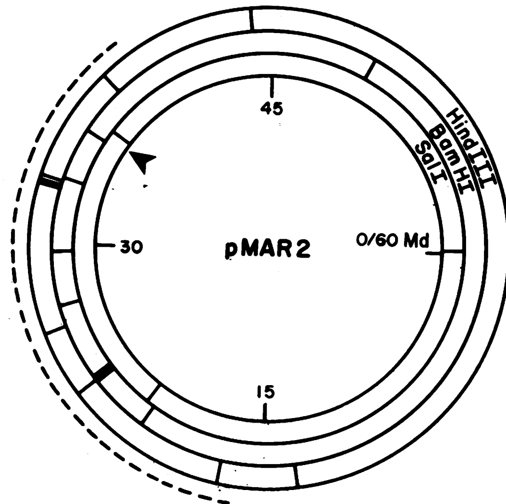

FIG. 1. Restriction endonuclease map of pMAR2. The region encompassed by pMAR15 is indicated by dashed line. The 1-kilobase *Bam*HI-*Sal*I fragment used as a probe is indicated by the arrow.

(Km^r) into either *Sal*I site. Digestion conditions which produced *Sal*I linear molecules of pMAR15 were determined empirically as described above. These linears were ligated with a *Sal*I fragment encoding Km^r originally derived from Tn*5*, transformed into *E. coli* HB101, and plated on L agar containing 200 μg of ampicillin and 50 μg of kanamycin per ml. Plasmids extracted from the resulting transformants were digested separately with *Bam*HI and *Hin*dIII. Digestion patterns were examined by agarose gel electrophoresis to determine in which *Sal*I site the Km^r fragment was inserted. pMAR15 with the Km^r fragment inserted into the *Sal*I site at ca. 36 MDa was designated pMAR17; pMAR19 consisted of pMAR15 with the Km^r fragment inserted into the *Sal*I site at ca. 21 MDa. Strain HB101 containing these mutant plasmids was tested for HEp-2 adherence. pMAR19 retained the ability to encode adherence to HEp-2 cells, whereas pMAR17 did not. These results indicated that the genes encoding the EAF span the *Sal*I site at 36 MDa.

DNA hybridization probe specific for EAF^+ strains. Because the region near the *Sal*I site at 36 MDa is apparently involved in HEp-2 adherence, the sensitivity and specificity of the 1-kilobase *Bam*HI-*Sal*I fragment to the left of this *Sal*I site as a DNA probe was evaluated by the colony blot method described by Moseley et al. (10). To prepare the probe, pMAR15 was digested with *Bam*HI and *Sal*I, and the fragments were separated by polyacrylamide gel electrophoresis. The isolated 1-kilobase *Bam*HI-*Sal*I fragment was electroeluted from the gel and purified by successive phenol, chloroform, and ether extractions. This fragment was labeled with [^{32}P]dATP by nick translation (13) and used to probe 28 EPEC, which had been tested for adherence to HEp-2 cells (Table 1). Hybridization to the probe correlated with the ability to adhere to HEp-2 cells.

Although the extent of the EAF gene(s) is not known, the localization of one region encoding this factor resulted in the identification of a DNA probe which appears sensitive and specific for these genes. The sample size in the present study was small; however, this DNA probe has also been used in an epidemiological survey of infantile gastroenteritis in Peru which confirmed its sensitivity and specificity in detecting HEp-2 adherent EPEC (11). Ongoing efforts to isolate and characterize these genes should enable us to accurately assess the importance of the EAF as a virulence factor of EPEC.

TABLE 1. HEp-2 adhesion and EAF probe reaction for EPEC of various serotypes[a]

Serotype	Strain no.	HEp-2 adhesion	EAF probe reaction
O26:NM	2184-76	−	−
	2262-79	−	−
O26:H11	1557-77	−	−
	1639-78	−	−
	3326-61	−	−
O55:NM	2374-73	−	−
	2362-75	+	+
O55:H6	2087-77	+	+
	1869-71	−	−
O55:H7	0036-78	−	−
	0660-79	−	−
O86a,b:H3b	5017-53	−	−
O111a,b:NM	2340-78	+	+
	2198-77	−	−
O111a,b:H2	3252-76	+	+
O111a,b:H12	2085-77	−	−
	0247-69	−	−
O119:H6	0659-79	+	+
	2395-80	+	+
	2450-80	+	+
O127a:H4	3715-67	−	−
	2749-75	−	−
O127a,b:NM	1092-80	+	+
	1104-80	+	+
O128a,c:NM	1791-79	−	−
	1381-73	−	−
O128a,c:H12	2520-81	−	−
	541-75	−	−

[a] Strains were isolated from human stool samples and were received from I. Kaye Wachsmuth of the Centers for Disease Control, Atlanta, Ga.

