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Construction and Analysis of Tn*phoA* Mutants of Enteropathogenic *Escherichia coli* Unable To Invade HEp-2 Cells

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Enteropathogenic *Escherichia coli* (EPEC) strains have recently been shown to invade tissue culture cells. We describe a set of 22 Tn*5* IS50$_L$::*phoA* (Tn*phoA*) insertion mutants of EPEC strain E2348-69 that are unable to invade HEp-2 cells. Each mutant was tested for the ability to adhere to and to induce the polymerization of actin in HEp-2 cells. Southern hybridization of plasmid and total DNA of each strain was performed to determine the location of each Tn*phoA* insert, and each Tn*phoA* insert along with flanking EPEC sequences was cloned. These studies resulted in the grouping of the mutants into five main categories. These include strains with plasmid and chromosomal insertions that alter adherence, chromosomal insertions that alter the ability to induce actin polymerization, and chromosomal insertions that do not affect adherence or actin polymerization. These studies indicate that genes affecting EPEC adherence may be located on both the plasmid and chromosome, that several genes are involved in the induction of actin polymerization in epithelial cells, and that EPEC invasion is a complex process involving multiple genetic loci.

Enteropathogenic *Escherichia coli* (EPEC) continue to be an important cause of infantile diarrhea in developing nations (8) and of occasional diarrheal outbreaks in day-care settings in developed countries (4). Although EPEC were the first *E. coli* shown to cause diarrhea (28), the pathogenesis of this infection remains poorly understood. EPEC continue to be identified on the basis of their association with specific serotypes (18), reflecting, perhaps, the lack of information available concerning the virulence factors of this organism.

Cravioto et al. were the first to show that EPEC are capable of adhering to tissue culture cells (6). EPEC of classic serotypes usually form distinctive microcolonies on the surface of epithelial tissue culture cells (26, 32). This so-called localized adherence is associated with the presence of a plasmid of 50 to 70 megadaltons. Loss of the large plasmid of EPEC strain E2348-69 is accompanied by loss of the ability to form microcolonies on the surface of HEp-2 cells (2) and by a reduction in virulence when fed to volunteers (20). Transfer of this plasmid to a nonadherent, nonpathogenic strain, *E. coli* HB101, confers the ability to adhere to HEp-2 cells (2). A DNA probe derived from the large plasmid of E2348-69, the EPEC adherence factor (EAF) probe, is highly sensitive and specific in recognizing EPEC strains capable of forming adherent microcolonies on tissue culture cells (26).

EPEC are capable of attaching to and effacing the microvillus surface of the intestinal epithelium in vivo (25). When viewed by electron microscopy, the bacteria are seen in very close proximity to the epithelial cells. Where bacteria are attached, microvilli are lost; the epithelial cells form cuplike pedestals upon which the bacteria rest. The underlying cytoskeleton of the epithelial cell is disorganized, with a proliferation of filamentous actin. The polymerization of actin at the sites of the attaching and effacing lesion forms the basis of a recently described diagnostic test for EPEC (16). Fluorescein isothiocyanate (FITC)-phalloidin, the fluorescein conjugate of a phallotoxin, binds specifically to polymerized actin and can be used to recognize EPEC by the fluorescence seen in the epithelial cells underlying the attached bacteria. This response has been termed fluorescence actin staining (FAS). Since enterohemorrhagic *E. coli* also cause the attaching and effacing lesions (11), they are also FAS positive (16).

The ability to cause the attaching and effacing lesion is not encoded on the EAF plasmid of E2348-69. The plasmid-cured derivative of this strain, which is unable to form adherent microcolonies on HEp-2 cells and is attenuated in virulence when fed to volunteers, retains the ability to induce the attaching and effacing lesions in human intestinal tissue culture (17). HB101 containing the EAF plasmid is unable to produce this effect (15).

Although EPEC have traditionally been considered to be noninvasive (8, 19, 28), accumulating evidence casts doubt on this assumption. From the earliest published electron micrographs of EPEC infection, bacteria have been observed within epithelial cells at the sites of the attaching and effacing lesion (36). Published studies showing intracellular EPEC have included rabbit models (25, 27), newborn pig models (25, 36), and gnotobiotic piglet models (38). The first published ultrastructural study of a human infected with EPEC also demonstrated intracellular organisms (39). These observations have led a number of investigators to study cellular invasion of EPEC in tissue culture. EPEC have been shown to be capable of invading HEp-2 cells in vitro (1) and to multiply within Henle-407 cells (23). In a quantitative study of HEp-2 cell invasion, EPEC strains that possessed the EAF were significantly more invasive than EPEC strains that lacked EAF. The most invasive EPEC strains were comparable to enteroinvasive *E. coli* in their ability to invade HEp-2 cells (7).

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To identify the genes required for EPEC invasion of HEp-2 cells, we used the transposon Tn*5* $IS50_L$::*phoA* (Tn*phoA*) (21) to generate a collection of noninvasive EPEC mutants. Since gene products necessary for cellular invasion are likely to be on the surface of the bacterium where they can interact with the epithelial target cells, we reasoned that this system would be ideal for identifying genes involved in EPEC pathogenesis.

