Properties of Adherence Factor Plasmids of Enteropathogenic *Escherichia coli* and the Effect of Host Strain on Expression of Adherence to HEp-2 Cells

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EPEC adherence factor (EAF) plasmids from three strains of enteropathogenic *Escherichia coli* (EPEC) – E2347/69 (O127:H6), E20517 (O111:H2) and E24582 (O142:H6) – were examined. The EAF plasmids were all marked with ampicillin resistance by transposition of Tn801 to give pDEP1, pDEP2 and pDEP11, respectively. All three plasmids showed incompatibility with an FI_{me} and an FIV plasmid and had some similarity in restriction enzyme digest patterns. Plasmid pDEP1 differed from pDEP2 and pDEP11 in being autotransferring and fertility-inhibition positive. An EAF probe consisting of a 1 kb *Bam*HI–*Sal*I restriction endonuclease fragment of the prototype EAF-associated plasmid pMAR2 hybridized to similar-sized *Sal*I–*Bam*HI fragments of pDEP1 and pDEP11 but to a different-sized fragment of plasmid pDEP2. Loss of the EAF plasmids from EPEC strains resulted in a marked reduction in the ability of these strains to adhere to HEp-2 cells. The EAF-plasmid-negative variants did not express a 94 kDa outer-membrane protein (OMP). When these EAF plasmids were reintroduced into EAF-plasmid-negative EPEC strains a high level of adherence equivalent to that of the parent EPEC strains was restored and a 94 kDa OMP was usually expressed. However, when EAF plasmids were transferred into *E. coli* K12 or non-EPEC *E. coli* the host strains either did not adhere or adhered poorly to the HEp-2 cells. These transconjugants did not express a 94 kDa OMP.

INTRODUCTION

The term enteropathogenic *Escherichia coli* (EPEC) is commonly used to describe *E. coli* of certain serotypes that have been epidemiologically implicated in diarrhoeal disease (Taylor, 1961). These *E. coli* are an important cause of diarrhoea in infants in developing countries and still cause sporadic cases and occasionally outbreaks of infantile enteritis in industrialized countries. EPEC do not have recognized virulence factors such as the ability to produce heat-stable or heat-labile enterotoxins or Vero cytotoxins or to invade the gut mucosa, and are generally identified by serotyping. The most common O serogroups are O55, O86, O111, O114, O125, O126, O127, O128 and O142 (Edelman & Levine, 1983; Gross & Rowe, 1985).

Cravioto *et al.* (1979) reported that 80% of EPEC strains adhered to HEp-2 cells and that this adherence was significantly more common among EPEC than among enterotoxigenic *E. coli* or *E. coli* isolates from healthy individuals. Three patterns of adherence to HeLa and HEp-2 cells have now been described; localized, diffuse and 'aggregative' (Scaletsky *et al.*, 1984; Nataro *et al.*, 1985*b*, 1987*a*). Most work has been done on strains which adhere to HEp-2 cells in a localized manner like E2347/69 and E2348/69 (Cravioto *et al.*, 1979). The ability of strain

Abbreviations: EPEC, enteropathogenic *Escherichia coli*; EAF, EPEC adherence factor; OMP, outer-membrane protein.

E2348/69 to adhere was shown to be coded for by a 60 MDa conjugative plasmid termed pMAR2 (Baldini *et al.*, 1983).

Certain properties of E2348/69 and its pMAR2-minus variant have been compared. In volunteer studies diarrhoea occurred in nine of ten healthy adults challenged with E2348/69 and in two of nine who received the plasmid-minus variant (Levine *et al.*, 1985). Knutton *et al.* (1987*b*) have compared the ability of E2348/69 and its pMAR2-negative derivative to adhere to HEP-2 cells, isolated human enterocytes and cultured human intestinal mucosal biopsy tissues. They found that the plasmid-cured derivative was non-adherent in the HEP-2 cell and isolated enterocyte assays after 3 h, but did exhibit some adherence to cultured mucosa after 9 to 12 h, producing identical lesions although not as extensive, as those produced by the parent strain. Chart *et al.* (1988) in a 6 h assay found that a plasmid-negative derivative of E2347/69 showed some ability to adhere to HEP-2 cells. An *E. coli* K12 strain containing pMAR2 adhered to HEP-2 cells but did not show the intimate attachment of the parent strain (Knutton *et al.*, 1987*a*). The latter authors concluded that plasmid pMAR2 coded for an adhesin, but that other, possibly chromosomally encoded factors were required for EPEC to achieve the characteristic mode of intimate cell attachment.

