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Attaching Effacement of the Rabbit Enterocyte Brush Border Is Encoded on a Single 96.5-Kilobase-Pair Plasmid in an Enteropathogenic *Escherichia coli* O111 Strain

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An enteropathogenic *Escherichia coli* (EPEC) O111 serotype a,b,H$^-$ strain carried the following four plasmids: pLV501 (96.5 kilobase pairs [kbp]) specifying resistance to chloramphenicol, tetracycline, and kanamycin; pLV502 (8 kbp) specifying ampicillin resistance; pLV503 (1.9 kbp) specifying streptomycin resistance; and pLV504 (80 kbp) with no resistance markers. This EPEC attached to HEp-2 cells to produce localized clumps of bacteria (localized adhesion) and attached intimately to the enterocyte surface, leading to loss of the brush border (attaching effacement). Plasmid pLV501 was also found to specify the ability to produce localized adhesion on HEp-2 cells and attaching effacement in a rabbit ileal explant model system. Restriction maps showed considerable dissimilarities between pLV501 and pMAR-2, an EPEC plasmid carrying the EPEC adherence factor (EAF) genes. Furthermore, pLV501 did not hybridize with the EAF probe, whereas pLV504 did. There was sequence homology between pLV501 and large plasmids in all seven other well-characterized EPEC, only five of which hybridized with the EAF probe. These findings indicate that pLV501 carries at least one of the genes responsible for production of the brush border damage characteristic of EPEC.

Escherichia coli produces gastroenteritis by at least five different mechanisms (16). Enterotoxigenic *E. coli* attach via fimbriae to the intestinal mucosa and elaborate heat-labile or heat-stable enterotoxins. Enteroinvasive *E. coli* invade the colonic mucosa, kill the enterocytes, and produce an inflammatory diarrhea. Enterohemorrhagic *E. coli* (EHEC) elaborate vero cytotoxins (VT) and produce damage to the ileal and colonic brush border (31). The pathogenic mechanism for enteroaggregative *E. coli* is less well understood (34).

Group five, enteropathogenic *E. coli* (EPEC), was first recognized by the association of certain O serogroups (e.g., O55, O111, O119, and O125) with outbreaks of gastroenteritis in the United Kingdom and the United States (7, 11, 26). Subsequently, EPEC have been recognized as a major cause of gastroenteritis in developing countries (22) and have been associated with diarrhea in rabbits (27), calves (12, 28), and cats (28). Following initial observations of rabbits (9) and later of infected children (29, 32, 33), it became apparent that EPEC cause enterocyte damage by brush border effacement, with pedestal and cup formation at the sites of bacterial attachment to the enterocyte membrane. These ultrastructural changes have been termed attaching effacement. Such ultrastructural changes have been found to be associated with a selective loss of functional brush border proteins (32), and diarrhea is most probably due to the resultant impairment of the digestive and absorptive capacities of the enterocytes.

It has been found that EPEC can adhere to human intestines and that the capacity to adhere is plasmid encoded (15). EPEC can also adhere to cultured cells such as HeLa and HEp-2 and can be subdivided into two classes on the basis of characteristic patterns of adherence (16). Class I EPEC exhibit localized adherence (LA) to cultured cells, in which large aggregates of bacteria are seen stuck to the cell surface. The ability of EPEC to exhibit LA to cultured cells has been found to be plasmid encoded (4, 24). Such plasmids encode the so-called EPEC adherence factor (EAF) (25); a DNA probe has been constructed from one of these (pMAR-2) and has been used in field trials to detect EPEC (23). However, it has been suggested that, although plasmid-encoded factors permit attachment of EPEC, chromosomally encoded gene products are required for full expression of enteropathogenicity (15).

Recently, we validated a model system in which the characteristic changes induced by EPEC can be followed in vitro by organ culture of rabbit intestinal explants (5, 10). In the present study, this approach was used to explore the role of plasmid-encoded gene products in the enteropathogenicity of EPEC.