MATERIALS AND METHODS

Bacterial strains, media, and tissue culture. EPEC O127:H6 strain E2348-69 was provided by B. Rowe, Colindale, London, England. Originally isolated from an outbreak of infantile diarrhea, this strain exhibits localized adherence (2) and the attaching and effacing lesion (25), is virulent in volunteer studies (19, 20), and invades HEp-2 cells (7). Its EAF plasmid-cured derivative, MAR20, exhibits the attaching and effacing lesion (17) but not localized adherence (2) and has reduced virulence in volunteer studies (20) and reduced invasion of HEp-2 cells (7). HB101(pMAR7) is a K12/B hybrid strain, transformed with a Tn*801*-marked EAF plasmid from E2348-69. It exhibits localized adherence (2) but not the attaching and effacing lesion (15). Bacteria were stored at −70°C in 2% tryptone–20% glycerol and grown in Trypticase (BBL Microbiology Systems) soy broth (TSB) or Luria broth or on Luria plates. Ampicillin (50 μg/ml), tetracycline (12.5 μg/ml), kanamycin (50 μg/ml), gentamicin (10 μg/ml), chloramphenicol (20 μg/ml), and 5-bromo-4-chloro-3-indolyl phosphate (X-P) (40 μg/ml) were added when indicated.

HEp-2 cells were maintained in RPMI 1640 medium supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml), L-glutamine (2 mM), and 10% fetal bovine serum.

Construction of Tn*phoA* mutants. To obtain a selectable marker for conjugation, plasmid pUR222 (30) was introduced into E2348-69 by electroporation with a Gene Pulser electroporater (Bio-Rad Laboratories, Richmond, Calif.) in 272 mM sucrose–3 mM potassium phosphate (pH 7.4)–15% glycerol at 2.5 kV/25 μF. The Tn*phoA*-bearing plasmid pRT291 (37) was introduced into the resulting transformant from SM10 (33) by conjugation. pUR222 was then cured from the resulting transconjugant by three passages on plates containing tetracycline and kanamycin but no ampicillin. Selection for mutants resulting from insertion of Tn*phoA* into EPEC DNA was accomplished by conjugal transfer of incompatible plasmid pPH1JI from MM294 into E2348-69(pRT291) with simultaneous selection for gentamicin and kanamycin (37). Transposon insertions that resulted in the production of fusion proteins directed by signal sequences out of the cytoplasm were detected by the blue appearance of colonies on plates containing X-P, an indicator dye for alkaline phosphatase activity. To minimize the inclusion of sibling clones, no more than three blue colonies were selected from each mating.

Screening Tn*phoA* mutants for invasive ability. All Tn*phoA* mutants that appeared blue on medium containing X-P were tested for invasive ability in a modification of the HEp-2 cell invasion assay (34). Since these mutants all contained plasmid pPH1JI, which encodes resistance to gentamicin, the invasion assays were performed with the related aminoglycoside amikacin. Preliminary work indicated that equivalent results were achieved with this substitution (data not shown). HEp-2 cells were grown overnight in 96-well microdilution plates at an initial density of 8×10^4 cells per well. The medium was replaced with RPMI 1640 without supplements. Each strain was grown in TSB overnight, diluted 1:20 in phosphate-buffered saline, and added to the HEp-2 cells in 0.01-ml portions. After 3 h at 37°C, the plates were washed three times with phosphate-buffered saline, the medium was replaced with RPMI 1640 containing amikacin (250 μg/ml), and the plates were incubated 1 h further. The plates were then washed once with phosphate-buffered saline, and the epithelial cells were lysed by treatment for 20 min with 0.2 ml of 1% Triton X-100 detergent. The lysate was diluted 10-fold in phosphate-buffered saline, and 0.1 ml was spread on L plates containing kanamycin for colony counting. E2348-69(pRT291) and SM10(pRT291) served as positive and negative controls, respectively, in each experiment. E2348-69(pRT291) yielded an average of 136 colonies per experiment, whereas SM10(pRT291) never gave more than 4 colonies. Strains that yielded three or fewer colonies in two separate experiments were passaged on medium containing kanamycin but no gentamicin to enrich for colonies cured of plasmid pPH1JI. After three to five passages, strains that had reverted to gentamicin sensitivity were stored and tested in triplicate in the gentamicin HEp-2 cell assay along with E2348-69 as a positive control as previously described (7). Each derivative less invasive than the parental strain was tested for auxotrophy on Bacto Minimal Agar Davis (Difco Laboratories, Detroit, Mich.) and for the presence of specific lipopolysaccharide with O127 antiserum (Difco) as recommended by the manufacturer.

Adherence and FAS assays. Each noninvasive Tn*phoA* mutant of E2348-69 was tested for the ability to form adherent microcolonies on the surface of HEp-2 cells essentially as described previously (6), except that assays were performed in chamber slides (Lab-Tek; Miles Scientific, Div. Miles Laboratories, Inc., Naperville, Ill.) in RPMI 1640 plus 1% D-mannose. For strains unable to form microcolonies in 3 h, the assay was extended to 6 h after replacing the medium at 3 h. FITC-phalloidin (Sigma Chemical Co., St. Louis, Mo.) was used essentially as described previously (16), except that the assay was performed in chamber slides and RPMI 1640 without additives served as the medium. Again, for strains that were negative at 3 h, the assay was extended to 6 h after replacement of the medium at 3 h. For both assays, strains HB101(pMAR7) and MAR20 served as controls.

Southern analysis. Plasmid DNA was extracted from each strain as described previously (3), separated by electrophoresis in 0.7% agarose, and transferred to nitrocellulose filters. A 2.2-kilobase (kb) *Bam*HI fragment that contains the kanamycin resistance gene found in Tn*phoA* was labeled with α-^{32}P by the random-primer method (9) and served as the DNA probe for hybridization by the method of Southern (35) under stringent conditions (50% formamide, 37°C, 750 mM NaCl; 65°C wash with 750 mM NaCl).

