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Characterization of Plasmids Encoding the Adherence Factor of Enteropathogenic *Escherichia coli*

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Volunteer studies have shown that a 60-megadalton plasmid is required for full virulence of the human enteropathogenic *Escherichia coli* (EPEC) strain E2348/69 (O127:H6). The plasmid, designated pMAR2, encodes localized adherence to HEp-2 cells in tissue culture via the adhesin known as the EPEC adherence factor (EAF). Using a DNA probe for the EAF, we have previously shown that these genes are specific for EPEC and are usually encoded on plasmids ranging from 55 to 65 megadaltons. In this study, Southern blot analysis and S1 nuclease homology determination reveal a high degree of sequence conservation among these plasmids, despite some variation in restriction maps. Phenotypic characterization of the prototype EAF plasmid pMAR2 reveals that the plasmid belongs to the group *Inc*FII and is negative for alpha-hemolysin, colicin, and aerobactin synthesis, as well as biochemical markers and antibiotic resistance. Regions encoding adherence to HEp-2 cells were localized by Tn*801* insertion mutagenesis. Adherence genes were then cloned as two distinct plasmid regions which confer the adherence phenotype only when complementing each other in *trans*.

Enteropathogenic *Escherichia coli* (EPEC) are diarrheogenic *E. coli* of certain generally accepted serotypes which do not elaborate the known heat-labile and heat-stable enterotoxins and do not invade the intestinal mucosa (10). Although the pathogenesis of EPEC diarrhea has yet to be completely elucidated, recent evidence has begun to illuminate features characteristic of the pathogen and the disease. The distinctive histopathologic lesion of EPEC infection suggests a probable role for bacterial adherence to the intestinal mucosa (25, 32). This lesion is characterized by close adherence of the bacterium to the enterocytes of the small bowel with effacement of the normal microvillar structure. Cravioto et al. (7) demonstrated that 80% of outbreak-associated EPEC adhered to HEp-2 cells in tissue cultures whereas this trait was uncommon in normal flora or enterotoxigenic *E. coli*. Baldini et al. (1) subsequently found that the genes encoding this adherence were present on a plasmid of 60 megadaltons (MDa) in one isolate of serotype O127:H6 (E2348/69). Studies with adult volunteers (18) showed that this plasmid (designated pMAR2) was necessary for full virulence of E2348/69. The name EPEC adherence factor (EAF) has been suggested for the putative plasmid-encoded factor conferring HEp-2 adherence and full virulence in humans (18).

Using a fragment from the plasmid pMAR2 as a genetic probe for HEp-2 adherence, we have demonstrated that this trait was most often found in EPEC serogroups frequently implicated in epidemic and sporadic EPEC diarrhea and that, among these serogroups, probe homology correlated with pathogenicity (21). We have also used the EAF probe to determine the genomic location of EAF-homologous genes and found that, although these genes are generally borne on plasmids ranging in size from 55 to 65 MDa, occasional strains show evidence of homologous chromosomal genes (22).

These data offer strong evidence to support the contention that the EAF plasmid is critical to the virulence of many EPEC serogroups. The present studies were undertaken to determine whether EAF plasmids represent a family of plasmids with conserved phenotypes. We sought such information for several reasons: (i) to screen for conserved plasmid-associated biochemical phenotypes which might simplify epidemiologic and clinical study of these pathogens; (ii) to identify phenotypes of possible clinical relevance, such as antibiotic resistance and aerobactin synthesis; (iii) to better understand the genetics of the EAF as a prelude to further characterization; and (iv) to elucidate features of the genetic natural history of this virulence plasmid. The results of the current study reveal that EAF plasmids are in fact closely related yet do not carry other phenotypes characteristic of *E. coli* virulence plasmids.

We have previously reported the mapping of the EAF plasmid, pMAR2, for the recognition sites of the restriction endonucleases *Hin*dIII, *Bam*HI, and *Sal*I (2) and localized adherence regions by deletion and insertion mutagenesis. Here, we report further localization and cloning studies which have allowed us to clone adherence regions onto stable plasmid vectors.

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MATERIALS AND METHODS

Strains and plasmids. EPEC strains from the United States were provided by I. Kaye Wachsmuth of the Centers for Disease Control, Atlanta, Ga. Strains from Peru were isolated in the course of a previous study in Lima (21). Isolates were also provided by Valeria Prado, University of Chile School of Medicine, Santiago, Chile; I. Sechter, Government Central Health Laboratories, Jerusalem, Israel; and Peter Echeverria, Armed Forces Institute of Pathology, Bangkok, Thailand. All strains were serotyped by standard methods (11).