The name EPEC adherence factor (EAF) has been applied to the adhesin of strain E2348/69 which contains plasmid pMAR2 (Levine *et al.*, 1985). A region of pMAR2 which is required for the expression of the EAF adhesin has been identified and a DNA fragment lying within this region has been used as a specific probe to detect EAF genes in *E. coli* (Nataro *et al.*, 1985*a*). By this means, it has been shown that EAF genes are present in strains which have the localized adherence phenotype (Nataro *et al.*, 1985*b*; Echeverria *et al.*, 1987; Chart *et al.*, 1988), and that these genes are usually encoded by plasmids varying in size from 55 to 70 MDa (Nataro *et al.*, 1985*b*). Strains carrying an EAF plasmid usually produce an outer-membrane protein (OMP) of 94 kDa (Levine *et al.*, 1985; Chart *et al.*, 1988). Antibodies to the 94 kDa OMP were detected in the sera of volunteers infected with E2348/69 suggesting that this protein might have a role in pathogenicity (Levine *et al.*, 1985). EAF-positive EPEC of serogroups O111, O119 and O142 possessed a protein of about 94 kDa that reacted with antibodies in these human sera (Levine *et al.*, 1985). However, an antiserum raised in rabbits to a HEP-2 adhesive strain of EPEC of serotype O111:H2 did not react with the 94 kDa OMP of all EPEC strains, indicating some interstrain variability in this protein (Chart *et al.*, 1988).

Two reports have described plasmids coding for both localized adherence and drug resistance. Two autotransferring adherence plasmids from *E. coli* strains of serogroup O55 coded for ampicillin resistance and ampicillin, chloramphenicol, kanamycin and sulphadiazine resistance, respectively (Laporta *et al.*, 1986). A plasmid transferred from an EPEC strain belonging to serotype O111:H⁻ encoded the expression of the O111-specific side-chain of the lipopolysaccharide as well as drug resistance and localized adherence (Riley *et al.*, 1987).

In the present study EAF plasmids were transferred from three EPEC strains of different serotypes to *E. coli* K12 and some of their properties compared. The expression of adhesion to HEP-2 cells when these EAF plasmids were transferred to *E. coli* of different serotypes were also compared to examine the relationship of plasmid and chromosomally encoded factors.

METHODS

Bacterial strains. The three EPEC strains used as sources of EAF plasmids were isolated from outbreaks of infantile gastroenteritis; E20517 and E2347/69 in the UK (Chart *et al.*, 1988) and strain E24582 in the Netherlands (Gerards *et al.*, 1984). Strain E24583 was isolated from the same outbreak as E24582 but did not adhere to HEP-2 cells (Table 1). Strain E2347/69 was isolated from the same outbreak as E2348/69, the strain from which the prototype EAF plasmid pMAR2 was isolated; these strains have identical properties. Strain E20517 was resistant to chloramphenicol, kanamycin, streptomycin, sulphonamides and tetracycline, E2347/69 to streptomycin and sulphonamides and E24582 and E24583 to ampicillin, chloramphenicol, kanamycin, streptomycin, sulphonamides and tetracycline. Table 1 also lists *E. coli* K12 and wild-type *E. coli*, which do not adhere to HEP-2 cells, that were used as recipients for the EAF plasmids.

Tests for resistance to antibacterial drugs and colicin E2 and for colicin, enterobactin and aerobactin production. Strains were tested for drug resistance by the methods of Anderson & Threlfall (1974). Colicin E2 resistance and colicinogeny were detected by the agar overlay method of Frédéricq (1957). The ability of strains to produce

Table 1. *Properties of E. coli strains*

Strain no.	Serotype	Source of strain*	Adherence to HEp-2 cells†		Reaction with EAF probe‡	Plasmid content (MDa)			
			3 h	6 h					
E20517	O111:H2	Outbreak	68	100	+	46	38	32	<2
60R556		58R689	1S	12S	—		30	24	<2
E2347/69	O127:H6	Outbreak	48	100	+	60			4.1
60R317		E2347/69	3S	13	—				4.1
E24582	O142:H6	Outbreak§	72	100	+	61	40		3.3
E24583			0	0	—		43		3.3
E27657	O128:H2	Infant with diarrhoea	0	2S	—	76			
E2722/1	O40:H3	Healthy infant	0	0	—	32		3	<3
E2698/1	O71:H48	Healthy infant	0	0	—	41			
E2939/3	O99:H4	Healthy infant	3S	7S	—	40	35	22	
14R519	O rough:H48	<i>E. coli</i> K12	0	0	—				None

* Outbreaks of infantile gastroenteritis; 58R689 is a chloramphenicol-, kanamycin- and tetracycline-sensitive derivative of E20517 with the same plasmid content as 60R556 plus the 46 MDa plasmid of E20517.