Total DNA was extracted from each strain by sodium dodecyl sulfate lysis, proteinase K treatment, phenol-chloroform extraction, and ethanol precipitation. Total DNA from each strain was digested overnight with restriction endonuclease *Mlu*I or *Eco*RV, neither of which recognizes sequences present within Tn*phoA*. After separation by electrophoresis in 0.5% agarose, Southern hybridization was performed with the kanamycin probe as above.

Cloning Tn*phoA* fragments. Plasmid pCVD433 was created from pACYC184 by inserting *Mlu*I linkers (New England BioLabs, Inc., Beverly, Mass.) into the *Eco*RV site. *Mlu*I digests of total DNA from each strain were ligated to *Mlu*I-digested pCVD433 and used to transform DH5α (Bethesda Research Laboratories, Inc., Gaithersburg, Md.),

TABLE 1. Characteristics of noninvasive TnphoA mutants of E2348-69

Strain	Invasive ability[a] (%)	Adherence[b]	FAS[c]	Location of insert (no. detected)[d]
E2348-69	100	LA	+	(0)
6-4-1(1)	0.44 ± 0.06	LA	Shadow	C (1)
6-8-1(1)	0.46 ± 0.03	DA6	+	P (1)
6-7-2(1)	0.72 ± 0.55	LA	Shadow	C (1)
8-1-1(3)	0.52 ± 0.17	LA	Shadow	C (1)
10-1-1(1)	2.05 ± 0.44	DA6	+	P (1)
10-5-1(1)	2.40 ± 0.26	LA	Shadow	C (1)
12-4-1(2)	1.40 ± 0.90	LA	Shadow	C (2)
12-4-1(3)	6.81 ± 4.72	LA	+	C (3)
14-2-1(1)	1.77 ± 0.91	LA	−	C (2)
14-5-1(2)	2.82 ± 0.28	LA	Shadow	C (2)
16-9-1(1)	0.51 ± 0.33	NA	+	C (1)
23-5-1(3)	5.34 ± 1.20	LA	+	C (1)
21-2-2(1)	2.26 ± 1.39	LA	+	C (1)
25-2-1(1)	0.43 ± 0.32	NA	+	C (1)
27-3-2(1)	0.34 ± 0.25	LA	−	C (2)
29-2-1(1)	0.14 ± 0.05	NA	−	P (1)
30-5-1(3)	2.78 ± 0.70	LA	+	C (1)
28-4-1(1)	0.71 ± 0.16	LA	Shadow	C (1)
31-6-1(1)	0.77 ± 0.50	DA6	+	P (1)
32-5-1(1)	0.97 ± 0.34	DA6	+	P (1)
32-5-1(2)	0.47 ± 0.04	DA6	+	P (1)
32-5-1(3)	0.38 ± 0.13	DA6	+	P (1)

[a] Mean ± standard deviation of triplicate CFU recovered in the HEp-2 gentamicin assay divided by the mean of E2348-69 parental control ($2.4 \times 10^4 \pm 1.3 \times 10^4$ CFU) × 100%. The original inoculum was 7×10^5 CFU.

[b] Phenotype observed in HEp-2 cell adherence assay. LA, Localized adherence at 3 h; DA6, diffuse adherence at 6 h; NA, nonadherent at 6 h relative to the control.

[c] Fluorescence actin staining observed in HEp-2 cells with FITC-phalloidin: +, bright fluorescence observed at sites of bacterial attachment; −, no fluorescence observed; Shadow, hazy shadow of fluorescence observed at sites of bacterial attachment (Fig. 1).

[d] Location of TnphoA insertions as determined by Southern hybridization of plasmid and total DNA. Number in parentheses is number of insertions detected. C, Chromosomal; P, plasmid.

selecting for kanamycin- and chloramphenicol-resistant transformants. In this manner, clones of *Mlu*I fragments containing every TnphoA insert were obtained.

Each clone was tested on agar containing X-P; a blue color indicates the production of a fusion protein with alkaline phosphatase activity by the clone. Limited restriction endonuclease maps for selected clones were constructed.

RESULTS

Construction of noninvasive mutants of E2348-69. A total of 339 blue transconjugants were selected from 351 separate mating experiments. After screening in the amikacin invasion assay, 29 preliminarily noninvasive mutants were identified. This number was reduced to 22 after passage to lose pPH1JI and testing in the gentamicin invasion assay. All 22 were prototrophic, as indicated by growth on minimal medium. No alterations in lipopolysaccharide were detected with O127 antiserum. The invasive ability of each mutant relative to the parental strain is shown in Table 1.

Adherence and FAS assays. After 3 h of incubation, 13 of the noninvasive derivatives showed typical microcolonies adherent to the surface of the HEp-2 cells. The nine strains that did not show typical microcolonies were tested after 6 h of incubation. Six strains showed adherence to the HEp-2 cells in a diffuse pattern at 6 h. Three strains remained nonadherent relative to the control.

Twelve of the noninvasive mutants showed typical bright fluorescence in the FAS assay by 6 h, including all but one of the poorly adherent strains (Fig. 1a and b). Of the 10 mutants with altered FAS, three showed no fluorescence (Fig. 1c and d), whereas seven showed an altered pattern with a hazy shadow of weak fluorescence in the area of attached bacteria (Fig. 1e and f).

Southern analysis. Hybridization of plasmid DNA with the kanamycin resistance gene probe revealed that seven of the noninvasive mutants had TnphoA insertions in the large EAF plasmid. Six of these seven were the same strains that showed a diffuse pattern of adherence after 6 h. An additional strain with a plasmid TnphoA insert was one of the three nonadherent strains.