E. coli HB101 is a laboratory strain previously described (4).

Strains containing plasmids of known incompatibility

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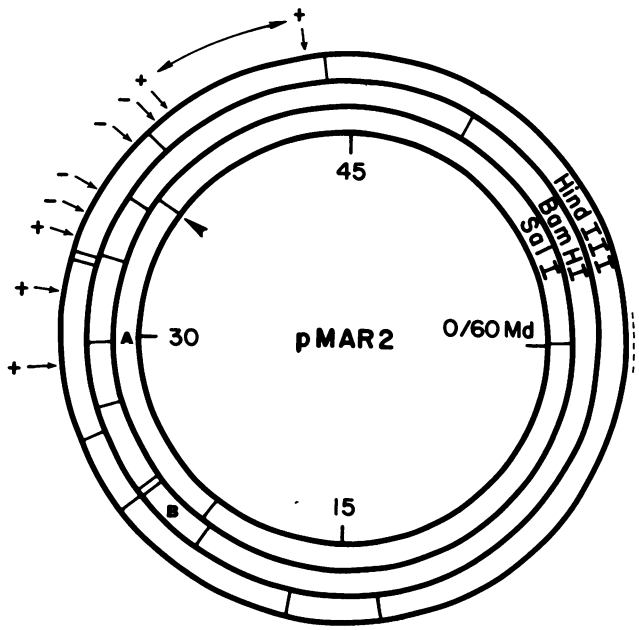

FIG. 1. Restriction map of the EAF plasmid pMAR2. The plasmid, derived from E2348/69, has been mapped for the recognition sites of the restriction endonucleases *Hin*dIII, *Bam*HI, and*Sal*I (2). The 23-kb *Sal*I fragment labeled A was used as a DNA probe in hybridization experiments against other EAF-positive strains (see text). Fragment B is a 5.0-kb *Bam*HI fragment which has undergone an apparent insertion of 3 kb in some EAF plasmids, including pJPN3 from *E. coli* 2087. The dashed arc at 0 MDa represents the sequences deleted in pJPN3. The + and − signs on the figure represent the HEp-2 adherence phenotypes of Tn*801* insertions at these sites. The arrow inside the plasmid map marks the site of kanamycin gene insertion which inactivated HEp-2 adherence in the deletion mutation pMAR15; the EAF probe consists of the 1-kb *Bam*HI-*Sal*I fragment flanking this site. The kanamycin-resistant derivative of pMAR2, pJPN11, was constructed by cloning kanamycin resistance genes into the *Sal*I site at 0 MDa on the map. This plasmid was mutagenized by random insertion of the transposon Tn*801* (Ap^r), transformed into *E. coli* HB101, and assayed for adherence to HEp-2 cells. Of 58 different insertion sites, half were located within the region indicated by the two-headed arrow; all of these retained adherence. All four adherence-negative mutations lay within one of the two contiguous *Hin*dIII fragments measuring 8.3 and 11 kb. Insertions in other areas of the plasmid (only a few indicated here) resulted in positive-adherence phenotypes. Md, Megadaltons.

groups were obtained from J. Crosa, University of Oregon School of the Health Sciences, Portland.

Rabbit diarrheogenic *E. coli* 1 (RDEC-1) was obtained from E. Boedeker, Walter Reed Army Institute of Research, Washington, D.C. *E. coli* 933 was obtained from A. D. O'Brien, Uniformed Services University of the Health Sciences.

Transposon mutagenesis was done by using the plasmid pMR5 (Tc^r, Km^r, Ap^r). This is a conjugative temperature-sensitive derivative of RP1 (24), carrying the ampicillin transposon Tn*801*.

Adherence regions were cloned into the vector pACYC 184 (Tc^r Cm^r) or pJBK68 (Tc^r), a 22-kilobase (kb) derivative of pRK290 previously described (14). Kanamycin resistance genes used in the construction of pJPN11 were encoded on a 1.4-kb *Sal*I fragment from the 7.7-kb plasmid pHU859 (Km^r Tc^r Ap^r). The latter plasmid is derived from pBR322 by the insertion of a *Hin*dIII fragment encoding kanamycin resistance genes of Tn*5*.

We have previously reported the construction of a 30-kb deletion mutation of pMAR2, designated pMAR15, which carries ampicillin resistance by virtue of Tn*801* and encodes adherence to HEp-2 cells via the EAF. This plasmid was derived by religation of *Sau*3A partial digestions. The HEp-2 adherence phenotype of this derivative was then inactivated by the cloning of kanamycin resistance genes into one of the two remaining *Sal*I sites (the site indicated by the arrow in Fig. 1). The resulting construction, pMAR17, was used for complementation analysis in the present study.