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LITERATURE CITED

1. **Baldini, M. M., J. B. Kaper, M. M. Levine, D. C. A. Candy, and H. W. Moon.** 1983. Plasmid-mediated adhesion in enteropathogenic *Escherichia coli*. J. Pediatr. Gastroenterol. Nutr. **2**:534–538.
2. **Birnboim, H. C., and J. Doly.** 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. **7**:1513–1523.
3. **Boyer, H. W., and D. Roulland-Dussoix.** 1969. A complementation analysis of the restriction and modification of DNA in *Escherichia coli*. J. Mol. Biol. **41**:459–472.
4. **Chang, A. C. Y., and S. N. Cohen.** 1978. Construction and characterization of amplifiable multicopy DNA cloning vehicles derived from the P15A cryptic miniplasmid. J. Bacteriol. **134**:1141–1156.
5. **Dagert, M., and S. D. Erlich.** 1979. Prolonged incubation in calcium chloride improves competence of *Escherichia coli* cells. Gene **6**:23–28.
6. **DuPont, H. L., S. B. Formal, R. B. Hornick, M. J. Snyder, J. P. Libonati, D. G. Sheahan, E. H. LaBrec, and J. P. Kalas.** 1971. Pathogenesis of *Escherichia coli* diarrhea. N. Engl. J. Med. **285**:1–9.
7. **Evans, D. G., R. P. Silver, D. J. Evans, Jr., D. G. Chase, and S. L. Gorbach.** 1975. Plasmid-controlled colonization factor associated with virulence in *Escherichia coli* enterotoxigenic for humans. Infect. Immun. **12**:656–667.

8. **Levine, M. M., and R. Edelman.** 1984. Enteropathogenic *Escherichia coli* of classical serotypes associated with infant diarrhea: epidemiology and pathogenesis. Epidemiol. Rev. **6**:31–51.
9. **Levine, M. M., J. P. Nataro, H. Karch, M. M. Baldini, J. B. Kaper, R. E. Black, M. L. Clements, and A. D. O'Brien.** 1985. The diarrheal response of man to some classical serotype enteropathogenic *Escherichia coli* is dependent on a plasmid encoding an enteroadhesiveness factor. J. Infect. Dis. **152**:550–559.
10. **Moseley, S., I. Huq, A. R. M. A. Alim, M. So, M. Samadpour-Motalebi, and S. Falkow.** 1980. Detection of enterotoxigenic *Escherichia coli* by DNA colony hybridization. J. Infect. Dis. **142**:892–898.
11. **Nataro, J. P., M. M. Baldini, J. B. Kaper, R. E. Black, N. Bravo, and M. M. Levine.** 1985. Detection of an adherence factor of enteropathogenic *Escherichia coli* using a DNA probe. J. Infect. Dis. **152**:560–565.
12. **Nataro, J. P., I. C. A. Scaletsky, J. B. Kaper, M. M. Levine, and L. R. Trabulsi.** 1985. Plasmid-mediated factors conferring diffuse and localized adherence of enteropathogenic *Escherichia coli*. Infect. Immun. **48**:378–383.
13. **Rigby, P. W., M. Dieckman, L. Rhodes, and P. Berg.** 1977. Labeling deoxyribonucleic acid to high specific activity *in vitro* by nick translation with DNA polymerase I. J. Mol. Biol. **113**:237–241.
14. **Rothbaum, R., A. J. McAdams, R. Giannella, and J. L. Partin.** 1982. A clinicopathologic study of enterocyte-adherent *Escherichia coli*: a cause of protracted diarrhea in infants. Gastroenterology **83**:441–454.
15. **Satterwhite, T. K., D. G. Evans, H. J. Dupont, and D. J. Evans, Jr.** 1978. Role of *Escherichia coli* colonization factor antigen in acute diarrhoea. Lancet **ii**:181–184.
16. **Smith, H. R., G. A. Willshaw, and B. Rowe.** 1982. Mapping of a plasmid, coding for colonization factor antigen I and heat-stable enterotoxin production, isolated from an enterotoxigenic strain of *Escherichia coli*. J. Bacteriol. **149**:264–275.
17. **Toledo, M. R. F., M. Alvariza, J. Murahovschi, S. R. T. S. Ramos, and L. R. Trabulsi.** 1983. Enteropathogenic *Escherichia coli* serotypes and endemic diarrhea in infants. Infect. Immun. **39**:586–589.