† The results are expressed as the percentage of HEp-2 cells with at least one localized cluster consisting of at least 50 adherent bacteria. When percentage is followed by S most cells showed a single cluster of less than 10 bacteria.

‡ Southern hybridization at 'high' stringency with a 1 kb *SalI*-*Bam*HI restriction endonuclease fragment of pMAR2, the prototype EAF plasmid (Baldini *et al.*, 1983).

§ Both isolates from the same outbreak.

siderophores was assessed by growing bacteria in Tris/succinate medium and testing for the phenolic siderophore enterobactin and the hydroxamate siderophore aerobactin as described by Griffiths *et al.* (1985).

HEp-2 cell adhesion. The ability of strains of *E. coli* to adhere to HEp-2 cells in the presence of D-mannose was determined using the method of Scotland *et al.* (1983). Either a 3 h incubation period for bacterial adherence, or a 6 h period in which the HEp-2 cells were washed after 3 h and the tissue culture medium replaced before continuing incubation for a further 3 h was used. In tests with *E. coli* K12, which grew less well in the tissue culture medium, the washing of the cells at 3 h was omitted, and tests were done in an atmosphere of 95% (v/v) air and 5% (v/v) CO₂.

Plasmid DNA studies. Plasmid DNA was partially purified from the wild-type strains and from *E. coli* K12 derivatives by the method of Birnboim & Doly (1979) and analysed by electrophoresis on vertical slab gels containing 0.6% (w/v) agarose (Sigma, type II); plasmids of known molecular masses were separated on the same gels. Purified plasmid DNA from *E. coli* K12 and wild-type strains was prepared by the alkaline SDS lysis method (Maniatis *et al.*, 1982) followed by centrifugation in caesium chloride-ethidium bromide density gradients. Plasmid DNA was treated with restriction enzymes *Eco*RV, *Pvu*II and *Sal*I plus *Bam*HI and fragments were separated by gel electrophoresis as described previously (Willshaw *et al.*, 1985).

DNA hybridization. The EAF probe consisted of a 1 kb *Bam*HI-*Sal*I restriction fragment isolated from plasmid pMAR22 which was derived from the plasmid pMAR2 (Nataro *et al.*, 1985a). It was labelled with deoxyadenosine 5'- α -[³⁵S]thiotriphosphate by the random primer method (Feinberg & Vogelstein, 1983). Strains were prepared for colony hybridization as described by Maniatis *et al.* (1982). Transfer of DNA fragments to nylon membranes (Hybond-N, Amersham) was by the method of Southern (1975). Hybridization and washing at 'high' stringency were done as described by Willshaw *et al.* (1985).

Fertility inhibition. Broth cultures of strains carrying an EAF plasmid and the F factor marked with tetracycline resistance, plasmid F-Tc (Anderson *et al.*, 1977) were tested with phage μ 2 in surface spot tests. Plasmids that cause fertility inhibition (fi⁺) inhibit synthesis of F fimbriae and therefore reduce or abolish visible lysis of the culture by phage μ 2 (Anderson & Threlfall, 1974).

Transfer of plasmids from EPEC. Strains E2347/69, E20517 and E24582 were tested for their ability to transfer drug resistance to *E. coli* K12 strain 14R519 by overnight mating of equal volumes of nutrient broth cultures containing approximately 10⁸ cells ml⁻¹. Transconjugants were selected on MacConkey agar plates containing the appropriate antibiotic and nalidixic acid to counter-select against the donor.

Strains E2347/69, E20517 and E24582 were tested for the presence of transfer factors by attempting to mobilize the non-autotransferring plasmids NTP2 and NTP107 into 14R519 (Anderson & Threlfall, 1974). Plasmid NTP2 codes for streptomycin and sulphonamide resistance and NTP107 for kanamycin resistance (McConnell *et al.*, 1980).

The F-Tc plasmid was transferred into E2347/69. This strain was used as a donor when F-Tc was transferred into 14R519. Transconjugants were examined for co-transfer of a plasmid coding for fertility inhibition. These transconjugants were then examined for their ability to adhere to HEp-2 cells, for hybridization with the EAF probe and for plasmid content.