MATERIALS AND METHODS

Bacterial strains. The enteropathogenic strain *E. coli* K798 was isolated from a child with gastroenteritis (32). It was serotyped as *E. coli* O111 a,b,H$^-$ at the Enteric Pathogens Division, Central Public Health Laboratory, Colindale, United Kingdom. It does not elaborate verotoxin, heat-labile enterotoxin, or heat-stable enterotoxin or carry the genes for their production, but it does show LA to HEp-2 cells. Seven more EPEC isolated from both outbreaks and sporadic cases were kindly provided by B. Rowe, Colindale, United Kingdom. For transformation experiments, the laboratory strain *E. coli* DH1 (F *recA1 endA1 gyrA96 thi-1 hsdR17* [r_K^- m_K^-] *supE44* λ^-) was employed as the recipient (17).

Media. Bacterial strains were stored at −70°C in 15% (vol/vol) glycerol broth until used. Bacteria were selected on nutrient agar containing one or more of the antibiotics kanamycin (40 mg liter^{-1}), streptomycin (25 mg liter^{-1}), ampicillin (100 mg liter^{-1}), tetracycline (12.5 mg liter^{-1}), and nalidixic acid (50 mg liter^{-1}).

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Plasmids, probes, conjugation, transformation, restriction endonuclease digestion, and DNA hybridization. Bacteria were grown in nutrient broth (10 ml) at 37°C in shaken culture for 18 h, transferred to two flasks of nutrient broth (500 ml) and grown to an absorbance of 0.8 at 660 nm. Plasmid DNA was extracted by the method of Humphreys et al. (13) and was separated by electrophoresis through 0.8% (wt/vol) agarose gels.

Conjugation was performed at both 37 and 30°C by broth or filter matings for 4 to 24 h. After extraction of plasmid DNA from cesium chloride gradients, transformation experiments were carried out by standard procedures (19).

Restriction endonucleases were obtained from Boehringer Mannheim (Lewes, Sussex, United Kingdom) and used according to the instructions of the manufacturer. DNA fragments were separated by electrophoresis through agarose gels (0.8%, wt/vol). DNA probes for EAF and EHEC (18) (obtained from M. Levine, University of Maryland, College Park, Md.) and VT1 and VT2 (obtained from H. Smith, Colindale, United Kingdom) were radioactively labeled and used in Southern transfer and whole colony blot DNA hybridization experiments as described previously (1). Appropriate positive controls consisting of known EPEC and EHEC expressing VT1 and VT2 (obtained from H. Smith and from M. Karmali, Toronto, Ontario, Canada) were also examined.

Adhesion to cultured cells. HEp-2 cells were grown to confluence on glass cover slips (diameter, 1 cm) in glass vials in Eagle minimal essential medium (MEM) supplemented with fetal calf serum (2%) at 37°C in CO_2 (7%) in air. The monolayers were washed three times with MEM and incubated for 3 h at 37°C with 50 μl of an 18-h nutrient broth culture of *E. coli* in 1 ml of MEM with or without D-mannose (0.5%, vol/vol). Following incubation, the monolayers were washed three times in MEM, fixed in absolute methanol, stained by Giemsa, and examined by light microscopy.

Organ culture of rabbit ileum. Multiple biopsy specimens of rabbit ileal mucosa were maintained in organ culture by a method (5, 10) modified from that of Browning and Trier (8). Briefly, adult New Zealand White rabbits that had been fasted overnight were anesthetized with Valium (Roche Products Ltd., Welwyn Garden City, United Kingdom) and Hypnorm (Crown Chemical Co. Ltd., Lamberhurst, United Kingdom). The ileum was exteriorized, and multiple mucosal biopsy specimens were taken with fine scissors. The biopsy specimens were then incubated for 10 min in modified organ culture medium (5, 10) (GIBCO, Paisley, United Kingdom) that contained the various bacteria (10^8 CFU ml^{-1}) to be tested. The fragments were then removed and placed on stainless steel grids, each supported to maintain contact with culture medium in sterile plastic dishes. The dishes were equilibrated and maintained throughout the culture period at 37°C in an atmosphere of 95% oxygen and 5% CO_2. After 24 h, the explants were fixed in cacodylate-buffered 2.5% (vol/vol) glutaraldehyde. They were then embedded, sectioned, stained with Reynold lead citrate and 1% (wt/vol) uranyl acetate, and examined by using a Philips 301 electron microscope. For each bacterial strain, at least three biopsy specimens were infected in each of three separate experiments.

