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Novel Invasion Determinant of Enteropathogenic *Escherichia coli* Plasmid pLV501 Encodes the Ability To Invade Intestinal Epithelial Cells and HEp-2 Cells

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An *Escherichia coli* K-12 transformant carrying 96.5-kb plasmid pLV501 from enteropathogenic *E. coli* (EPEC) strain K798 is able to produce the same characteristic attaching-effacing lesions in a rabbit ileal biopsy explant model as its parent strain. Cloned *Eco*RI-*Sal*I DNA restriction fragments from this plasmid failed to reproduce the attaching-effacing lesions, but one recombinant plasmid, pLV527, containing 4.5 kb of pLV501 DNA, conferred on *E. coli* DH1 transformants the ability to invade enterocytes in the rabbit explant model. DH1(pLV527) was also able to adhere to and invade HEp-2 cells. The relative invasive ability of DH1(pLV527) was quantified by recovery of internalized bacteria following gentamicin treatment of infected HEp-2 monolayers. DH1(pLV527) was 1,000-fold more invasive than DH1 carrying pBR322 or a recombinant plasmid which had no physiological effect on ileal biopsy explants but was less invasive than an enteroinvasive *E. coli* strain or a transformant carrying the cloned invasion genes of *Shigella flexneri*. Invasion by DH1(pLV501) could also be detected but occurred at a level 30 times lower than that by DH1(pLV527). Colony hybridization of the pLV527 insert against a panel of 49 EPEC and related strains revealed that only 11 contained pLV527-hybridizing sequences; thus, the invasion determinant is not an essential component of the attachment-effacement pathogenic mechanism. One pLV527-hybridizing strain displayed both attachment-effacement and invasiveness in the rabbit ileal biopsy explant model. No significant hybridization was observed to non-EPEC invasive pathogenic enteric bacteria, indicating that the invasion determinant encoded on pLV527 is distinct from those used by these organisms.

Although the role of enteropathogenic *Escherichia coli* (EPEC) in contributing to outbreaks of diarrhea in developed countries is declining, EPEC continues to be a major cause of acute infantile diarrhea in the Third World (31, 32). Despite intensive studies, the mechanisms involved in the pathogenesis of EPEC infection still remain relatively unclear, compared with those of enterotoxigenic *E. coli* and enteroinvasive *E. coli* (EIEC).

Intestinal biopsies from infected patients and experimentally infected animals reveal characteristic lesions, with adherent bacteria and elongation and subsequent effacement of the microvilli of the brush border (26, 35). Moon et al. (21) described this effect as attaching-effacing, and the term is now used to describe those strains, generally of the classic EPEC serotypes, which are capable of this form of microvillous damage. In the areas of effacement, the bacteria appear in intimate association with the enterocyte membrane, which is cupped around the organisms and frequently raised beneath adherent bacteria in pedestal structures (34). Polymerized actin from the cytoskeletal microfilaments becomes concentrated in the apical cytoplasm beneath adherent bacteria, and fluorescence staining of this actin has been used as a diagnostic test for EPEC infection (16). Lesions virtually identical to these have been reproduced by use of organ culture techniques (4, 17).

Generally, EPEC are considered to be noninvasive in vivo and do not produce a positive reaction in the Serény test, used to assay for EIEC. In some histopathological studies, bacteria have occasionally been observed within enterocytes at or near regions of brush border effacement (21, 26), while an early report by Staley et al. (29) described internalization of an O55 strain in a gnotobiotic piglet model. Using 13 EPEC strains in a similar model, Tzipori et al. (33) also found one O55 strain that was invasive, but for the most part these observations have been considered to have little relevance to the overall pathogenicity of EPEC.

Several studies have used monolayers of tissue culture cell lines, such as HEp-2 and HeLa, to investigate the adherence of EPEC, in particular the localized adherence phenotype associated with the presence of EPEC adherence factor (EAF) sequences (2). It was recently demonstrated that at least some EPEC strains are able to invade these cells. Internalized bacteria have been identified by both electron microscopy (1, 19) and the recovery of invading bacteria following gentamicin treatment of infected monolayers (7).

We previously reported that pLV501, a 96.5-kb plasmid from EPEC strain K798, is capable of producing the same attaching-effacing lesions in a rabbit ileal biopsy explant model as its parent when transformed into an *E. coli* K-12 host (10). The findings presented in this paper were the result of attempts to localize the genes responsible for the attachment-effacement phenotype by cloning restriction fragments from this plasmid. While such genes were not identified, an apparently novel determinant which confers an invasive capacity on K-12 transformants was cloned.