Media. All strains were stored on L-agar slants before testing. Antibiotic media consisted of L-agar plates with the following concentrations of antibiotics (micrograms per milliliter): ampicillin, 200; tetracycline, 30; chloramphenicol, 20; kanamycin, 50.

pJPN11 construction. The EPEC strain E2348/69 carries two plasmids: the 60-MDa EAF plasmid pMAR2 and a cryptic plasmid of 5 MDa. Insertion into one of three *Sal*I sites causes inactivation of the adherence genes (2). To isolate the plasmid and monitor its passage into other *E. coli* hosts, we sought to introduce a fragment conferring antibiotic resistance into one of the other *Sal*I sites. Purified plasmid DNA from E2348/69 was partially digested with *Sal*I to produce large linear molecules, which were then ligated with the 1.4-kb *Sal*I fragment of pHU859, encoding resistance to kanamycin. Ligated DNA was transformed into HB101, and kanamycin-resistant transformants were tested for adherence to HEp-2 cells. One recombinant plasmid, designated pJPN11, was shown to be a derivative of the native plasmid pMAR2, but with kanamycin resistance genes cloned into a *Sal*I site not lying within the adherence region. The site of this insertion is at 0 MDa in Fig. 1.

Cloning. Restriction endonucleases and DNA ligase were used according to the instructions of the manufacturer (Bethesda Research Laboratories, Gaithersburg, Md.). Partial digestions were done at 0°C for empirically determined periods.

Conjugation and transformation. Conjugation was done at 37°C by broth matings or by filter matings for 4 h on Nuclepore membranes (Nuclepore Corp., Pleasanton, Calif.). Transformation into the strain HB101 was done by the calcium chloride method of Dagert and Ehrlich (9).

Transposon mutagenesis. Plasmids were marked with the ampicillin transposon Tn*801* by using the temperature-sensitive donor plasmid pMR5 by the method of Quackenbush and Falkow (23). pMR5 was introduced by conjugation into the recipient strain at 30°C and subsequently cured by multiple passage at 44°C. Insertions were detected by selecting for ampicillin resistance and scoring for tetracycline sensitivity.

S1 nuclease plasmid homology determination. The degree of sequence homology between the EAF plasmid pMAR2 and other *E. coli* plasmids was tested by the S1 nuclease procedure of Crosa et al. (8). Plasmid DNA was labeled by direct iodination with ^{125}I by the method of Selin et al. (28). Labeled plasmid was hybridized against whole-cell DNA from plasmid-bearing *E. coli* prepared by the method of Brenner et al. (5).

Insertion sequence detection. The EAF plasmid pMAR2 was tested for the presence of common *E. coli* insertion sequences by Southern blot hybridization (see below). Plasmid DNA was extracted, digested with the endonuclease *Bam*HI, separated by agarose gel electrophoresis, and transferred to nitrocellulose. Probes of insertion sequences (IS)

IS*1* and IS*50* were provided by D. Berg of Washington University, St. Louis, Mo. IS*2*, IS*4*, and IS*5* were provided by D. Dykhuizen of Washington University. Gamma-delta (IS*1000*) sequences were provided by M. Guyer of Genex, Inc., Gaithersburg, Md.

Southern blot analysis. Plasmid DNA was extracted by the method of Birnboim and Doly (3), separated by electrophoresis through 0.7% agarose gels, and transferred to nitrocellulose paper by the method of Southern (31). Plasmid DNA used for probe preparation was extracted by the method of Birnboim and Doly and purified by cesium chloride-ethidium bromide equilibrium density gradient centrifugation. DNA probes were purified by restriction digestion, agarose gel electrophoresis, and electroelution (19). Probes were labeled by using [^{32}P]dATP by nick translation (19).

Analysis of plasmid phenotypes. The assay for colicin production was done by the method of Smith and Huggins (29) with *E. coli* C600 as the indicator strain. Assays for biochemical markers were done by the API Rapid CHE system (Analytab Products, Plainview, N.Y.), which assesses the fermentation of 49 carbohydrates and their derivatives. Alpha-hemolysin was assayed by plating on Trypticase Soy Agar II plates with 5% defibrinated sheep blood (BBL Microbiology Systems, Cockeysville, Md.). Plasmid incompatibility was determined by filter and broth matings of standard incompatibility plasmids into EAF-positive strains. Aerobactin gene detection by colony hybridization was kindly done by J. Crosa of the University of Oregon School of the Health Sciences.

EAF plasmids were tested for the presence of antibiotic resistance genes by transforming *E. coli* HB101 with plasmid DNA from EAF-positive strains and plating on L-agar plates containing ampicillin, kanamycin, tetracycline, or chloramphenicol. Transformants were tested for the presence of EAF plasmids by colony hybridization (20) with the EAF probe.