RESULTS

EPEC strain K798 was found to carry the following four plasmids: pLV501 (96.5 kilobase pairs [kbp]), pLV502 (8 kbp), pLV503 (1.9 kbp), and pLV504 (80 kbp). Despite numerous mating experiments at 30 and 37°C both in liquid media and on filters, transconjugants containing individual plasmids were not obtained. Therefore, purified plasmid DNA was obtained and transformed into *E. coli* DH1. Plasmid pLV501 was found to encode resistance to tetracycline, chloramphenicol, and kanamycin, whereas pLV502 and pLV503 encoded resistance to ampicillin and streptomycin, respectively. pLV504 apparently has no resistance marker, and we have been unable to maintain it stably in *E.*

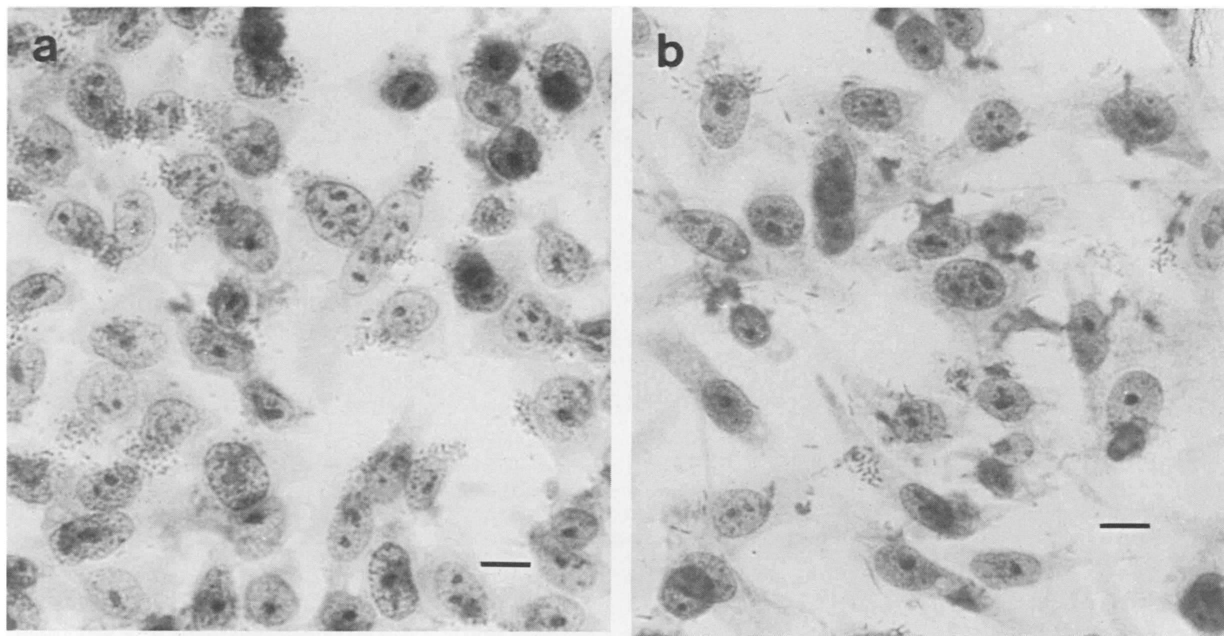


FIG. 1. Mannose-resistant LA to HEp-2 cells. (a) Parental EPEC (K798), showing large clumps of LA. Bar = 20 μm. (b) *E. coli* DH1 C590 carrying pLV501, showing smaller clumps of LA. Bar = 20 μm.