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MATERIALS AND METHODS

Bacterial strains. EPEC strain K798, serotype O111:H−, and the *E. coli* DH1 transformant carrying plasmid pLV501 from this strain have been described previously (10, 30). *E. coli* DH1 (F⁻ *recA1 endA1 gyrA96 thi-1 hsdR17* [r_K^- m_K^+] *supE44* λ^-) was used as a host for transformation. HB101 (pHS4108) carries a cosmid encoding the invasion genes of *Shigella flexneri* and was generously provided by P. Sansonetti, Pasteur Institute, Paris, France (18). EI314 is an EIEC strain (serogroup O124) and was kindly provided by R. M. Robins-Browne, University of Melbourne (25). Other strains of various EPEC serogroups and other enteric pathogens used for colony hybridization are listed with their sources in Table 1.

Growth conditions. Bacteria were grown in liquid cultures in 2.5% (wt/vol) nutrient broth (Amersham, Bury, England) at 37°C with constant shaking in the presence of appropriate antibiotics (Sigma, Poole, England). For use in HEp-2 adhesion and invasion assays, 0.5% (wt/vol) D-mannose was added to the medium. HB101(pHS4108) was grown in 3% Trypticase soy broth (GIBCO BRL, Paisley, Scotland) to avoid cosmid loss (27a).

Plasmid purification and DNA manipulations. Plasmid DNA was isolated by the method of Humphreys et al. (12) and then subjected to cesium chloride density centrifugation.

Restriction endonucleases were obtained from Boehringer Mannheim (Lewes, England) or P & S Biochemicals (Liverpool, England) and were used with the manufacturer's recommended buffer. T4 DNA ligase and calf intestinal alkaline phosphatase were obtained from Boehringer Mannheim. DNA ligations were performed at 16°C for 16 h. DNA restriction fragments were separated by electrophoresis through 0.7% agarose gels. Fragments for use as DNA probes were separated on 1% low-melting-point agarose gels (BRL, Gaithersburg, Md.) and, after excision, were labelled in situ with [^{32}P]dCTP (ICN Radiochemicals, Irvine, Calif.) by use of a random-primer hexanucleotide labelling kit (Boehringer Mannheim).

Plasmid DNA was transformed into *E. coli* made competent for DNA uptake by the method of Cohen et al. (5).

Colony hybridization. Single colonies of bacteria were applied with toothpicks directly to sterile nitrocellulose filters (Schleicher and Schuell, Dassel, Germany) overlaid on a fresh nutrient agar plate and were allowed to grow overnight at 37°C. The colonies were lysed by laying the filters on Whatman 3MM paper (Whatman, Maidstone, England) soaked sequentially in (i) 0.5 N NaOH, twice for 5 min each time, (ii) 1 M Tris-HCl (pH 8.0), twice for 5 min each time, and (iii) neutralizing solution (1.5 M NaCl, 1 M Tris-HCl [pH 8.0]) once for 5 min. The filters were air dried and baked under vacuum at 80°C for 2 h. Hybridization with the labelled probe was done as described by Sambrook et al. (27) with 50% deionized formamide in the hybridization solution and at a temperature of 42°C.

Organ culture of rabbit ileal biopsies. The methods for organ culturing and infection of rabbit ileal biopsies have been described previously (8).

HEp-2 adhesion and invasion assays. HEp-2 cells were maintained in Eagle's minimal essential medium (MEM) (GIBCO BRL) supplemented with 5% (vol/vol) fetal calf serum (Sera-lab, Sussex, England) and 0.15% (wt/vol) Na_2HCO_3 and with added penicillin (10^5 U liter^{-1}) and streptomycin (100 mg liter^{-1}). For assays, cells were grown in the same medium to confluent monolayers on 10-mm-diameter coverslips in glass vials (12 by 40 mm). The coverslips were washed three times with MEM and infected with 50 μl of an overnight culture of bacteria in 1 ml of the medium lacking antibiotics. For adhesion assays, after 3 h the monolayers were washed with MEM, fixed in methanol, and stained with a 10% solution of Giemsa (BDH) prior to

TABLE 1. Hybridization of EPEC and related strains with the EAF probe and the pLV527 insert