RESULTS

S1 nuclease homology determination. We have previously shown that EAF genes are usually encoded on plasmids of 55 to 65 MDa (22). To determine whether these plasmids represent a closely related family or have only the EAF phenotype in common, we used the enzyme S1 nuclease to compare the degree of genetic relatedness of pMAR2 with several other *E. coli* plasmids. The EAF plasmid pJPN11 was labeled by incorporation of ^{125}I and hybridized against whole-cell DNA extracted from plasmid-bearing *E. coli*. Results are listed in Table 1. EAF plasmids demonstrated a high degree of sequence homology by this method, ranging from 53 to 79%. *Inc*FI and *Inc*FII plasmids were 41 and 43% homologous to pMAR2, respectively, as would be expected from the conservation of *tra* and other functions among plasmids of the same incompatibility group (12). In contrast, pMAR2 was only 4 and 6% homologous to *E. coli* plasmids of groups *Inc*A and *Inc*P, respectively. pMAR2 was 18% homologous to the enterohemorrhagic *E. coli* strain 933, which contains a 70-MDa plasmid thought to play a role in intestinal colonization (15). pMAR2 demonstrated 50% homology to rabbit diarrheogenic *E. coli* 1 (RDEC-1); this strain has been shown to carry an 85-MDa plasmid encoding pili which mediate adherence to the intestinal mucosa of rabbits (6).

Analysis of restriction patterns of EAF plasmids. S1 homology data suggest that EAF plasmids display sequence conservation, yet the variable size of the EAF plasmids implies that restriction maps might vary considerably. Because all EAF-positive strains in our collection possessed multiple plasmids and specific isolation of EAF plasmids from each of these would be prohibitive, we used a Southern blot hybridization method for restriction analysis. The probe in this series of experiments consisted of a ^{32}P-labeled 23-kb *Sal*I fragment of pMAR2 (fragment A in Fig. 1), shown to carry a portion of the adherence genes (2). The previously described EAF probe represents the 1-kb *Bam*HI-*Sal*I fragment at one end of the 23-kb region as indicated in Fig. 1. In addition to carrying the EAF probe sequences, this fragment represents the largest restriction fragment of pMAR2 which hybridized solely with the EAF plasmids in undigested plasmid profiles (data not shown).

TABLE 1. DNA homology of *E. coli* plasmids with the EAF plasmid pMAR2

Strain	Homology with EAF probe	% Homology with pMAR2
2087 (United States)	+	79.6
23/254 (Peru)	+	67.0
Ch6 (Chile)	+	61.0
659 (United States)	+	55.1
64/28 (Israel)	+	53.3
RDEC-1	−	49.7
933 (EHEC)[a]	−	18.1
X209 (*Inc*FI)	−	41.1
RI (*Inc*FII)	−	42.6
RP4 (*Inc*P)	−	6.3
RA1 (*Inc*A)	−	4.3

[a] EHEC, Enterohemorrhagic *E. coli*.

Twenty-nine EAF probe-positive *E. coli* strains of different serotypes and from different geographic regions were analyzed (Table 2). Total plasmid DNA extracts were digested with the restriction endonuclease *Bam*HI or *Hin*dIII, yielding a mixed population of fragments representing all of the plasmids of the strain. Restricted DNA was separated by agarose gel electrophoresis, transferred to nitrocellulose, and hybridized with the labeled 23-kb fragment.

The results revealed that there was substantial sequence conservation among EAF plasmids in the region studied, indicated by the fact that every isolate had several restriction fragments homologous to the labeled fragment. At least four distinct restriction patterns were produced by *Bam*HI digestion (Table 1). Fragments of 6.2, 6.0, 4.0, and 3.9 kb were conserved in all plasmids. In many of the plasmids, the 5.0-kb fragment seen in pMAR2 had undergone an apparent insertion of 3.0 kb, yet this event was found in several areas of the world and across different serotypes. Greater variation was seen in the location of *Hin*dIII sites (data not shown).

Southern blot analysis of EAF plasmids by using the EAF probe. We assumed that although *Bam*HI and *Hin*dIII recognition sites were somewhat divergent within the region homologous to the 23-kb probe, they would be significantly less divergent in the region encoding the EAF. We tested this hypothesis by examining EAF plasmids for conservation of the 1-kb *Bam*HI-*Sal*I fragment which serves as the EAF probe. Total plasmid DNA from six EAF-positive strains from the United States was digested simultaneously with both *Bam*HI and *Sal*I, separated by electrophoresis, blotted, and hybridized with the EAF probe. Only one of the six isolates we examined carried EAF probe-homologous genes on a 1-kb restriction fragment; the other isolates carried these genes on fragments of approximately 4 kb (Fig. 2). In