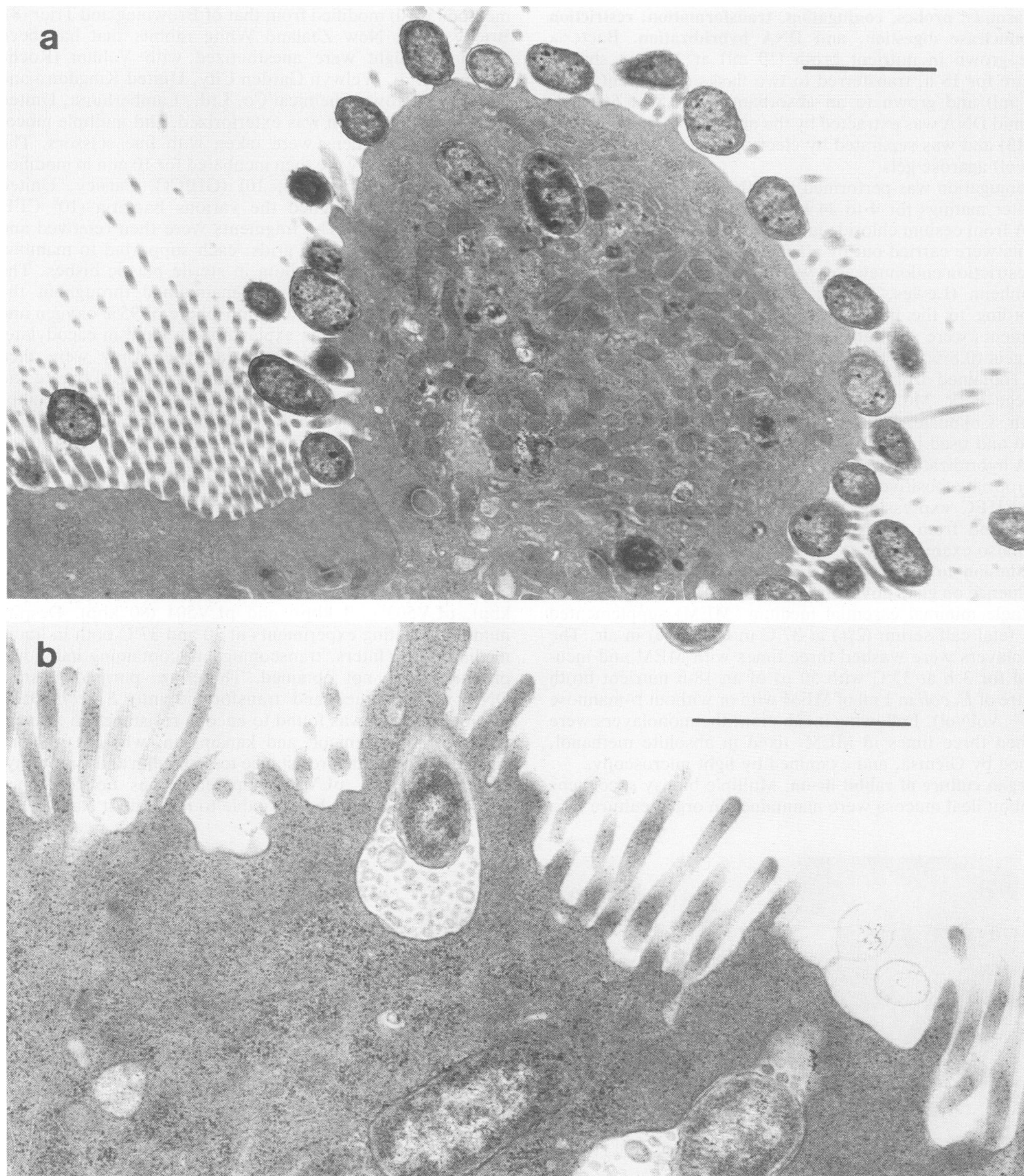