Strain	Serogroup[a]	Source[b]	Hybridization with:	
			EAF probe	pLV527 insert
E2348	O127	PHL	+	−
E74/68	O128	PHL	−	−
E380/69	O114	PHL	+	−
E851/71	O142	PHL	+	−
E16501	O111	PHL	+	−
E19895	O111	PHL	−	−
E22803	O111	PHL	+	+
E27256	O111	PHL	−	−
E27688	O111	PHL	−	−
E29322	O111	PHL	−	−
C287	O111	ALD	−	−
C288	O126	ALD	−	−
C323	O111	ALD	−	−
C408	O111 (VT1⁺)	MRI	−	+
C412	O111 (VT1⁺)	MRI	−	+
C413	O111 (VT1⁺)	MRI	−	+
C727	O111	ALD	−	−
C736	O114	ALD	−	−
D11	ND	BAN	−	−
D12	ND	BAN	−	−
D13	ND	BAN	−	+
D14	ND	BAN	−	+
D15	ND	BAN	−	−
D16	ND	BAN	−	−
D17	ND	BAN	−	−
D18	ND	BAN	−	−
D19	ND	BAN	−	−
D20	O128	ALD	−	−
D21	O142	ALD	−	−
D22	O114	ALD	−	−
D23	O128	ALD	−	−
D24	O125	ALD	−	−
D46	NG	THI	+	+
D48	NG	THI	+	−
D50	O119	THI	+	−
D51	O111	THI	+	−
D53	O142	THI	+	+
D55	O127	THI	+	+
D67	O125	ALD	−	−
D96	O114	ALD	−	−
D103	O126	ALD	−	−
D162	O142	ALD	−	−
D163	O111	ALD	−	−
D164	O114	ALD	−	+
D165	O126	ALD	−	−
D226	O111	ALD	−	−
B/M 369	O86	IND	−	−
C-35	O86	IND	−	+
RDEC-1	O15	RAB	−	−

[a] ND, not determined; NG, nongroupable.

[b] PHL, sporadic and epidemic isolates from the Central Public Health Laboratory, Colindale, England (B. Rowe); ALD, sporadic isolates from Alder Hey Children's Hospital, Liverpool, England; MRI, verotoxin-producing strains isolated from calves with diarrhea at the Moredun Research Institute, Edinburgh, Scotland; BAN, isolates from infants with severe diarrhea in Bangladesh; THI, EAF⁺ isolates from infants with severe diarrhea in Thailand (P. Echeverria); IND, sporadic diarrheal isolates from Calcutta, India (R. Pal); RAB, attaching-effacing rabbit enteric pathogen.

examination by light microscopy. The invasiveness of bacteria was quantified by the method of Sansonetti et al. (28). After 3 h of infection, extracellular bacteria were killed by the addition of gentamicin to MEM to a final concentration of 25 $\mu g\ ml^{-1}$ (sufficient to eliminate the recovery of all strains and transformants in the absence of HEp-2 cells). The monolayers were incubated for an additional 1 h, washed five times with MEM, and lysed by the addition of 0.5 ml of 0.5% (wt/vol) sodium deoxycholate. Serial dilutions of the resulting suspension were plated on the appropriate selective medium.

RESULTS

Cloning of pLV501 restriction fragments. DNA fragments resulting from an *Eco*RI-*Sal*I restriction digest of pLV501 were cloned into similarly treated pBR322 DNA. The resulting recombinant plasmids were transformed into *E. coli* DH1 and selected by plating on medium containing ampicillin. A total of 15 different clones, the inserts of which accounted for 65% of the total pLV501 DNA, were obtained. None of the transformants was resistant to tetracycline, kanamycin, or chloramphenicol, indicating that the antibiotic resistance genes encoded by pLV501 had not been cloned in a functional form.

Analysis of clones in the rabbit ileal biopsy explant model. All of the transformants containing recombinant plasmids were assayed individually in the rabbit explant model, under conditions which had produced attachment and effacement with transformant DH1(pLV501). None of the clones was able to produce this type of damage, the majority failing to adhere to the brush border. On the other hand, the transformant carrying pLV527, with a 4.5-kb DNA insert, displayed invasive behavior. After incubation for 6 h, bacteria were seen adhering to the microvillus tips, with isolated organisms intercalating between the microvilli (Fig. 1A). At 24 h, the bacteria had apparently moved to the base of the microvilli, causing some distortion and disruption of the brush border structure but no effacement. Within the cytoplasm of individual enterocytes there were numerous bacteria, those near the cell membrane apparently located within discrete vesicles. Bacteria that had penetrated deeper tended to mass together, with possible fusion of the vesicles (Fig. 1B).

Examination of sections at a higher magnification confirmed that at least some of the vesicles were membrane bound and also indicated that bacterial division had occurred following internalization (Fig. 1C). After 36 to 48 h postinfection, we found areas of the sections in which all bacteria visible were internalized, being surmounted by an apparently normal brush border (Fig. 1D).