TABLE 2. *Bam*HI fragments of EAF plasmids homologous with 23-kb pMAR2 fragment

Source	EPEC strain (serotype)	Homology with *Bam*HI fragment of size (kb):									
		22	20	18	9.0	8.0	6.2	6.0	5.0	4.0	3.9
United Kingdom	E2348 (O127:H6)		+				+	+	+	+	+
United States	2087 (O55:H6)		+			+	+	+		+	+
	1104 (O127:NM)		+			+	+	+		+	+
	2309 (O111:H2)		+			+	+	+		+	+
	2430 (O111:NM)		+			+	+	+		+	+
	2450 (O119:H6)		+			+	+	+		+	+
	659 (O119:H6)		+				+	+	+	+	+
	2395 (O119:H6)		+			+	+	+		+	+
	2362 (O55:NM)		+			+	+	+		+	+
	3252 (O111:H2)		+				+	+	+	+	+
Peru	081 (O111:NM)		+				+	+	+	+	+
	090 (O119:H6)		+				+	+	+	+	+
	056 (O55:NM)		+				+	+	+	+	+
	105 (O119:H6)		+				+	+	+	+	+
Chile	C30 (O86:?)		+			+	+	+		+	+
	C11 (O111:?)		+				+	+	+	+	+
	C5 (O55:?)		+			+	+	+		+	+
	C1 (O119:?)		+				+	+	+	+	+
	C22 (O55:?)		+			+	+	+		+	+
Israel	90-510 (O55:H6)	+					+	+	+	+	+
	92-3080 (O86:NM)			+			+	+	+	+	+
	7398 (O119:H6)				+	+	+	+		+	+
	74-695 (O126:NM)		+				+	+		+	+
	106-74 (O127:NM)		+				+	+		+	+
	73-254 (O142:H34)		+				+	+		+	+
South Africa	6515 (O126:H?)				+	+	+	+		+	+
	10783 (O126:H?)		+				+	+		+	+
Thailand	40/28 (O119:?)				+	+	+	+		+	+
	58/28 (O119:?)				+	+	+	+		+	+

contrast to the hybridization studies using the 23-kb probe, the size of the EAF probe-homologous fragment did correlate with the serotype of the U.S. strains. Isolates of serotype O111:H2 or O111:NM carried these homologous sequences on 4.2-kb *Bam*HI-*Sal*I fragments, O119:H6 isolates carried them on 4.0-kb fragments, and only the O55:H6 strain carried these genes on the 1.0-kb fragment characteristic of pMAR2 (from the O127:H6 English isolate E2348/69). One of the O111:H2 isolates, 2309, does not adhere to HEp-2 cells despite carrying a 60-MDa plasmid homologous to both the EAF probe and the 23-kb *Sal*I fragment used in the above experiments. Although we assumed that this negative-adherence phenotype was caused by mutation in the adherence genes, our analyses failed to show any unique restriction pattern.

Mapping of EAF plasmid pJPN3. The above experiments describe a family of plasmids with a significant degree of sequence conservation, yet with some variation in restriction map. To study this phenomenon in detail, we identified and mapped the EAF plasmid from the O55:H6 strain 2087, isolated from the stool of an infant with diarrhea in the United States in 1977. The two large plasmids of this strain were marked with ampicillin transposon Tn*801*, which was donated from the temperature-sensitive plasmid pMR5. Plasmid DNA from transposon insertion derivatives was transformed into HB101 and transformants were tested for adherence to HEp-2 cells. The EAF plasmid, pJPN3, was found to be 55 MDa and to differ from the *Hin*dIII, *Bam*HI, and *Sal*I restriction maps of pMAR2 in only two locations. A large deletion was identified including the *Sal*I site at 60 MDa on a map of pMAR2 (dashed arc on Fig. 1), and an apparent insertion of approximately 3 kb was seen in the 5-kb *Bam*HI fragment at 19 to 22 MDa on the pMAR2 map (fragment B). The insertion lies within the region recognized by the 23-kb *Sal*I fragment probe. Although the nature of the inserted sequences is unknown, conservation of this insertion presumably accounts for the presence of the 5-kb *Bam*HI fragment in some EAF plasmids but not in others (Table 2).

EAF plasmid phenotype characterization. We next sought to identify phenotypes which might be encoded by the EAF plasmid. E2348/69, its plasmid-cured derivative MAR20, and HB101 with and without pJPN11 were tested for certain phenotypes known to be characteristic of *E. coli* plasmids. The EAF plasmid was found to be negative for colicin, aerobactin, and alpha-hemolysin production, as well as 49 fermentations by the API Rapid CHE system (Analytab).

The EAF-positive EPEC isolates from various parts of the world were quite frequently resistant to a variety of clinically important antibiotics. Of the 29 EAF-positive EPEC listed in Table 2, 41% were resistant to kanamycin, 83% were resistant to ampicillin, 38% were resistant to chloramphenicol, 28% were resistant to tetracycline, and 80% were resistant to sulfonamides. We hypothesized that the genetic variability of EAF plasmids observed in the above analyses might have been partly caused by the insertion of transposable antibiotic resistance genes. To test for the presence of antibiotic resistance on EAF plasmids, plasmid DNA was extracted from the 29 EAF-positive strains listed in Table 2, transformed into *E. coli* HB101 and plated on L agar containing ampicillin, tetracycline, kanamycin, or chloramphenicol. Representative transformants were hybridized by colony blot with the EAF probe. Although all of these strains carried R factors for at least one antibiotic, none of the resistances were found on the EAF plasmids.