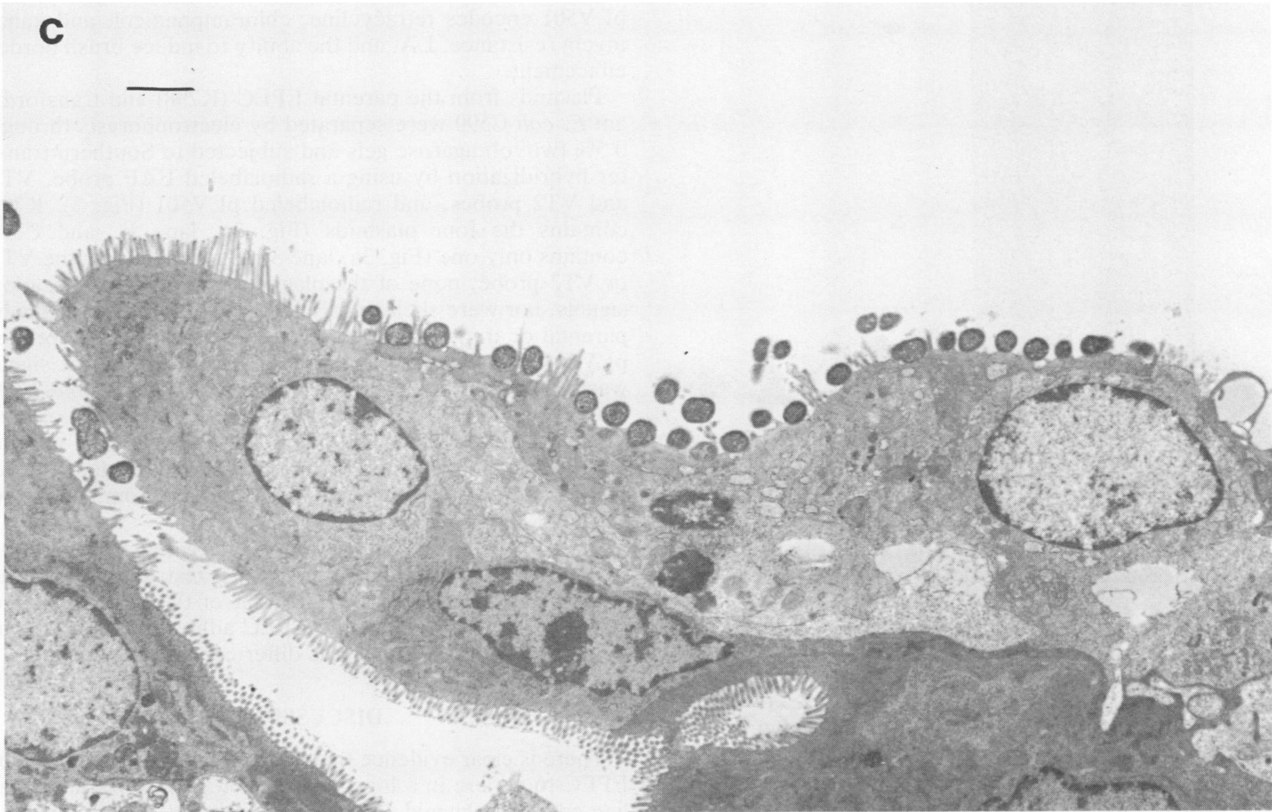