Southern analysis of total DNA from each of the 22 noninvasive mutants digested with *Mlu*I and *Eco*RV revealed that 17 of the mutants contained single TnphoA insertions, 4 had two insertions, and 1 mutant had three TnphoA insertions.

Cloning and categorization of TnphoA insertions. All 28 *Mlu*I fragments containing TnphoA insertions from the 22 noninvasive derivatives were cloned in DH5α. Twenty-two clones were blue on X-P medium. Each of the five mutants with more than one TnphoA insert yielded only one blue clone.

By using data from the adherence and FAS phenotypes of each strain, the location of the TnphoA insertions (plasmid or chromosomal), and restriction endonuclease mapping of cloned DNA from these strains, the 22 noninvasive mutants of E2348-69 could be grouped into five main categories (Table 2).

The six strains in category 1 had abnormal adherence to HEp-2 cells, displaying little adherence at 3 h and a diffuse pattern at 6 h. These mutants had TnphoA insertions localized to a small region of approximately 450 base pairs in the 90-kb EAF plasmid (Fig. 2a). Three of these mutants [32-5-1(1), 32-5-1(2), and 32-5-1(3)] were isolated from the same mating and may therefore be related. An additional mutant [29-2-1(1)] possessed a TnphoA insertion in the same region but did not adhere to HEp-2 cells at all, even after 6 h of

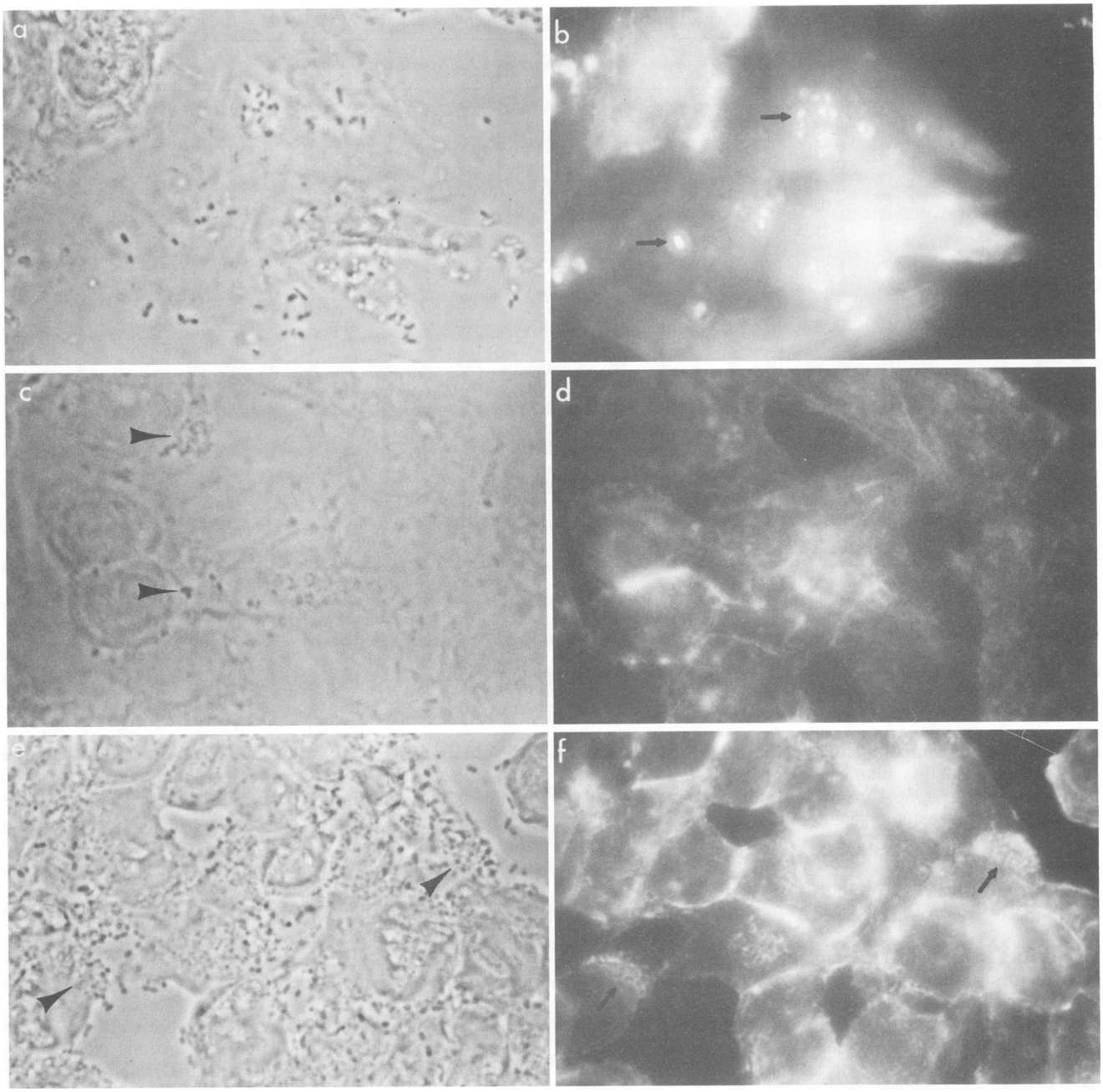

FIG. 1. Photomicrographs of representative mutants in an FITC-phalloidin assay for fluorescence actin staining. Magnification, ×760. (a and b) Phase-contrast (a) and fluorescent (b) views of mutant 32-5-1(1) from category 1, displaying bright fluorescence (arrows) underlying bacteria despite low amounts of bacteria present. (c and d) Phase-contrast (c) and fluorescent (d) views of mutant 27-3-2(1) from category 4, which displays no fluorescence in areas underlying bacteria (arrowheads). (e and f) Phase-contrast (e) and fluorescent (f) views of mutant 28-4-1(1) from category 2, showing weak hazy shadows of fluorescence (arrows) corresponding to areas of heavy bacterial adherence (arrowheads).

incubation. Unlike the six category 1 mutants, 29-2-1(1) did not cause fluorescence in HEp-2 cells when tested with FITC-phalloidin. This strain has been placed in category 1A. Cloned DNA from each of these mutants was tested for hybridization with the EAF probe (26); none hybridized.