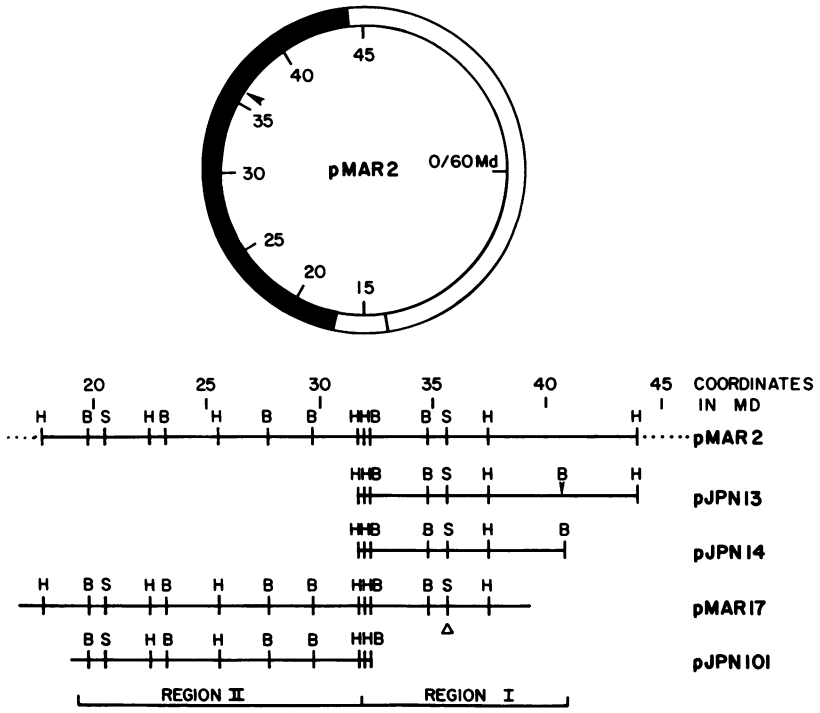

FIG. 2. Cloning of HEp-2 adherence regions. Areas involved in the HEp-2 adherence function of pMAR2 were cloned as two separate regions which complemented in *trans*. The circular map respresents the *Hin*dIII (H) map of pMAR2. The shaded area is linearized below, with corresponding map coordinates. pJPN13 represents the 8.3-, 11-, and 0.8-kb *Hin*dIII fragments (the first two shown to be essential to adherence in the Tn*801* experiments) cloned into pACYC184. These insert fragments were cloned from a Tn*801* derivative to maintain positive selection of the desired 11-kb fragment. The site of Tn*801* insertion is indicated by the arrow; the transposon carries a *Bam*HI (B) site. pJPN14 is a derivative of pJPN13 lacking a 5-kb *Bam*HI fragment, which extends from the *Bam*HI site of the transposon to the site in the vector pACYC184. The latter site, not shown in the figure, lies just to the right of the *Hin*dIII site. Neither pJPN13 nor pJPN14 confers HEp-2 adherence alone. pMAR17 is the 30-kb derivative of pMAR2 previously described. Although it contains all the sequences required for expression of adherence, the function has been lost by the cloning of kanamycin resistance genes into the *Sal*I (S) site indicated by the triangle. pJPN101 is a derivative of pMAR17 which, although it does not confer HEp-2 adherence by itself, is able to complement pJPN13 or pJPN14. The inserts of pJPN14 and pJPN101 are designated EAF regions I and II, respectively. MD or Md, Megadaltons.

The incompatibility group of the EAF plasmids was determined by conjugating reference incompatibility plasmids into spontaneous streptomycin-resistant mutants of EAF-positive strain E2348/69 or 2087 (O55:H6). Only the reference plasmid of the incompatibility group *Inc*FII displayed marked instability in both recipients.

Insertion sequence detection. Our experience with pMAR2 suggests that this plasmid may be subject to recombinational events. Previous experiments give evidence that the plasmid may occasionally be integrated into the chromosome (22). The propensity of pMAR15 to form spontaneous deletions (see below) would also be explained by genetic recombination. To determine a possible means by which these events may occur, we probed the EAF plasmid pJPN11 for the presence of genes representing common *E. coli* insertion sequences. Probes carrying sequences native to IS*1*, IS*2*, IS*4*, IS*5*, IS*50*, and gamma-delta failed to show homology with the EAF plasmid.