Quantification of invasiveness of DH1(pLV527). To quantify invasion by using HEp-2 monolayers, we found it necessary to establish that DH1(pLV527) could adhere to these cells. Giemsa staining of monolayers after 3 h of incubation with the bacteria showed that the transformant adhered in a diffuse manner (Fig. 2). For the invasion assays, two negative controls, DH1(pBR322) and DH1(pLV524), were included. The latter contains another of the recombinant plasmids obtained from the cloning of pLV501 restriction fragments; this plasmid incorporates a 1.4-kb *Eco*RI-*Sal*I insert but has no effect on rabbit ileal biopsy explants. With both of these controls, the residual recovery of bacteria could not be eliminated but occurred at a very low level, <200 CFU per coverslip (Table 2). DH1(pLV527) was significantly more invasive ($P < 0.005$, Student's t test) than either control, being recovered in approximately 1,000-fold greater numbers than DH1(pLV524). In this system, invasion by transformant DH1(pLV501) could also be detected at a level significantly higher than that by the controls ($P < 0.01$), but the recovery of DH1(pLV501) was 30-fold lower than that of DH1(pLV527). In contrast, DH1(pLV527) was approximately 10-fold less invasive than "professional" invasive organisms, such as EI314, an EIEC strain, or *E. coli* K-12 strain HB101 carrying pHS4108, a cosmid encoding the invasion genes of *S. flexneri*.

Hybridization of pLV527 with EPEC strains. It was considered possible that the invasion determinant encoded on pLV527 constituted part of an overall pathway leading to the attaching-effacing lesions of EPEC-induced damage. If this were so, it could be predicted that such sequences would be common to all EPEC strains. A total of 49 known EPEC and related strains, including those isolated from infants with diarrhea and in whom strains of EPEC serogroups were the sole incriminated pathogens, were probed on colony blots with the labelled DNA insert from pLV527. The results are presented in Table 1. Also included are the results from the probing of these strains with the EAF probe of Baldini et al. (3), as Donnenberg et al. (7) found that EAF^+ strains were significantly more invasive for tissue culture cells than EAF^- strains. Of the strains tested, only 11 hybridized to the pLV527 insert. These were from fairly diverse sources and included three strains isolated in Thailand and two isolated in Bangladesh. One of the two strains isolated in Calcutta, India, and displaying mannose-resistant hemagglutinating activity (22) hybridized to pLV527, while only one strain isolated from a sporadic case of infantile diarrhea in Liverpool hybridized. pLV527 also hybridized to all three of the verocytotoxin-producing isolates from calves with diarrhea, although two of these, C412 and C413, have very similar plasmid profiles and may represent separate isolates of the same strain. No relationship was evident between hybridization to pLV527 or the EAF probe and serogroup.

pLV527 was also tested against a limited range of other enteric invasive pathogens on colony blots. No hybridization was found to two strains of EIEC, two of *Shigella sonnei*, one of *S. flexneri*, four of *Yersinia enterocolitica*, and one of *Yersinia pseudotuberculosis*, although weak hybridization was observed to one of five strains of *Salmonella* species (data not shown).

Effect of pLV527-hybridizing strain D46 in the rabbit ileal biopsy explant model. The rabbit ileal biopsy explant model was used to study the effects of at least 20 of the EPEC strains listed in Table 1, including 4 of those which hybridized to pLV527. Of these, pLV527-hybridizing strain D46 was unique in that explant sections showed not only the characteristic attaching-effacing lesions of EPEC but also large-scale invasion of the enterocytes (Fig. 3). As with DH1(pLV527), the internalized bacteria were contained within vesicles. The point of entry into the enterocytes was seemingly at the areas of effacement of the brush border, with subsequent migration away from the enterocyte membrane toward the lamina propria.

DISCUSSION

We previously demonstrated that pLV501, a 96.5-kb plasmid from an EPEC strain, encodes sufficient genes to cause attaching-effacing lesions in a rabbit ileal biopsy explant model (10). This result is at variance with those of Knutton et al. (15), who found that pMAR2, the prototype EAF plasmid from strain E2348, did not confer this capacity to