The seven strains in category 2 each had altered fluorescence in the FAS assay, displaying a shadow pattern. All of these strains had chromosomal Tn*phoA* insertions located within a 13.7-kb *Mlu*I fragment (Fig. 2b). In addition, cloned plasmid DNA from each of these mutants hybridized with a 1.1-kb DNA probe (data not shown) that is highly sensitive and specific in identifying attaching and effacing *E. coli* (A. E. Jerse, J. Yu, B. D. Tall, and J. B. Kaper, submitted for publication). No other clones hybridized with this probe. Within category 2, there were two clusters of insertions. Five strains had Tn*phoA* insertions within 2.2 kb near one end of the fragment. Clones from these strains were blue on media containing X-P, indicating the production of a fusion protein with alkaline phosphatase activity. These five strains each had Tn*phoA* inserts within an *Eco*RV fragment of

TABLE 2. Characteristics of noninvasive strains grouped in six categories

Category (n^a)	Adherence[b]	FAS[c]	TnphoA location[d]	MluI fragment size (kb)[e]
1 (6)	DA6	+	P	14.2
1A (1)	NA	−	P	14.2
2 (7)	LA	Shadow	C	13.7[f]
3 (2)	NA	+	C	10.5
4 (2)	LA	−	C	Varied[f]
5 (4)	LA	+	C	Varied[f]

[a] Number of mutants in each category.
[b] See footnote *b* of Table 1.
[c] See footnote *c* of Table 1.
[d] C, Chromosomal; P, plasmid.
[e] Approximate size of *Mlu*I fragment containing Tn*phoA* insert. Data are derived from Southern hybridization and restriction analysis of cloned DNA. Actual fragments are 7.7 kb larger due the presence of Tn*phoA*.
[f] Some mutants in this category had more than one Tn*phoA* insertion.

approximately 10 kb identified by Southern hybridization. Two strains had insertions located closer to the other end of the *Mlu*I fragment. Clones from these two were white on media containing X-P. Southern hybridization of *Eco*RV digests of total DNA from these strains identified a common 9-kb fragment containing Tn*phoA*.

Category 3 includes the two strains with chromosomal Tn*phoA* insertions that virtually eliminated their ability to adhere to HEp-2 cells in the presence of 1% D-mannose but did not affect FAS for the rare bacteria seen associated with cells in this assay. Restriction maps of cloned DNA from these mutants indicate that the Tn*phoA* insertions are located approximately 300 base pairs apart (Fig. 2c).

The two strains in category 4 adhered normally but produced no observable fluorescence in the FAS assay. Each of these mutants had two chromosomal Tn*phoA* insertions. Although a clone containing 12.2 kb of insert DNA was obtained from each strain, which in each case was blue on X-P medium, extensive restriction endonuclease mapping revealed that these fragments had different digestion patterns. Therefore it must be concluded that these mutants do not contain Tn*phoA* insertions in the same fragment.

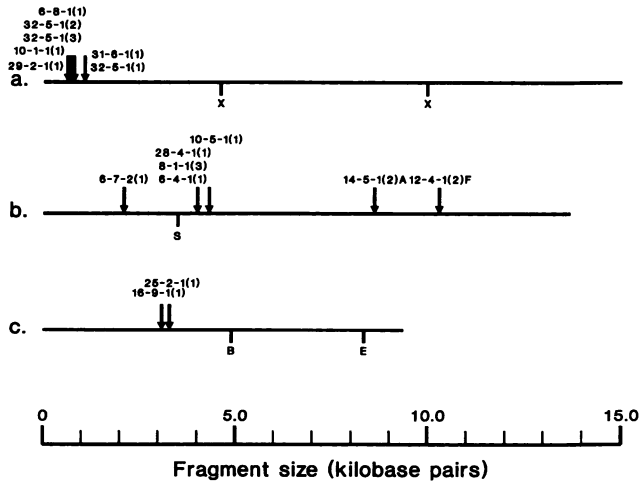

FIG. 2. Limited maps of cloned *Mlu*I fragments containing Tn*phoA* insertions from mutants in categories 1 (a), 2 (b), and 3 (c). The arrows depict the location of the Tn*phoA* insertions in each strain. Recognition sites for selected restriction endonucleases are also indicated: X, *Xma*III; S, *Sal*I; B, *Bam*HI; E, *Eco*RI. The direction of transcription of *phoA* is from left to right for all mutants except 14-5-1(2)A.

The final category includes the four strains with chromosomal insertions, normal adherence, and normal fluorescence in the FAS assay. The heterogeneity in this group is demonstrated by the variation in the size of the *Mlu*I and *Eco*RV fragments containing Tn*phoA* inserts.