Derivatives of E20517 and E24582 which had lost tetracycline resistance were selected by replica plating. Plasmids in these two tetracycline-sensitive derivatives were marked with the ampicillin-resistance transposon Tn801 using a temperature-sensitive derivative of plasmid RP1 (pMR5) (Robinson *et al.*, 1980). Plasmid F-Tc was transferred into the wild-type strain and used to mobilize the Tn801-carrying plasmids into 14R519. The transconjugants were tested for their ability to adhere to HEp-2 cells, for hybridization with the EAF probe and for plasmid content.

The transconjugants from the crosses where the tetracycline-sensitive derivatives of E20517 and E2347/69 were used as donors were also tested for co-transfer of streptomycin and sulphonamide resistance. Transconjugants carrying EAF plasmids from all three crosses were tested for tube agglutination with O111-, O127- and O142-specific antisera to see if they expressed the O-polysaccharide side-chains.

Transfer of EAF plasmids to E. coli of different serotypes. The Tn801-marked EAF plasmids were transferred to a variety of *E. coli* recipients by overnight crosses as previously described, using as donor strains *E. coli* K12 carrying an autotransferring EAF plasmid or a non-autotransferring EAF plasmid and the R factor F-Tc to mobilize the EAF plasmid. Recipients were selected on MacConkey agar plates containing ampicillin ($100 \mu\text{g ml}^{-1}$) and colicin E2 (10^3 units ml^{-1}), to counter-select against the donor. The ampicillin-resistant transconjugants were tested for the presence of the EAF plasmid by DNA probe tests, for their ability to adhere to HEp-2 cells and for plasmid DNA content.

Incompatibility tests. The EAF plasmid from E2347/69 was marked with ampicillin resistance by Tn801 transposition as described by Robinson *et al.* (1980). Drug-resistance plasmids of the F group were used in incompatibility tests: F-Tc, incompatibility group (inc.) FI; TP160, inc. FI_{mc} (Anderson *et al.*, 1977); R1-16, inc. FII (Frydman & Meynell, 1969); and TP129, inc. FIV (Hedges & Datta, 1972). They were transferred into *E. coli* K12 strains carrying the Tn801-marked EAF plasmids to test the ability of the two plasmids to co-exist (Smith *et al.*, 1983). Five to eight hundred colonies were tested for stability of the plasmids.

Isolation of HEp-2 non-adherent variants of EPEC. Variants of E2347/69 and E20517 which had lost the EAF plasmid were isolated by transferring an incompatible drug-resistance plasmid TP129 into these EPEC. Transconjugants were examined for loss of the unmarked or marked EAF plasmid by testing for drug resistance, then HEp-2 adhesion and plasmid DNA content. A colony without the R factor was then isolated by looking for drug-sensitive segregants.

Isolation of outer membranes. Bacterial outer membranes were prepared as described previously (Chart & Griffiths, 1985) using the protease inhibitor PMSF (Chart *et al.*, 1988) and proteins separated by SDS-PAGE by the method of Laemmli (1970). Proteins were stacked using 4.5% (w/v) acrylamide gels and separated in 10% (w/v) acrylamide gels. Outer membranes were prepared on at least three occasions and each preparation was separated by SDS-PAGE at least twice.

Immunoblotting. Immunoblotting was done as described previously (Chart *et al.*, 1988) with the exception that OMPs were separated on SDS-PAGE gels with a 10% (w/v) acrylamide separation gel, and profiles transferred using a constant current of 0.2 A (16 h). An antiserum prepared against *E. coli* E20513 and containing antibodies to the 94 kDa protein was used for immunoblotting as described previously (Chart *et al.*, 1988).

Isolation of lipopolysaccharide. Lipopolysaccharide (LPS) for SDS-PAGE was prepared from strains of *E. coli* by proteinase K digestion (Hitchcock & Brown, 1983). LPS profiles analysed by SDS-PAGE were stained by a silver technique (Tsai & Frasch, 1982). The LPS from 200 μg (wet wt) of whole bacterial cells was applied to each lane for SDS-PAGE.

RESULTS

Properties of EPEC strains

The three strains E2347/69, E20517 and E24582 all showed localized adherence to HEp-2 cells, hybridized with the EAF probe and expressed an OMP of 94 kDa (Table 1; Fig. 1, lanes 1, 2 and 3). All three EPEC were drug resistant and produced enterobactin, but did not produce colicin or aerobactin. They were resistant to colicin E2.

Transfer of plasmids from EPEC

The drug-resistance markers of E2347/69, E20517 and E24582 were not autotransferable. E20517 and E24582 both contained transfer factors but these did not code for HEp-2 adherence or hybridize with the EAF probe. The transfer factor from E20517 was a 30 to 32 MDa plasmid and that from E24582, a plasmid of about 40 MDa.