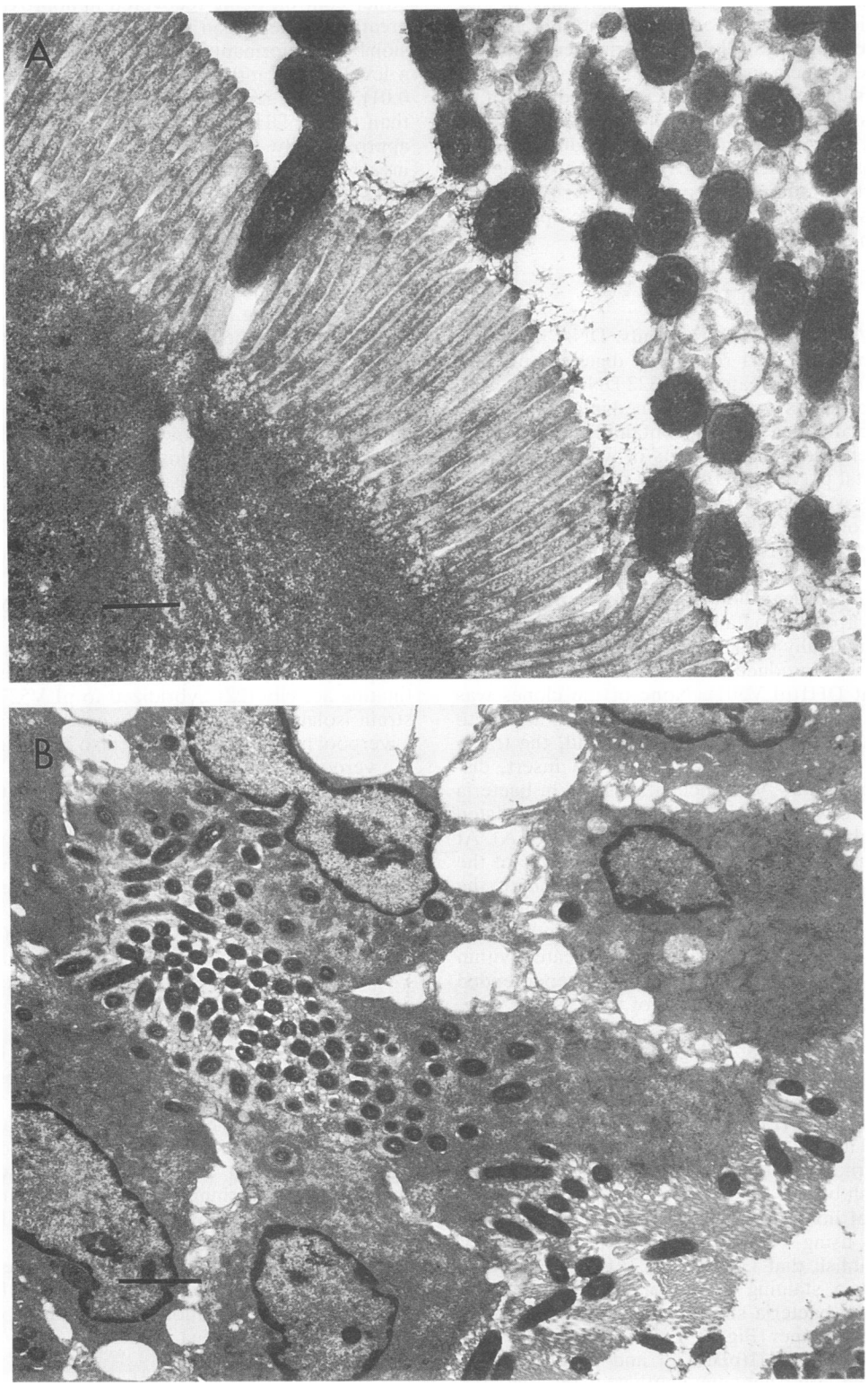

FIG. 1. (A) Electron micrograph of a section of a rabbit ileal biopsy explant 6 h after infection with transformant DH1(pLV527). Bacteria are adherent to the tips of the microvilli, causing slight localized distortion, and are capable of penetrating between the microvilli. Bar, 1 μm. (B) Similar section 24 h after infection with DH1(pLV527). Several bacteria are in intimate contact with the enterocyte membrane, causing apparent distortion of the microvilli but little brush border effacement. Large numbers of bacteria are present within the enterocytes, the majority being in one fused vesicle. Bar, 4 μm. (C) Section of a rabbit ileal biopsy explant 48 h after infection with DH1(pLV527). There is evidence that the vesicles enclosing the bacteria are membrane bound, and several bacteria appear to be undergoing cell division. Bar, 2 μm. (D) Similar section showing bacteria distributed throughout the enterocytes, with a normal overlying brush border. Bar, 4 μm.