FIG. 2. The effect of bacteria on the brush border of rabbit ileal explant fragments in organ culture. (a) *E. coli* DH1 C590 carrying pLV501, showing brush border effacement after 24 h in culture. Bar = 2 μm. (b) *E. coli* DH1 C590 carrying pLV501, showing apparent invasion into the enterocyte after 24 h in culture. Bar = 1 μm. (c) Parental enteropathogenic *E. coli* O111 (K798), showing brush border effacement and cup and pedestal formation at the sites of attachment after 24 h in culture. Bar = 2 μm. (d) *E. coli* DH1 C588 carrying pLV502, showing intact brush border and numerous bacteria after 48 h in culture. Bar = 2 μm.

coli DH1. The following three transformants were selected for further study: *E. coli* C588 containing pLV502, *E. coli* C590 containing pLV501, and *E. coli* C592 containing both pLV501 and pLV502.

Transformants *E. coli* C590 and C592 as well as the parental EPEC (K798) each showed LA to HEp-2 cells (Fig. 1), in both the absence and presence of D-mannose. The parental EPEC (K798) produced large numbers of localized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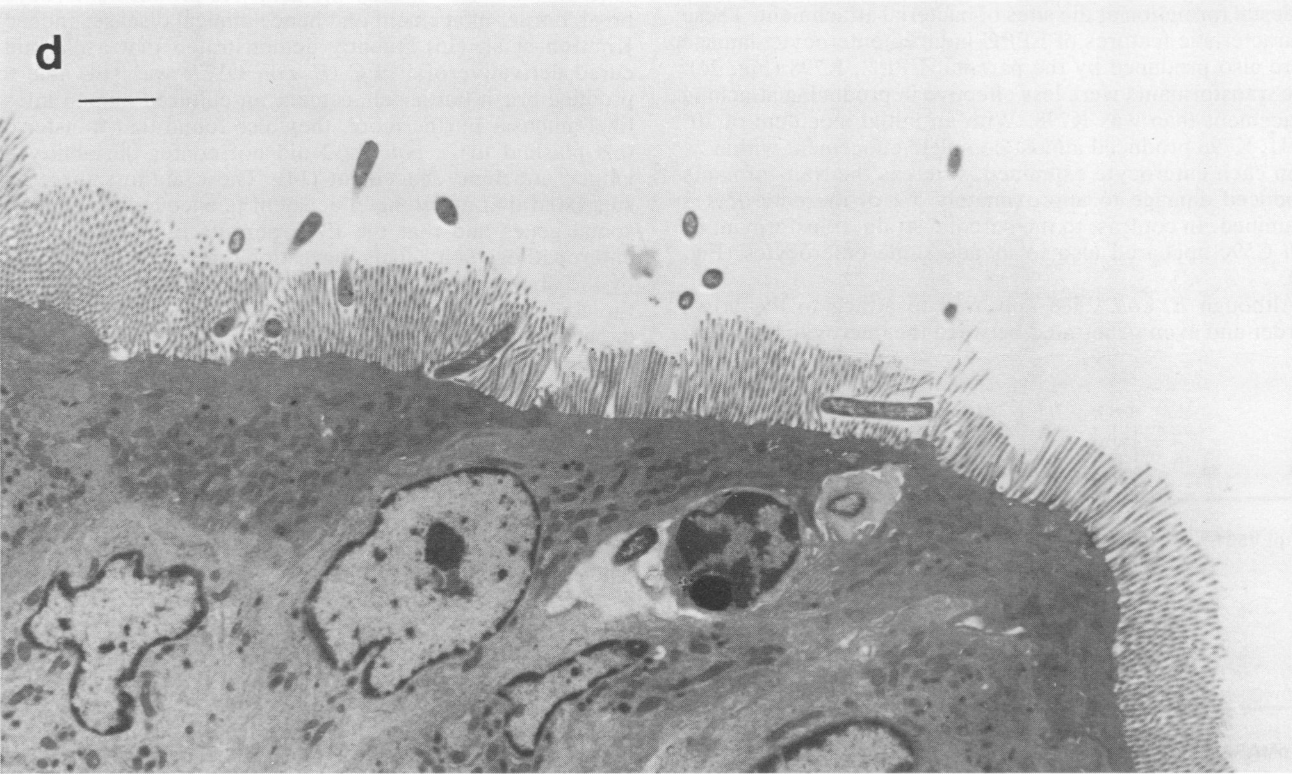
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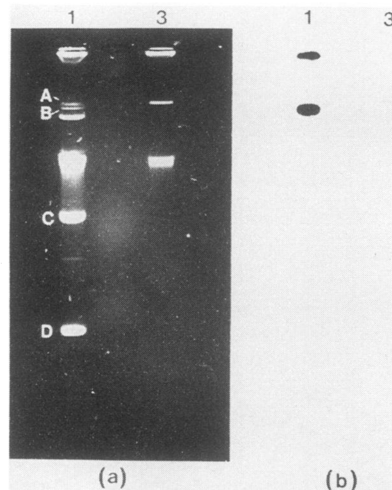

FIG. 3. Plasmids of EPEC O111 (K598) and transformant *E. coli* C590 and Southern hybridization using the EAF probe. (a) Plasmids of K798 (lane 1): pLV501 (A), pLV504 (B), pLV502 (C), and pLV503 (D). The bands between B and the chromosome and D and C are alternative forms of C and D, respectively. Transformant *E. coli* C590 (lane 3) contains pLV501 alone. (b) Southern hybridization using the EAF probe against K798 (lane 1) and C590 (lane 3).

clumps of bacteria (Fig. 1a). In general, C590 produced fewer clumps of LA, each comprising fewer bacteria (Fig. 1b). In contrast, transformant *E. coli* C588 adhered to HEp-2 cells in a diffuse fashion only in the absence of mannose.