Fig. 1. SDS-PAGE profiles of OMPs of wild-type EPEC. Strains E2347/69 (O127:H6) (lane 1), E20517 (O111:H2) (lane 2) and E24582 (O142:H6) (lane 3) carrying pDEP1, pDEP2 and pDEP11, respectively, showing the expression of an OMP of 94 kDa by each strain (arrowed). Strains 60R317 (lane 4) and 60R556 (lane 5) were derived by loss of pDEP1 and pDEP2 from the respective parent EPEC strains and did not express a 94 kDa protein. Strain E24583 (O142:H6), which lacked pDEP11, expressed several proteins in the 94 kDa region of the gel (lane 6), complicating the identification of a 94 kDa OMP (lane 6). The protein load per lane was 30 µg. Protein molecular mass standards are shown on the right-hand side of the Figure.

The autotransferring plasmid F-Tc was used to mobilize plasmids from E2347/69 into *E. coli* K12. Six of ten transconjugants were *fi*⁺. Two *fi*⁺ transconjugants, which both hybridized with the EAF probe, were examined further. Adherence to HEp-2 cells was examined after 6 h when cells showed a small number of clusters of less than 10 bacteria. The transconjugants contained a 60 MDa plasmid as well as the F-Tc plasmid. No co-transfer of streptomycin or sulphonamide resistance occurred.

Tetracycline-sensitive derivatives of E20517 (58R689) and E24582 (60R718) were obtained by replica plating. Strain 58R689 was also sensitive to chloramphenicol and kanamycin but was still resistant to streptomycin and sulphonamides, while 60R718 had also lost resistance to ampicillin, chloramphenicol, kanamycin and sulphonamides. Plasmids in these strains were marked with ampicillin resistance by transposition of Tn801. Plasmid F-Tc was then transferred into the EPEC strains and used to mobilize the Tn801-marked plasmids. The *E. coli* K12 transconjugants from the crosses with both EPEC strains adhered to 3 to 5% of HEp-2 cells with clusters of less than 10 bacteria and hybridized with the EAF probe. The EAF plasmid from E20517 (pDEP2) which was marked with Tn801 had a molecular mass of about 50 MDa (Table 2). No co-transfer of streptomycin or sulphonamide resistance occurred. The K12 transconju-

Table 2. *Properties of Tn801-marked EAF plasmids*

	EAF plasmids		
	pDEP1	pDEP2	pDEP11
Source of plasmid	E2347/69	E20517	E24582
Mol. mass (MDa)	60	50	61
f ⁺	+ve	-ve	-ve
Autotransferability	+ve	-ve	-ve
Incompatibility group	FI _{inc} and FIV	FI _{inc} and FIV	FI _{inc} and FIV

gant from the cross with strain 60R718 carried a 40 MDa transfer factor, a 3.3 MDa plasmid and a 61 MDa plasmid (pDEP11) as well as plasmid F-Tc. Plasmid pDEP11 was shown to be the EAF plasmid by Southern blotting with the EAF probe (result not shown).

None of the *E. coli* K12 transconjugants carrying an EAF plasmid expressed a 94 kDa OMP (Fig. 2, lanes 2 to 4). None of them reacted with the O111-, O127- or O142-specific antisera. Long-chain LPS was not detected by SDS-PAGE of proteinase-K-digested *E. coli* K12 containing pDEP2.

Properties of EAF plasmids

The EAF plasmids from E2347/69 and E20517 were obtained in strains separately from the F-Tc factor by segregation experiments. A strain carrying pDEP11 and the 3.3 MDa plasmid present in the original strain E24582 was obtained after crosses into a wild-type strain and subsequently *E. coli* K12 (see next section). The EAF plasmid from E2347/69 was marked with *Tn801* as previously described (pDEP1). Plasmid pDEP1 was autotransferring and able to mobilize the non-autotransferring factors NTP2 and NTP107 in crosses from *E. coli* K12 to *E. coli* K12, while pDEP2 and pDEP11 were non-autotransferring. Plasmid pDEP1 was f⁺ while pDEP2 and pDEP11 were not (Table 2).

The plasmids were tested for incompatibility with F-like plasmids. All the plasmids showed dual incompatibility with plasmids of incompatibility groups FIV and FI_{inc}, although there was no incompatibility with the plasmid F-Tc of incompatibility group FI. All the EAF plasmids were stable with a plasmid of incompatibility group FII.