DISCUSSION

We used the transposon Tn*phoA* to produce a collection of mutants in EPEC strain E2348-69 that are unable to invade HEp-2 cells. Although the importance of invasion in EPEC pathogenesis remains uncertain, we reasoned that the genes necessary for this process in vitro might include some of the important virulence genes necessary for the production of disease in vivo. For example, the current two-stage theory of EPEC pathogenesis proposes that plasmid-mediated factors allow EPEC to come in contact with the epithelial cell, whereas chromosomally mediated factors allow the bacteria to efface microvilli and become intimately associated with the epithelial cell membrane (17). We hypothesized that the same factors might be necessary for the invasion of epithelial cells in vitro. Since surface products are likely to be necessary for these events, we felt that Tn*phoA* mutagenesis could be used to identify these factors.

Using this approach, we isolated 22 Tn*phoA* mutants that had lost their ability to invade HEp-2 cells. The proportion of Tn*phoA* mutants that were noninvasive (22 of 339, 6.5%) is remarkably similar to that reported by Finlay et al. (42 of 626, 6.7%) in their study of Tn*phoA* mutants of *Salmonella choleraesuis* (10). As predicted, our mutants included those with insertions in plasmid genes that affect adherence as well as chromosomal genes that affect the induction in epithelial cells of actin polymerization (a feature closely correlated with attaching and effacing [16]).

The mutants we identified with altered adherence and Tn*phoA* insertions in the EAF plasmid (category 1) were not entirely nonadherent. When the incubation period was extended to 6 h, they did adhere to HEp-2 cells. They did not, however, form the microcolonies characteristic of localized adherence. For lack of a better term, we have described the adherence pattern as diffuse. We do not wish to imply, however, that the adherence of these mutants is identical to that of diffusely adherent *E. coli* (32).

Interestingly, we also identified mutants with chromosomal insertions that eliminate localized adherence (category 3). Since the ability to form adherent microcolonies has been shown to be plasmid associated, this may seem contradictory, yet there is precedence for this observation. First, although strain HB101 transformed with the EAF plasmid does form adherent microcolonies (2), it does so poorly in comparison with wild-type EPEC (unpublished observations). This suggests the presence in EPEC of additional chromosomal factors that augment adherence. Second, it has been demonstrated in enterotoxigenic *E. coli* that, although adherence is plasmid-associated, the structural genes for the adhesins CS1 and CS2 are not found on the plasmid. Instead, the plasmid encodes a regulatory element, *rns*, that allows expression of these genes (5). A similar situation could exist in EPEC. Perhaps HB101 also contains chromosomal genes that act in concert with the EAF plasmid to promote localized adherence.

The analysis of cloned DNA from mutants with altered ability to induce actin polymerization suggests that more than one locus is involved in this process. Altered shadow

patterns of fluorescence with FITC-phalloidin were localized to two regions of a 13.7-kb *Mlu*I fragment (category 2), whereas total elimination of phalloidin binding is localized to different *Mlu*I fragments (category 4). This suggests that the production of the attaching and effacing lesion may be dependent on the cooperation of several genes. The presence of two Tn*phoA* inserts in both mutants of category 4 is an unfortunate confounding factor. We are currently attempting to separate these mutations by inserting them individually into wild-type E2348-69 with a suicide vector.

One mutant remains difficult to explain. 29-2-1(1) contains a single plasmid Tn*phoA* insertion that is located in close proximity to insertions in mutants that adhere in a diffuse pattern only after 6 h (category 1). Yet this mutant adhered no better than the negative control after 6 h, and, unlike the other category 1 mutants, was negative in the FAS assay. It is possible that due to the extremely poor adherence of this strain, insufficient bacteria were present to detect a positive FAS response. Whether this mutant contains a Tn*phoA* insertion in a gene central to both adherence and actin polymerization, or whether the phenotype observed is due to inadvertent additional mutations that have occurred during strain construction, is not known at this time. These questions may be answered by moving the mutated plasmid from this strain into a plasmid-cured EPEC and observing the effect on adherence and FITC-phalloidin binding. These studies are currently in progress.

The nature of the Tn*phoA* insertions in the mutants in category five remains unclear. The variation in the observed sizes of *Mlu*I and *Eco*RV fragments containing Tn*phoA* insertions suggests that this group is heterogeneous. Thus several additional chromosomal genes may be involved in EPEC invasion. Alternatively, the possibility that these represent mutations in genes only indirectly involved in invasion must be considered. It should be noted, however, that trivial factors such as auxotrophy or increased susceptibility to Triton X-100 or aminoglycosides were not responsible for loss of invasive ability in these strains.

The analysis of EPEC mutants that are unable to invade epithelial cells illustrates both similarities and differences between this and other invasive pathogens. EPEC, like *Shigella* spp. (31), contain large plasmids that are important for invasion (7). Also like EPEC, *Shigella* spp. contain chromosomal genes involved in invasion. In the case of *Shigella* spp., a temperature-sensitive repressor of the plasmid invasion genes is located on the chromosome (22). The *inv* gene, a single locus found in *Yersinia pseudotuberculosis* and *Y. enterocolitica* and encoding a 103-kilodalton protein (14), can impart the invasive phenotype when transferred to noninvasive *E. coli* (13). However, even in *Yersinia* spp., the genetics of invasion are more complex. Although *inv* is sufficient for invasion, it appears not to be necessary (29). Other loci, including *ail* (24) and unidentified chromosomal and plasmid loci (12), appear to be involved. When we employed the strategy of Isberg and Falkow (13) to the study of EPEC invasion, we were unable to identify a single locus capable of conferring the invasive phenotype. Such was the experience of Finlay et al. in their investigation of *S. choleraesuis* invasion (10). In contrast to our experience, these authors found that adherence and invasion were closely linked. In *S. choleraesuis*, Tn*phoA* mutants unable to transcytose an epithelial barrier were also unable to adhere and invade. Finlay et al. also found that mutants unable to transcytose often possessed altered lipopolysaccharide that could be detected with specific antisera. We did not find the same to be true for noninvasive EPEC mutants.