The effects of the transformant bacteria on the brush border were examined in the rabbit ileal explant model system. Both *E. coli* C590 and C592 produced areas of brush border effacement within 24 h (Fig. 2a), with cup and pedestal formation at the sites of bacterial attachment. These characteristic features of EPEC-induced enterocyte damage were also produced by the parental EPEC, K798 (Fig. 2c). The transformants were less effective in producing attaching effacement than was K798. With an initial inoculum of 10^8 CFU, K798 produced almost complete effacement within 24 h on each enterocyte examined, whereas the transformants produced damage to approximately 5% of the enterocytes examined. In contrast to the parental strain, transformant *E. coli* C590 appeared also to invade some enterocytes (Fig. 2b).

Although *E. coli* C588 appeared to attach to the brush border and even penetrated between the microvilli (Fig. 2d),

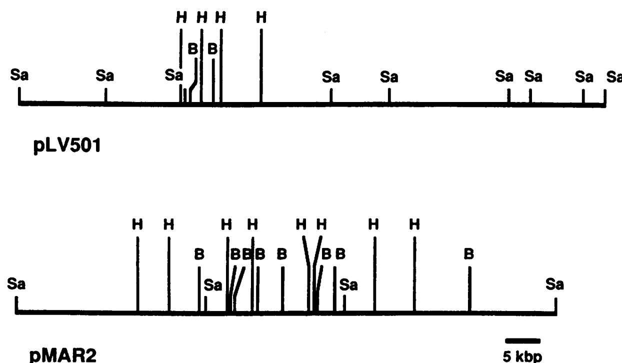

FIG. 4. Restriction map of pLV501 compared with that of pMAR-2 (23). B, *Bam*HI; H, *Hin*dIII; Sa, *Sal*I.

this transformant did not induce brush border effacement even after 48 h of incubation. *E. coli* DH1 alone had no effect on the brush border (data not shown). Thus, it appears that pLV501 encodes tetracycline, chloramphenicol, and kanamycin resistance, LA, and the ability to induce brush border effacement.

Plasmids from the parental EPEC (K798) and transformant *E. coli* C590 were separated by electrophoresis through 0.5% (wt/vol) agarose gels and subjected to Southern transfer hybridization by using a radiolabeled EAF probe, VT1 and VT2 probes, and radiolabeled pLV501 (Fig. 3). K798 contains the four plasmids (Fig. 3a, lane 1), and C590 contains only one (Fig. 3a, lane 3). By using either the VT1 or VT2 probe, none of the plasmids showed hybridization signals, nor were such signals seen in whole colony blots of parental or transformant *E. coli*. By using the EAF probe, pLV504 in K798 showed a hybridization signal; this signal was absent from the extract from transformant *E. coli* C590, which contains pLV501 (Fig. 3b). Thus, strain C590 carries pLV501, produces LA on HEp-2 cells, and is also able to induce attaching effacement to rabbit ileal explants. This plasmid does not contain DNA sequences homologous to VT1, VT2, or EAF.

In order to begin localization of the gene(s) on pLV501 responsible for enteropathogenicity, a restriction map was constructed and compared with that of the EPEC plasmid pMAR-2, which specifies the EPEC adhesion factor. It can be seen that the two plasmids differ considerably (Fig. 4).