Plasmid DNA prepared from *E. coli* K12 strains carrying the *Tn801*-marked EAF plasmids pDEP1, pDEP2 or pDEP11 was digested with restriction enzymes (Fig. 3*a*). The strain carrying pDEP11 also harboured the 3.3 MDa plasmid present in the original strain. Comparison of the fragment patterns obtained after digestion with *Bam*HI plus *Sal*I showed that the plasmids were distinguishable from each other, as expected from differences in their molecular mass and genetic properties. There were at least 12 fragments larger than 0.6 kb and several of these appeared to be of similar size in all the plasmids. To identify the region hybridizing to the EAF probe, separated plasmid DNA restriction fragments were blotted onto nylon membranes and probed with the 1 kb *Bam*HI-*Sal*I DNA fragment of pMAR2 under high stringency conditions (Fig. 3*b*). As expected plasmid pDEP1 contained a 1 kb *Sal*I-*Bam*HI fragment homologous to the probe fragment and this fragment was also present in pDEP11. However, the EAF probe hybridized to a larger fragment (4 kb) in *Sal*I plus *Bam*HI digests of pDEP2 and no 1 kb fragment was detected (Fig. 3*b*, tracks 1 to 4). In both pDEP1 and pDEP11, the EAF probe hybridized with a 5.9 kb *Eco*RV fragment (tracks 5 and 7) and the *Pvu*II fragments detected by the probe were also of similar size (6.1 kb in pDEP1 and 6.4 kb in pDEP11; tracks 8 and 10). *Eco*RV and *Pvu*II digests of pDEP2 showed two hybridizing bands: these were *Eco*RV fragments of 5.8 kb and 2.8 kb and *Pvu*II fragments of 5.4 kb and 3.9 kb (tracks 6 and 9). The target of the EAF probe in pDEP2 appeared to differ in the presence of some restriction enzyme sites from that in pDEP1 and pDEP11, but not to the extent that would affect hybridization under stringent conditions. This was not due to the presence of the transposon marker, since identical results were obtained with digests of plasmid DNA from the wild-type strain 58R689 containing the unmarked EAF plasmid.

Fig. 2. SDS-PAGE profiles of OMPs of transconjugants carrying plasmids pDEP1, pDEP2 and pDEP11. *E. coli* K12 strain 14R519 harbouring pDEP1, pDEP2 and pDEP11 (lanes 2 to 4, respectively) did not contain an OMP of 94 kDa. The profile of the plasmid-free K12 host is shown in lane 1. Strain 60R556 (O111:H2) carrying pDEP1 and strain 60R317 (O127:H6) carrying pDEP2 expressed an OMP of 94 kDa (arrowed in lanes 5 and 6) whereas strain 60R556 carrying pDEP11 did not express a 94 kDa OMP (lane 7). Strain E27657 (O128:H2) carrying pDEP1 did not express an OMP of 94 kDa (lane 8); however, this strain carrying pDEP2 did express a 94 kDa OMP (lane 9). The protein load per lane was 30 µg. Protein molecular mass standards are shown on the right-hand side of the Figure.

Isolation of HEP-2 non-adherent derivatives of EPEC

A variant of E2347/69 which had lost the EAF plasmid was obtained by transferring the FIV plasmid TP129 into the parent strain. Colonies of E2347/69 (TP129) were examined for loss of HEP-2 adherence and loss of ability to hybridize with the EAF probe. An EAF-plasmid-negative colony was obtained with reduced ability to adhere to HEP-2 cells and TP129 was lost from this colony to give derivative 60R317 (Table 1). In a similar way TP129 was introduced into the tetracycline-sensitive derivative of E20517, which contained pDEP2. Colonies were tested for loss of ampicillin resistance. An ampicillin-sensitive segregant had reduced ability to adhere to HEP-2 cells, and had lost the plasmid which coded for EAF. TP129 was lost from this strain to give derivative 60R556 (Table 1). 60R556 still expressed the O111-specific side-chain of the LPS. Neither of the EAF-plasmid-negative variants expressed the 94 kDa OMP (Table 3; Fig. 1, lanes 4 to 6).

Fig. 3. Restriction enzyme digests and Southern blot analyses of EAF plasmids from EPEC strains. (a) Restriction enzyme digests. Fragments of purified plasmid DNA were separated by electrophoresis on a 1% (w/v) agarose gel (Willshaw *et al.*, 1985). Track 1 shows a *Sa*II + *Bam*HI digest of pMAR22 to indicate the 1 kb EAF probe fragment (arrowed). Tracks 2 to 4 *Sa*II + *Bam*HI digests of pDEP1, pDEP2 and pDEP11; tracks 5 to 7 *Eco*RV digests of pDEP1, pDEP2 and pDEP11; tracks 8 to 10 *Pvu*II digests of pDEP1, pDEP2 and pDEP11. Track 11 shows phage λ DNA digested with *Eco*RI + *Hind*III, with sizes of fragments (kb) on the right-hand side. (b) Autoradiogram showing hybridization of the 35 S-labelled EAF probe with a Southern blot of the gel shown in (a). Tracks 1 to 10 are as above.