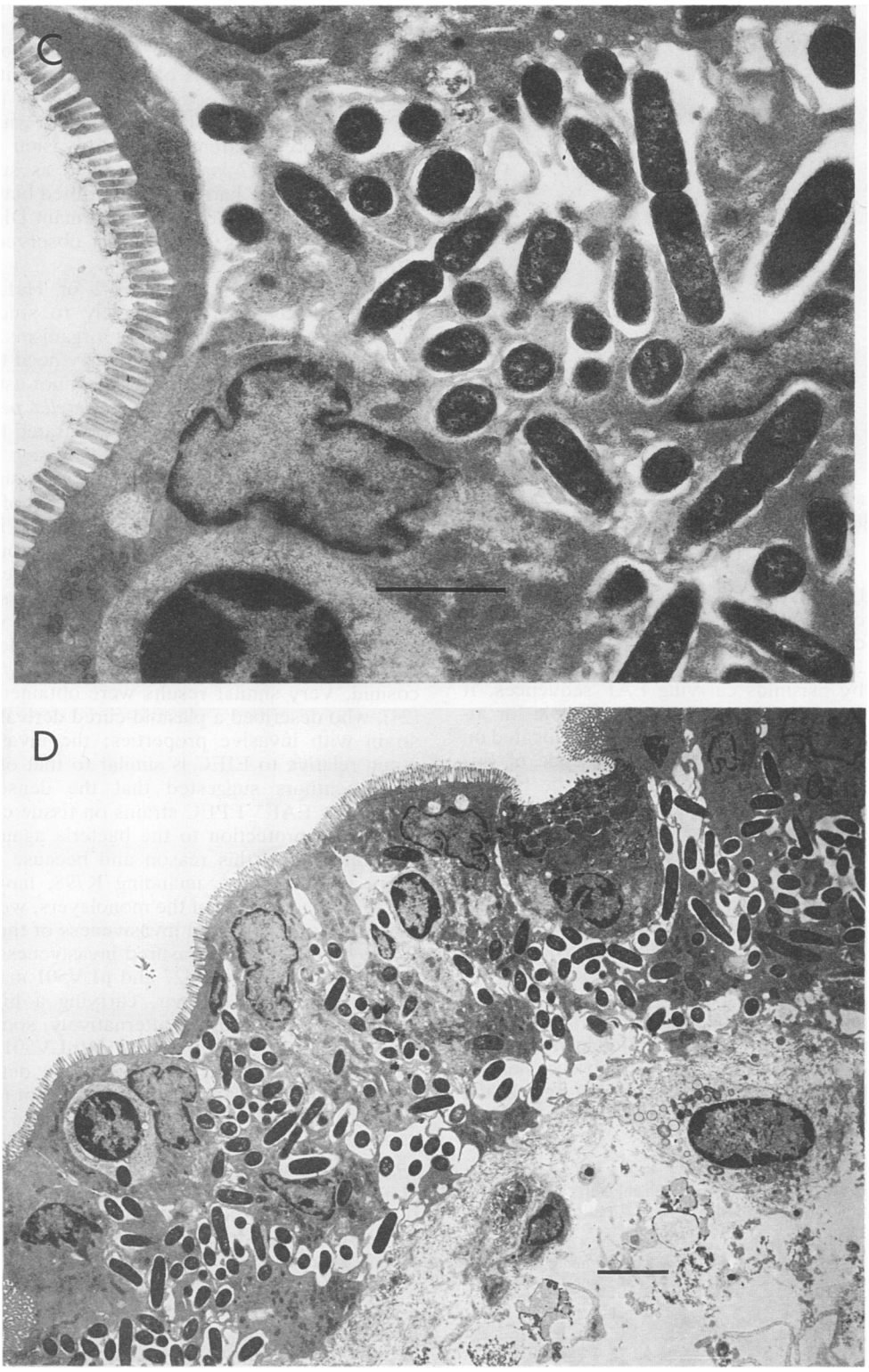

FIG. 1—*Continued.*

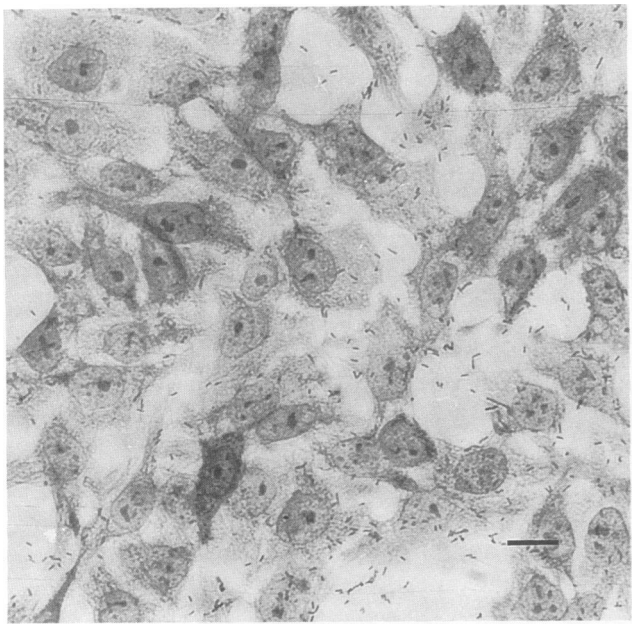

FIG. 2. Diffuse adherence of transformant DH1(pLV527) to HEp-2 cells. Bar, 20 μm.