DISCUSSION

There is clear evidence that genes specifying the ability of EPEC to adhere in a localized fashion to cultured mammalian cells are plasmid encoded, but it is not certain whether such plasmids also specify the ability to produce enterocyte brush border effacement and hence clinical disease. Indeed, Knutton et al. (15) recently demonstrated that a plasmid-cured derivative of EPEC (*E. coli* O127) was still able to produce brush border effacement on cultured human intestinal mucosa. Furthermore, they also found that transfer of this plasmid to *E. coli* K-12 did not confer the ability to induce attaching effacement (14). These authors therefore suggested that attaching effacement is encoded by chromosomal genes and that the EAF permits full expression of enteropathogenicity. This suggestion is given further support by the observations that the same plasmid-cured EPEC lost the ability to produce LA on HEp-2 cells (4) and produced either mild or no diarrhea in newborn piglets (4) or in adult human volunteers (17). Consequently, it is clear that plasmid-specified factors are necessary for the complete expression of the enteropathogenicity trait in vivo.

By transforming *E. coli* DH1, we have demonstrated that plasmid pLV501 extracted from an EPEC (O111) specifies all the factors necessary to produce LA on cultured mammalian cells and to produce enterocyte brush border effacement in an ileal explant model system. Although C590 was less efficient than the parental EPEC (K798) in producing LA, clumps of bacteria adhering to HEp-2 cells in the presence of mannose were clearly visible. That pLV501 did not contain DNA sequences homologous to the EAF probe implies that there are at least two sets of genes encoding LA. It is noteworthy that Scotland et al. recently described strains of EPEC that produce LA but do not carry DNA sequences homologous to the EAF probe (30). There is as yet no evidence that *E. coli* DH1(pLV501) can reproduce the EPEC disease in animals. Indeed, by analogy with our previous

experience in introducing the genes responsible for the expression of *E. coli* K1 capsule into an attenuated laboratory strain of *E. coli* (2), it is conceivable that it might not reproduce EPEC in animals because of decreased survival in the gut. In addition, C590 was far less efficient than the parental EPEC in producing attaching effacement and LA. Nevertheless, the use of the ileal explant model system has clearly demonstrated that plasmid-specified factors are able to produce enterocyte brush border damage characteristic of EPEC at least in vitro. Clearly, it would be of interest to determine whether cotransformation of *E. coli* DH1 with pLV501 and an EAF plasmid allows more complete expression of enteropathogenicity on cultured ileal mucosa. Our findings would be reconcilable with those of Knutton et al. (14) if the genes specifying attaching effacement could, like EAF genes, be present on either a plasmid or a chromosome (24).

In addition to producing attaching effacement, transformant *E. coli* C590 occasionally appeared to invade the enterocytes. Clearly, it is difficult to state unequivocally from electron microscopic examination of ultrathin sections that these bacteria are located inside enterocytes. We have never observed such invasion by the parental EPEC (K798) in over 100 separate experiments (5, 10) or in the original infection (32). However, others have observed that EPEC can invade cultured cells (3, 21), and invasion of enterocytes has been noted during naturally occurring infections (33).

In addition to producing attaching effacement, transformant *E. coli* C590 demonstrated LA on HEp-2 cells, yet plasmid pLV501 does not hybridize with the EAF probe and has a completely different restriction map from pMAR-2. The relationship between LA and the newly described enteroadherent aggregative *E. coli* (34), which does not contain EAF, remains clouded, since differences in methodology apparently alter the adhesion observed (20).

Recently, some strains of *E. coli* O111 serotype H^- have been designated EHEC rather than EPEC (6). Although our isolate is *E. coli* O111 H^-, we feel that it is an EPEC rather than an EHEC for the following reasons. First, the disease in the child from whom the isolate came presented as a small rather than large bowel diarrhea, and the stool did not contain either leukocytes or erythrocytes (32). Second, unlike EHEC, K798 does not elaborate VT and does not contain genes homologous to the VT1, VT2, or EHEC probes. Finally, EHEC produce attaching effacement limited to the ileum and colon (31), whereas K798 also produces this effect on the duodenum and jejunum (10).

The finding that plasmid pLV501 specifies both the ability to adhere to cultured cells in a localized fashion and to produce brush border effacement means that the genes responsible for these properties are more easily accessible than if they were present on the chromosome. Work is currently under way to clone such genes and to examine their gene products in order to further explore the mechanism of EPEC enteropathogenicity.

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