Transfer of EAF plasmids into E. coli of different serotypes

The recipient strains included an EAF-negative strain of EPEC serotype O128:H2, EAF-plasmid-negative variants of *E. coli* O111:H2, and O127:H6, all of which showed slight adherence to HEp-2 cells, and also *E. coli* of serotypes O40:H3, O71:H48 and O99:H4 isolated from healthy individuals (Table 3). After transfer of the EAF plasmid its presence in the recipient was confirmed by plasmid DNA content and recipient strains were positive in colony hybridization with the EAF probe. When the transconjugants of EPEC serotypes were examined for adherence to HEp-2 cells all showed a high level of adherence (Table 3). Strains of serotypes O111:H2 and O127:H6 carrying pDEP1 and pDEP2, respectively, expressed OMPs of 94 kDa (Table 3; Fig. 2, lanes 5 and 6) which reacted with the antiserum raised to the 94 kDa OMP of strain E20513 (see Methods). Variant 60R556 carrying pDEP11 was HEp-2 adherent (Table 3) but an OMP of 94 kDa was not expressed (Fig. 2, lane 7) and an immunoblot reaction was not detected. Strain E27657 (O128:H2) carrying pDEP1 did not express a protein of 94 kDa and an immunoblot reaction was not observed (Table 3; Fig. 2, lane 8). When pDEP2 was introduced into E27657 a 94 kDa OMP was expressed which reacted with the antiserum (Table 3; Fig. 2, lane 9); similarly, pDEP11 in E27657 also expressed an OMP of 94 kDa but this protein did not react by immunoblot (Table 3, profile not shown).

The EAF plasmids in the *E. coli* from healthy individuals either did not confer the ability to adhere to HEp-2 cells or resulted in adhesion at a low level. None of the strains expressed the 94 kDa OMP and an immunoblot reaction was not detected (Table 3). When the plasmids were transferred out of these *E. coli* into *E. coli* K12 and then into strain 60R317, the ability to adhere in large numbers to HEp-2 cells was restored to this strain.

Table 3. *Properties of E. coli strains containing Tn801-marked EAF plasmids*

Host strain		Plasmid present	Adherence to HEP-2 cells†		Reaction with EAF probe‡	Presence of a 94 kDa protein	Immunoblot reaction with 94 kDa protein
No.	Serotype		3 h	6 h			
14R519	O rough:H48	None	0	0	—	—	—
		pDEP1	NT	19S	+	—	—
		pDEP2	NT	5S	+	—	—
		pDEP11	NT	3S	+	—	—
60R556	O111:H2	None	0	12S	—	—	—
E20517	O111:H2	pDEP1	64	100	+	+	+
60R556	O111:H2	pDEP2	68	100	+	+	+
		pDEP11	35	100	+	—	—
60R317	O127:H6	None	3S	13	—	—	—
E2347/69	O127:H6	pDEP1	48	100	+	+	+
60R317	O127:H6	pDEP2	10	100	+	+	+
E27657	O128:H2	None	0	2S	—	—	—
		pDEP1	36	100	+	—	—
		pDEP2	67	100	+	+	+
		pDEP11	43	100	+	+	—
E2722/1	O40:H3	None	0	0	—	—	—
		pDEP1	1S	1S	+	—	—
		pDEP2	1S	0	+	—	—
E2698/1	O71:H48	None	0	0	—	—	—
		dDEP1	0	0	+	—	—
		pDEP2	0	0	+	—	—
E2939/3	O99:H4	None	3S	7S	—	—	—
		pDEP1	3S	4S	+	—	—
		pDEP2	0	1S	+	—	—

NT, Not tested.

†,‡ See corresponding footnotes to Table 1.