The construction and preliminary analysis of noninvasive EPEC Tn*phoA* mutants provides a foundation for the study of the molecular basis of invasion and of the pathogenesis of EPEC infection. The identification of noninvasive derivatives that have reduced adherence suggests a role for initial adherence in EPEC invasion and provides a valuable tool for the ultimate identification of the genes responsible for the characteristic localized adherence of this organism. The location of the Tn*phoA* insertions in mutants with altered adherence suggests that genes from both the EAF plasmid and the chromosome may be involved. The identification of EPEC mutants unable to induce the polymerization of actin likewise suggests that this property is necessary for EPEC invasion and should enable the characterization of the genes and gene products involved in this phenomenon. Finally, the identification of noninvasive derivatives that have normal adherence and actin polymerization properties suggests that other factors are necessary for EPEC invasion and provides further evidence of the complexity of this organism. Future studies will be directed at the molecular analysis of the mutants described in the present report.

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LITERATURE CITED

1. **Andrade, J. R. C., V. F. DaVeiga, M. R. DeSanta Rosa, and I. Suassuna.** 1989. An endocytic process in HEp-2 cells induced by enteropathogenic *Escherichia coli*. J. Med. Microbiol. **28**:49–57.
2. **Baldini, M. M., J. B. Kaper, M. M. Levine, D. C. A. Candy, and H. W. Moon.** 1983. Plasmid-mediated adhesion in enteropathogenic *Escherichia coli*. J. Pediatr. Gastroenterol. Nutr. **2**:534–538.
3. **Birnboim, H. C., and J. Doly.** 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. **7**:1513–1523.
4. **Bower, J. R., B. L. Congeni, T. G. Cleary, R. T. Stone, A. Wanger, B. E. Murray, J. J. Mathewson, and L. K. Pickering.** 1989. *Escherichia coli* O114:nonmotile as a pathogen in an outbreak of severe diarrhea associated with a day care center. J. Infect. Dis. **160**:243–247.
5. **Caron, J., L. M. Coffield, and J. R. Scott.** 1989. A plasmid-encoded regulatory gene, *rns*, required for expression of the CS1 and CS2 adhesins of enterotoxigenic *Escherichia coli*. Proc. Natl. Acad. Sci. USA **86**:963–967.
6. **Cravioto, A., R. J. Gross, S. M. Scotland, and B. Rowe.** 1979. An adhesive factor found in strains of *Escherichia coli* belonging to the traditional infantile enteropathogenic serotypes. Curr. Microbiol. **3**:95–99.
7. **Donnenberg, M. S., A. Donohue-Rolfe, and G. T. Keusch.** 1989. Epithelial cell invasion: an overlooked property of enteropathogenic *Escherichia coli* (EPEC) associated with the EPEC adherence factor. J. Infect. Dis. **160**:452–459.
8. **Edelman, R., and M. M. Levine.** 1983. Summary of a workshop on enteropathogenic *Escherichia coli*. J. Infect. Dis. **147**:1108–1118.
9. **Feinberg, A. P., and B. Vogelstein.** 1983. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. **132**:6–13.
10. **Finlay, B. B., M. N. Starnbach, C. L. Francis, B. A. D. Stocker, S. Chatfield, G. Dougan, and S. Falkow.** 1988. Identification and characterization of Tn*phoA* mutants of *Salmonella* which are unable to pass through a polarized MDCK epithelial cell monolayer. Mol. Microbiol. **2**:757–766.
11. **Francis, D. H., J. E. Collins, and J. R. Duimstra.** 1986. Infection

of gnotobiotic pigs with an *Escherichia coli* O157:H7 strain associated with an outbreak of hemorrhagic colitis. Infect. Immun. **51**:953–956.