transformants and suggested that the genes for attachment-effacement were chromosomally encoded. It therefore appears that the location of such genes may vary between strains, although the available evidence suggests that they are not encoded by plasmids carrying EAF sequences. It seems highly probable that the gene(s) responsible for attachment-effacement should be the same whether located on the chromosome or the plasmid, so the presence of this gene(s) on pLV501 should theoretically make the task of localizing and cloning this gene(s) much easier. That none of the recombinant plasmids produced from the cloning of restriction fragments of pLV501 resulted in attachment-effacement is perhaps not surprising. In identifying the chromosomally encoded *eae* gene, Jerse et al. (14) found that a cosmid encoding the *eae* gene product and 11 other proteins was insufficient to produce attachment-effacement, almost certainly indicating that at least two loci were involved in a complex pathway.

The invasive determinant encoded by pLV527 may represent a single component in the attachment-effacement pathway. Limited enterocyte invasion has been an occasionally noted effect of EPEC infection (21, 26, 29), but against the background of gross microvillous destruction by the attaching-effacing properties of these organisms, it appears to contribute little, if at all, to the overall pathogenesis of the disease. Additionally, the clinical syndrome manifested by EPEC infection differs markedly from the dysenterylike illness associated with invasive pathogens such as *Shigella* and *Yersinia* spp., with the absence of blood, mucus, and neutrophils in the stools of infected patients (11). In many ileal biopsy explant sections infected with the parent strain K798, there was no evidence of bacteria within enterocytes; it is therefore apparent that the invasion determinant encoded by pLV527 is not expressed as such in K798. A difference that we had previously noted between the effects of infection with K798 and transformant DH1(pLV501) was that the latter was occasionally observed intracellularly (10).

TABLE 2. Invasive capacity of bacteria measured as the recovery of internalized organisms from HEp-2 cell monolayers[a]

Strain or transformant	No. of CFU recovered/10-mm coverslip (mean ± SD)
EI314 (EIEC)	$(1.1 \pm 0.4) \times 10^6$
HB101(pHS4108)	$(2.2 \pm 0.4) \times 10^6$
DH1(pLV501)	$(3.4 \pm 0.8) \times 10^3$
DH1(pLV527)	$(1.1 \pm 0.4) \times 10^5$
DH1(pLV524)	$(1.2 \pm 0.8) \times 10^2$
DH1(pBR322)	$(1.7 \pm 1.2) \times 10^2$

[a] Values are the results of duplicate assays performed at least twice. The original inoculum was 2×10^7 to 5×10^7 bacteria in 1 ml of MEM.

The gentamicin-treated HEp-2 or HeLa cell invasion assay has been used extensively to study the invasion characteristics of a variety of organisms. However, the results obtained from this assay may need to be interpreted with a degree of caution, as bacteria not usually considered invasive pathogens, such as *Bordetella pertussis* and *Edwardsiella tarda*, have been demonstrated to be invasive in this assay (9, 13). We used this assay to quantify the invasiveness of transformants previously shown to be invasive for rabbit enterocytes relative to that of known invasive bacteria and confirmed the low-level invasiveness of DH1(pLV501). Donnenberg et al. (7) found that EAF^+ EPEC strains were generally more invasive for HEp-2 cells than EIEC strains, whereas EAF^- EPEC strains were less invasive. We showed that DH1(pLV527), which obviously lacks EAF sequences, was at least 10-fold less invasive than EIEC or a transformant carrying the *S. flexneri* invasion cosmid. Very similar results were obtained by Riley et al. (24), who described a plasmid-cured derivative of an EPEC strain with invasive properties; the invasiveness of this strain relative to EIEC is similar to that of DH1(pLV527). These authors suggested that the dense microcolonies formed by EAF^+ EPEC strains on tissue culture cells may offer some protection to the bacteria against the effect of gentamicin. For this reason and because of the fact that many EPEC strains, including K798, have a detrimental effect on the integrity of the monolayers, we did not attempt to quantify the potential invasiveness of the parent strain.

The differences in measured invasiveness between transformants carrying pLV527 and pLV501 may be due to the dose effect of the former, carrying a high-copy-number recombinant plasmid, or, alternatively, some form of weak repression may be acting in DH1(pLV501). In the parent strain, the expression of the invasion determinant is not evident phenotypically. The EPEC strain (B171) described by Riley et al. (24) only becomes invasive when cured of a 54-MDa plasmid. This plasmid has also been shown to be unusual in that it encodes the lipopolysaccharide (LPS) O-antigenic polysaccharide (23). It is consistent with the results of these authors and our own results obtained with *E. coli* K-12 (rough) transformants to postulate that wild-type EPEC strains carry chromosomally encoded or plasmid-encoded invasion determinants, the effects of which are counteracted in vivo by the O-antigenic side chains of LPS. In support of this hypothesis, it is intriguing that the one EPEC strain, D46, which we showed to be grossly invasive in the rabbit ileal biopsy explant model, has a nongroupable LPS type.