Tn*801* mutagenesis. We have previously reported partial localization of EAF regions of pMAR2 (2). Unfortunately, the adherence-positive deletion mutant in these studies (pMAR15) was prone to deletion of the majority of its sequences, maintaining only ampicillin resistance and replication functions. It was therefore necessary to clone the adherence regions into a stable vector. To identify restriction fragments which would be good candidates for cloning, we used a method of insertional mutagenesis using the transposon Tn*801* (Ap^r). Of 200 Tn*801* insertions, 58 different patterns were detected by digestion of the plasmids with *Bam*HI and *Hin*dIII. Transposition displayed marked regional specificity; approximately half of the insertions occurred in an 11-kb region (Fig. 1) and did not affect adherence. Of the remainder, four insertions were found to abolish the adherence phenotype.

Cloning of EAF regions. All four inactivating insertions were found to lie within one of two contiguous *Hin*dIII fragments of 11 and 8.3 kb. These data suggested that the cloning of these two fragments together would be sufficient to confer the adherence phenotype. To maintain positive selection in cloning this region, the fragments were taken from an adherence-positive pJPN11::Tn*801* derivative which carried the transposon on the 11-kb *Hin*dIII fragment. Plasmid DNA was partially digested with *Hin*dIII, and fragments were cloned into the *Hin*dIII site of the vector pACYC184 (Tc^r Cm^r). Insertion into this vector site inactivates the tetracycline resistance gene. Chloramphenicol- and ampicillin-resistant colonies were then scored for the presence of the 8.3-kb *Hin*dIII fragments by colony hybridization with the EAF probe (the sequences for which are part of this fragment). One such recombinant clone was found. Although this construction, pJPN13, carried the 11-kb, 8.3-kb, and also the adjacent 0.8-kb *Hin*dIII fragment, it did not confer HEp-2 adherence in HB101.

We assumed that the failure to successfully clone the

regions necessary for HEp-2 adherence on the 11- and 8.3-kb fragments was caused by the presence of additional required genes in another region of the plasmid, not detected by Tn*801* mutagenesis. To test this hypothesis, we attempted to complement the missing genes in *trans* by using the previously described pMAR17, a stable derivative of the 30-kb deletion mutant pMAR15 in which the adherence genes are inactivated at the *Sal*I site (see Fig. 2) (2). pMAR17 was transformed into strain HB101(pJPN13), and the transformants were assayed for the property of HEp-2 adherence. Although HB101 with pJPN13 or pMAR17 alone was incapable of adhering to HEp-2 cells, the phenotype was restored when these two plasmids were present together.

pJPN13 was further reduced in size by partially digesting with *Bam*HI and religating to produce deletion mutations. A 5-kb *Bam*HI fragment extending from the *Bam*HI site of transposon Tn*801* to the *Bam*HI site of the vector pACYC184 (Fig. 2) was found to be nonessential to adherence upon complementation with pJPN101. This 24-kb derivative of pJPN13 missing the 5-kb *Bam*HI fragment was designated pJPN14.

To localize regions providing the critical complementation to pJPN14, random partial *Sau*3A fragments of pMAR17 were subcloned into the *Bgl*II site of vector pJBK68. Recombinant molecules were transformed into HB101(pJPN13) and the transformants were assayed for HEp-2 adherence. The smallest recombinant found to complement pJPN13 (or pJPN14) to confer adherence carried an insert of 18 kb. This clone, which contained less than 2 kb of overlap with pJPN13, was designated pJPN101. For ease of reference, the cloned region of pJPN14 has been designated adherence region I and the insert of pJPN101 constitutes adherence region II.

DISCUSSION

Epidemiologic studies and studies with human volunteers provide compelling evidence that plasmids which encode localized adherence to HEp-2 cells via the EAF are important in the virulence of EPEC (18, 21). Previous work with a sensitive and specific DNA probe for EAF revealed that these genes are usually found on plasmids of 55 to 65 MDa (22). In this study, we investigated whether these plasmids represent a closely related family, such as the 120- to 140-MDa virulence plasmids of *Shigella* sp. and enteroinvasive *E. coli* (13), or whether they are instead unrelated plasmids which simply carry a common phenotype, as is the case with heat-stable enterotoxin plasmids of enterotoxigenic *E. coli* (30). By Southern blot analysis and S1 nuclease studies, we show that EAF plasmids represent a family which displays a significant conservation of sequences with some divergence of restriction sites. This divergence may in part be caused by the insertion and deletion of DNA segments within the plasmids as has been previously described for *E. coli* R factors (27) or by rearrangements within the plasmid. The presence of a limited number of *Bam*HI patterns independent of serotype or geographic origin is consistent with the latter theory. Divergence of restriction sites was observed even in the region of the EAF probe, although these differences showed evidence of clonality.

EAF plasmids were shown to be 50 to 90% homologous by using S1 nuclease. This is a greater degree of homology than would be expected if the plasmids were of the same incompatibility group yet encoded widely differing functions (12). In support of this contention, pMAR2 was only 41 and 43% homologous to plasmids of *Inc* groups FI and FII, respectively. The relatedness of the EAF plasmid to the RDEC-1 adherence plasmid (50%) is presumably caused by the presence of uncharacterized shared phenotypes. RDEC-1 gives rise to the characteristic EPEC histopathologic lesion in the small bowel and has been proposed as a model for human EPEC infection (6). RDEC-1, however, is EAF probe negative and does not adhere to HEp-2 cells (J. Nataro, unpublished observations).