12. **Isberg, R. R.** 1989. Determinants of thermoinducible cell binding and plasmid-encoded cellular penetration detected in the absence of the *Yersinia pseudotuberculosis* invasin protein. Infect. Immun. **57**:1998–2005.
13. **Isberg, R. R., and S. Falkow.** 1985. A single genetic locus encoded by *Yersinia pseudotuberculosis* permits invasion of cultured animal cells by *Escherichia coli* K12. Nature (London) **317**:262–264.
14. **Isberg, R. R., D. L. Voorhis, and S. Falkow.** 1987. Identification of invasin: a protein that allows enteric bacteria to penetrate cultured mammalian cells. Cell **50**:769–778.
15. **Knutton, S., M. M. Baldini, J. B. Kaper, and A. S. McNeish.** 1987. Role of plasmid-encoded adherence factors in adhesion of enteropathogenic *Escherichia coli* to HEp-2 cells. Infect. Immun. **55**:78–85.
16. **Knutton, S., T. Baldwin, P. H. Williams, and A. S. McNeish.** 1989. Actin accumulation at sites of bacterial adhesion to tissue culture cells: basis of a new diagnostic test for enteropathogenic and enterohemorrhagic *Escherichia coli*. Infect. Immun. **57**: 1290–1298.
17. **Knutton, S., D. R. Lloyd, and A. S. McNeish.** 1987. Adhesion of enteropathogenic *Escherichia coli* to human intestinal enterocytes and cultured human intestinal mucosa. Infect. Immun. **55**:69–71.
18. **Levine, M. M.** 1987. *Escherichia coli* that cause diarrhea: enterotoxigenic, enteropathogenic, enteroinvasive, and enteroadherent. J. Infect. Dis. **155**:377–389.
19. **Levine, M. M., E. J. Bergquist, D. R. Nalin, D. H. Waterman, R. B. Hornick, C. R. Young, S. Sotman, and B. Rowe.** 1978. *Escherichia coli* strains that cause diarrhea but do not produce heat-labile or heat-stable enterotoxins and are noninvasive. Lancet **i**:119–122.
20. **Levine, M. M., J. P. Nataro, H. Karch, M. M. Baldini, J. B. Kaper, R. E. Black, M. L. Clements, and A. D. O'Brien.** 1985. The diarrheal response of humans to some classic serotypes of enteropathogenic *Escherichia coli* is dependent on a plasmid encoding an enteroadhesiveness factor. J. Infect. Dis. **152**: 550–559.
21. **Manoil, C., and J. Beckwith.** 1985. Tn*phoA*: a transposon probe for export signals. Proc. Natl. Acad. Sci. USA **82**:8129–8133.
22. **Maurelli, A. T., and P. Sansonetti.** 1988. Identification of a chromosomal gene controlling temperature-regulated expression of *Shigella* virulence. Proc. Natl. Acad. Sci. USA **85**: 2820–2824.
23. **Miliotis, M. D., H. J. Koornhof, and J. I. Phillips.** 1989. Invasive potential of noncytotoxic enteropathogenic *Escherichia coli* in an in vitro Henle 407 cell model. Infect. Immun. **57**:1928–1935.
24. **Miller, V. L., and S. Falkow.** 1988. Evidence for two genetic loci in *Yersinia enterocolitica* that can promote invasion of HEp-2 cells. Infect. Immun. **56**:1242–1248.
25. **Moon, H. W., S. C. Whipp, R. A. Argenzio, M. M. Levine, and R. A. Giannella.** 1983. Attaching and effacing activities of rabbit and human enteropathogenic *Escherichia coli* in pig and rabbit intestines. Infect. Immun. **41**:1340–1351.
26. **Nataro, J. P., M. M. Baldini, J. B. Kaper, R. E. Black, N. Bravo, and M. M. Levine.** 1985. Detection of an adherence factor of enteropathogenic *Escherichia coli* with a DNA probe. J. Infect. Dis. **152**:560–565.
27. **Polotsky, Y. E., E. M. Dragunskaya, V. G. Seliverstova, T. A. Avdeeva, M. G. Chakhutinskaya, I. Ketyi, A. Vertenyi, B. Ralovich, L. Emody, L. Malovics, N. V. Safonova, E. S. Snigirevskaya, and E. I. Karyagina.** 1977. Pathogenic effect of enterotoxigenic *Escherichia coli* and *Escherichia coli* causing infantile diarrhea. Acta Microbiol. Acad. Sci. Hung. **24**:221–236.
28. **Robins-Browne, R. M.** 1987. Traditional enteropathogenic *Escherichia coli* of infantile diarrhea. Rev. Infect. Dis. **9**:28–53.
29. **Rosqvist, R., M. Skurnik, and H. Wolf-Watz.** 1988. Increased virulence of *Yersinia pseudotuberculosis* by two independent mutations. Nature (London) **334**:522–525.
30. **Ruther, U., M. Koenen, K. Otto, and B. Muller-Hill.** 1981. pUR222, a vector for cloning and rapid chemical sequencing of DNA. Nucleic Acids Res. **9**:4087–4098.
31. **Sansonetti, P. J., T. L. Hale, G. J. Dammin, C. Kapfer, H. H. Collins, Jr., and S. B. Formal.** 1983. Alteration of the pathogenicity of *Escherichia coli* K12 after transfer of plasmid and chromosomal DNA from *Shigella flexneri*. Infect. Immun. **39**: 1392–1402.
32. **Scaletsky, I. C. A., M. L. M. Lilva, and L. R. Trabulsi.** 1984. Distinctive patterns of adherence of enteropathogenic *Escherichia coli* to HeLa cells. Infect. Immun. **45**:534–536.
33. **Simon, R., U. Priefer, and A. Puhler.** 1983. A broad host range mobilization system for *in vivo* genetic engineering: transposon mutagenesis in gram negative bacteria. Biotechnology **1**:784–791.
34. **Small, P. L. C., R. R. Isberg, and S. Falkow.** 1987. Comparison of the ability of enteroinvasive *Escherichia coli*, *Salmonella typhimurium*, *Yersinia pseudotuberculosis*, and *Yersinia enterocolitica* to enter and replicate within HEp-2 cells. Infect. Immun. **55**:1674–1679.
35. **Southern, E. M.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98**:503–517.
36. **Staley, T. E., E. W. Jones, and L. D. Corley.** 1969. Attachment and penetration of *Escherichia coli* into intestinal epithelium of the ileum in newborn pigs. Am. J. Pathol. **56**:371–392.
37. **Taylor, R. K., C. Manoil, and J. J. Mekalanos.** 1989. Broad-host-range vectors for delivery of Tn*phoA*: use in genetic analysis of secreted virulence determinants of *Vibrio cholerae*. J. Bacteriol. **171**:1870–1878.
38. **Tzipori, S., R. M. Robins-Browne, G. Gonis, J. Hayes, M. Withers, and E. McCartney.** 1985. Enteropathogenic *Escherichia coli* enteritis: evaluation of the gnotobiotic piglet as a model of human infection. Gut **26**:570–578.
39. **Ulshen, M. H., and J. L. Rollo.** 1980. Pathogenesis of *Escherichia coli* gastroenteritis in man—another mechanism. N. Engl. J. Med. **302**:99–101.