DISCUSSION

The EAF plasmid pDEP1 differed from the other two EAF plasmids in being *fit*⁺ and autotransferring. However, the EAF plasmids were all incompatible with an *FI*_{me} plasmid TP160 and an FIV plasmid TP129 but showed no incompatibility with plasmid F-Tc. The genes and sites required for autonomous replication and its control constitute the basic replicons of plasmids. Several replicons are common in plasmids belonging to *inc F* groups (Couturier *et al.*, 1988) which may account for the dual incompatibility. The replicons present in F factor, an *FI*_{me} plasmid and plasmid pDEP1 are different (Couturier *et al.*, 1988; W. Maas, personal communication), which may account for the finding that the present EAF plasmids were compatible with plasmid F-Tc but incompatible with plasmid TP160. Nataro *et al.* (1987*b*) reported that EAF plasmid pMAR2, which was isolated from a strain from the same outbreak as the strain yielding plasmid pDEP1, was incompatible with plasmid R1 of the FII incompatibility group when the experiment was done in the wild-type strain. In contrast, plasmid pDEP1 was compatible with plasmid R1-16, a derivative of R1, in *E. coli* K12 although its properties were otherwise identical to the published properties of pMAR2 (Baldini *et al.*, 1983; Nataro *et al.*, 1987*a*). We cannot explain this discrepancy. None of the EAF plasmids we transferred encoded drug resistance or the expression of O antigens in contrast to the findings of Laporta *et al.* (1986) and Riley *et al.* (1987). Plasmid pDEP2 from an EPEC of serotype O111:H2 with a molecular mass of about 46 MDa was smaller than the 54 MDa plasmid transferred from a strain of serotype O111:H⁻ by Riley *et al.* (1987).

It was recently reported that strains belonging to a number of traditional EPEC serogroups, and carrying EAF plasmids, adhered avidly to HEP-2 cells and generally expressed an OMP of

94 kDa. Also, EPEC strains cured of an EAF plasmid exhibited greatly reduced HEp-2 adhesion, and lacked a 94 kDa OMP (Chart *et al.*, 1988). The presently observed inability of K12 and non-EPEC transconjugants to express full HEp-2 adhesion, correlated with the lack of expression of detectable levels of a 94 kDa OMP, suggests that HEp-2 adherent EPEC may require additional genetic information to that encoded by an EAF plasmid for the complete assembly of the HEp-2 adhesion mechanism. EPEC strains 60R556, 60R317 and E27657, which adhere only weakly to HEp-2 cells, became fully HEp-2 adhesive regardless of which EAF plasmid (pDEP1, 2 or 11) they received. Nevertheless, plasmids pDEP1 and pDEP2 appear to be different from pDEP11 since 94 kDa proteins were expressed by recipients of pDEP1 and pDEP2, and these proteins were antigenically similar. In contrast, strain 60R556 carrying pDEP11 did not express a 94 kDa OMP and E27657 carrying pDEP11 produced a 94 kDa OMP that did not react with the antiserum. Knutton *et al.* (1987*a, b*) have suggested that EPEC adhesion involves a two step process consisting of an initial binding to the eukaryotic cell surface, possibly mediated by a putative fimbrial adhesin, followed by attachment involving close cell-to-cell contact. Thus, full HEp-2 adhesion may be mediated by two distinct genetic determinants, one of which is associated with an EAF plasmid and the other possibly chromosomally located.

The absence of a 94 kDa OMP in K12 and non-EPEC transconjugants suggests that these strains were unable to synthesize this protein, which might explain the observed reduction in HEp-2 adhesion. In contrast, the inability of HEp-2 adherent strain 60R556 carrying pDEP11 and strain E27657 carrying pDEP1 to express an OMP of 94 kDa is more difficult to explain. Proteolytic degradation might be responsible (Chart *et al.*, 1988), although outer membranes were prepared in the presence of a protease inhibitor. However, if indeed a 94 kDa OMP was not being expressed by these strains, a role for this OMP in HEp-2 adhesion is put into question.

Identification of EPEC by serotyping or HEp-2 adherence ability poses problems either because of the non-availability of diagnostic antisera or the technical problems associated with tissue culture. An alternative method of screening for EPEC by using a DNA probe for EAF has been proposed. Two surveys showed complete correlation between the presence of the EAF genes and ability to adhere to HEp-2 or HeLa cells (Nataro *et al.*, 1985*a*; Chatkaemorakot *et al.*, 1987). In a recent survey Levine *et al.* (1988) isolated some non-EPEC O group strains which hybridized with the DNA probe for EAF. These strains were as common in controls as in patients suggesting that they might not be pathogenic. It was not stated if they adhered to HEp-2 cells. We have shown that *E. coli* K12 or some non-EPEC *E. coli* strains carrying the EAF plasmid do not adhere to HEp-2 tissue culture cells. Thus it is possible that HEp-2 non-adherent, non-EPEC wild-type strains exist and that they will be detected when strains are screened with the DNA probe for EAF.

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