The fact that the pLV527 insert hybridized to the DNAs of only 22% of the EPEC and related strains tested tends to imply that this determinant is not an essential component of

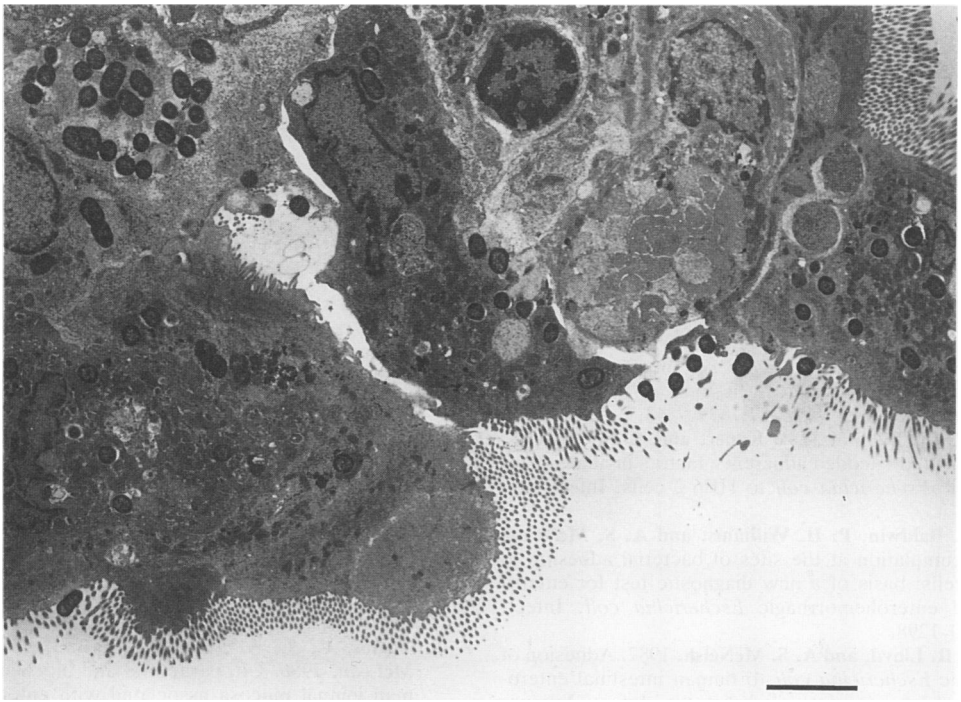

FIG. 3. Section of a rabbit ileal biopsy explant 24 h after infection with pLV527-hybridizing strain D46. Effacement of the brush border is evident, with elongation of adjacent microvilli, but bacteria are also present within the cytoplasm of the enterocytes. Bar, 4 μm.

the attachment-effacement pathway. That Donnenberg et al. (6) found E2348, a strain which does not hybridize to pLV527, to be invasive is problematic. Tn*phoA* insertions in strain E2348 identified both plasmid-encoded and chromosomally encoded genes which were involved in the invasion process. One insertion mapped close to the *eae* gene and also destroyed the attaching-effacing ability, indicating a close linkage between attachment-effacement and invasion in this strain. It seems probable that at least some EPEC strains contain two distinct invasion determinants, one homologous to that encoded by pLV527 and one linked to the *eae* gene, neither of which is generally manifested phenotypically. A precedent for two independent invasion systems exists with the genetically distinct *inv* and *ail* loci of *Y. enterocolitica* (20).

The invasion determinant encoded by pLV527 may represent an accessory plasmid-borne virulence determinant which has been acquired by a number of EPEC strains, although the potential benefits to the bacteria of this novel determinant seem limited, as evidence from sections of infected ileal biopsy explants indicates that while some cell division occurs, the bacteria are unable to escape from their enclosing vacuole. The size of the DNA insert (4.5 kb) in pLV527 mitagates against a multigenic process of the type exemplified by *Shigella* and EIEC invasion. Since DH1 (pLV527) can both adhere to and invade HEp-2 cells, either the cloned fragment encodes a separate adhesin(s) and a separate invasin(s) or, as with the *Y. enterocolitica inv* gene, a single gene product has both functions. However, the lack of hybridization to any of the common invasive enteric pathogens indicates that the genetic determinants cloned on pLV527 potentially encode a novel mechanism for invasion.

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