One EPEC strain which did not adhere to HEp-2 cells was nevertheless EAF probe positive and carried a plasmid closely related to pMAR2. This fact is presumably due to a mutation in the adherence genes, although no unique restriction pattern was observed here. Other similar plasmids may occasionally confuse the comparison of probe data with HEp-2 adherence. The significance of such strains in disease is unknown.

As EAF is apparently encoded by a significantly conserved virulence plasmid of EPEC, we sought to identify other phenotypes carried on this plasmid. A phenotype other than HEp-2 adherence could be used as an easier method of laboratory screening for EAF or may even be of significance in pathogenesis. The EAF plasmid was not found to encode biochemical markers, transposable antibiotic resistance, colicin production, or alpha-hemolysin production in the strains examined. Although Laporta et al. (17) have recently reported the finding of EAF plasmids encoding antibiotic resistance in O55:NM and O55:H6 strains from Brazil, this appears to be an unusual phenomenon.

Scaletsky et al. (26) have reported that EAF-bearing *E. coli* belong to characteristic biotypes on the basis of sugar fermentations. Two alternative explanations for these data are that the fermentations are encoded on the plasmids themselves or that EAF strains belong to certain clones recognizable by chromosomal markers. Because we observed no plasmid-mediated carbohydrate fermentation, our data support the latter hypothesis.

Williams and Roberts (33) have speculated that the plasmid-mediated siderophore aerobactin may play a role in the virulence of some EPEC. Here we show that if indeed aerobactin is shown to be characteristic of EPEC strains, these genes will probably be shown to be present on the chromosome or on plasmids other than the EAF plasmid.

Previous reports (2) have described the mapping of the EAF plasmid and partial localization of the EAF genes by deletion and insertion mutagenesis. However, the deletion mutation derived in these earlier experiments (pMAR15) was found to be extremely unstable and inadequate for further cloning and localization studies. A kanamycin-resistant derivative of pMAR2, pJPN11, was subjected to insertion mutagenesis by using the ampicillin transposon Tn*801*, and mutations were scored for HEp-2 adherence. Adherence regions were found to lie within two contiguous *Hin*dIII fragments (Fig. 1). These fragments were cloned by partial digestion and random ligation into the vector pACYC184. Unfortunately, this construction, pJPN13, did not confer adherence in *E. coli* HB101.

We next attempted to complement the missing adherence regions of pJPN13 by using a previously described adherence-negative mutation, pMAR17, in which adherence was lost by insertional inactivation. When pJPN13 and pMAR17 were present within the same *E. coli* cell, adherence was restored. The critical adherence region of pMAR17 not present in pJPN13 was identified by randomly cloning fragments of pMAR17 into the vector pJBK68. One recombinant was found which complemented pJPN13 with only 2 kb of overlapping DNA. We thus succeeded in identifying two

regions of the plasmid pMAR2, neither of which was sufficient to confer adherence alone, but which were capable of conferring adherence when present together in the same cell. The insert of a subclone of pJPN13, pJPN14, was designated EAF region I, and the insert of pJPN101 was designated EAF region II. The total of the two inserts comprises 35 kb of plasmid DNA.

Studies with human volunteers have shown that the EAF plasmid pMAR2 is associated with the ability of the strain E2348/69 to cause diarrhea, yet we do not have evidence that it is the adherence genes themselves which mediate this virulence or that the genes for this adhesin are entirely encoded by the plasmid. Because humans remain the only model for EPEC infection, thorough genetic analyses of the EAF plasmids must be done before these studies can again be undertaken.

The work of Knutton et al. (16) suggests the presence of ruthenium red-staining fimbriae associated with pMAR2-bearing E2348/69. Unfortunately, although *E. coli* HB101 adheres to HEp-2 cells when carrying EAF plasmids, the fimbriae have not as yet been demonstrated in this host. Knutton et al. also provide in vitro evidence that the adhesin may mediate a more distant attachment than the characteristically close EPEC adherence to mucosal cells, perhaps providing initial colonization potential. The fact that a large area of DNA is required for encoding the EAF, as well as the fact that at least two operons function in *trans*, is consistent with the hypothesis that the EAF is fimbrial. In studies with the enterotoxigenic *E. coli* pilus CFA/I, Willshaw et al. (34) demonstrated that fimbrial sequences were encoded on two plasmid regions of 5.2 and 1.4 MDa, separated by 25 MDa, which operated in *trans*.

Further experiments with the EAF region I and II clones have allowed us to better localize EAF regions and to begin peptide analysis. Future work will focus on the structural products of these genes and their application to potential vaccine development.

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