INFECTION AND IMMUNITY, May 1995, p. 2070–2074
0019-9567/95/$04.00+0

Vol. 63, No. 5

# NOTES

## Investigation of Enterohemorrhagic *Escherichia coli* O157:H7 Adherence Characteristics and Invasion Potential Reveals a New Attachment Pattern Shared by Intestinal *E. coli*

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Received 9 November 1994/Returned for modification 5 January 1995/Accepted 1 February 1995

**In this study, the interactions of enterohemorrhagic *Escherichia coli* (EHEC) O157 strains with human ileocecal (HCT-8) epithelial cells and HEp-2 cells were examined. EHEC adhered to, but did not invade, HCT-8 cells by the localized adherence mechanism and a heretofore unrecognized pattern which we called log jam. The log jam formation was (i) not observed on HEp-2 cells, (ii) independent of the EHEC *eaeA* gene required for localized adherence, and (iii) shared by pathogenic and nonpathogenic *E. coli* strains but not K-12 strains. The log jam phenotype may represent a basal means by which *E. coli* bacteria attach to the human intestine.**

Epithelial cells in culture serve as models to evaluate the mechanisms by which enteric pathogens interact with eukaryotic host cells in vivo. Epithelial cell lines that have been used to analyze the adherence of pathogenic *Escherichia coli* include HeLa (human cervix), Caco-2 (human colon), Henle 407 (human small intestine), HEp-2 (human larynx), and $T_{84}$ (human colon). Adherence patterns of *E. coli* to these cell types have been described as diffuse, localized, and aggregative, patterns that correlate with distinct subgroups of diarrhea-causing *E. coli* (16, 17, 20).

One well-described phenotype of adherence to HEp-2 cells is that of localized adherence (LA) by enteropathogenic *E. coli* (EPEC). This adherence pattern involves the accumulation or condensation of filamentous actin (F-actin) at the site of bacterial attachment. The condensation of F-actin is indicative of the tight bacterial association with the eukaryotic cell and correlates with the capacity of the bacteria to induce an attaching-and-effacing lesion in vivo (11). The fluorescence actin staining (FAS) phenotype of EPEC is associated with localized adherence and microcolony formation. The FAS adherence phenotype is easily discernible by a fluorescein-conjugated phalloidin stain of epithelial cells to which bacteria are bound (11).

At the time we undertook this study, no consensus had been reached in the literature about the nature of the adherence phenotype of enterohemorrhagic *E. coli* (EHEC). There were reports of FAS-positive adherence of EHEC on some, but not all, cell lines (11). Recent data indicate that one biotype of *Citrobacter freundii* and some strains of *Hafnia alvei* are, like EPEC strains, FAS positive (1, 3, 4, 11). All of these FAS-positive organisms carry the *eaeA* gene, a locus required for polymerization of F-actin noted when these species of bacteria adhere to epithelial cells (4, 5, 9). EHEC strains have been shown to be probe positive for the *eaeA* locus and to cause the attaching-and-effacing lesion in piglet intestines (7, 21). More recently, there has been a report that EHEC bacteria invade tissue culture cells (18). The purpose of this investigation was to compare and characterize the interaction (adherence pattern and potential invasive ability) of EHEC strains with human ileocecal HCT-8 cells and with HEp-2 human laryngeal cells in a quest for a consistent and relevant in vitro model of EHEC adherence and attaching-and-effacing lesion formation. In the course of this investigation, we serendipitously observed a previously unrecognized attachment phenotype on the HCT-8 cells, but not on HEp-2 cells. We named this pattern log jam adherence for reasons described below.

The adherence of *E. coli* to either HEp-2 or HCT-8 cells was assessed by the method of Cravioto et al. (2), with modifications. In a preliminary study, no differences in the levels of adherence were observed when static overnight, shaking overnight, or shaking logarithmic bacterial cultures were used in the adherence assay (data not shown). Therefore, for routine adherence assays, all bacterial cultures were grown statically in Luria broth to saturation. Bacteria suspended in adherence medium (Eagle minimum essential medium [BioWhittaker, Rockville, Md.] with 1% mannose) were inoculated at a multiplicity of infection of 100:1 onto semiconfluent monolayers of epithelial cells grown on eight-well plastic chamber slides (LabTek, Naperville, Ill.). Bacteria and cells were incubated for 2.5 h, washed once with sterile 10 mM phosphate-buffered saline (PBS) without calcium or magnesium (pH 7.4), and overlaid with fresh adherence medium for an additional 2.5 h. These incubation times were determined to be optimal for visualization of EHEC adherence and the FAS phenotype. The monolayers were fixed and stained with fluorescein isothiocyanate-phalloidin (Sigma, St. Louis, Mo.) or modified Giemsa stain (Sigma) for microscopic evaluation.

Several EHEC strains tested adhered to HEp-2 cells significantly more than the DH5α control after 5 h (data not shown). Among these EHEC strains, O157:H7 strain 86-24 (8) adhered at high levels most consistently and was, therefore, selected as the EHEC standard for all subsequent studies. Wild-type

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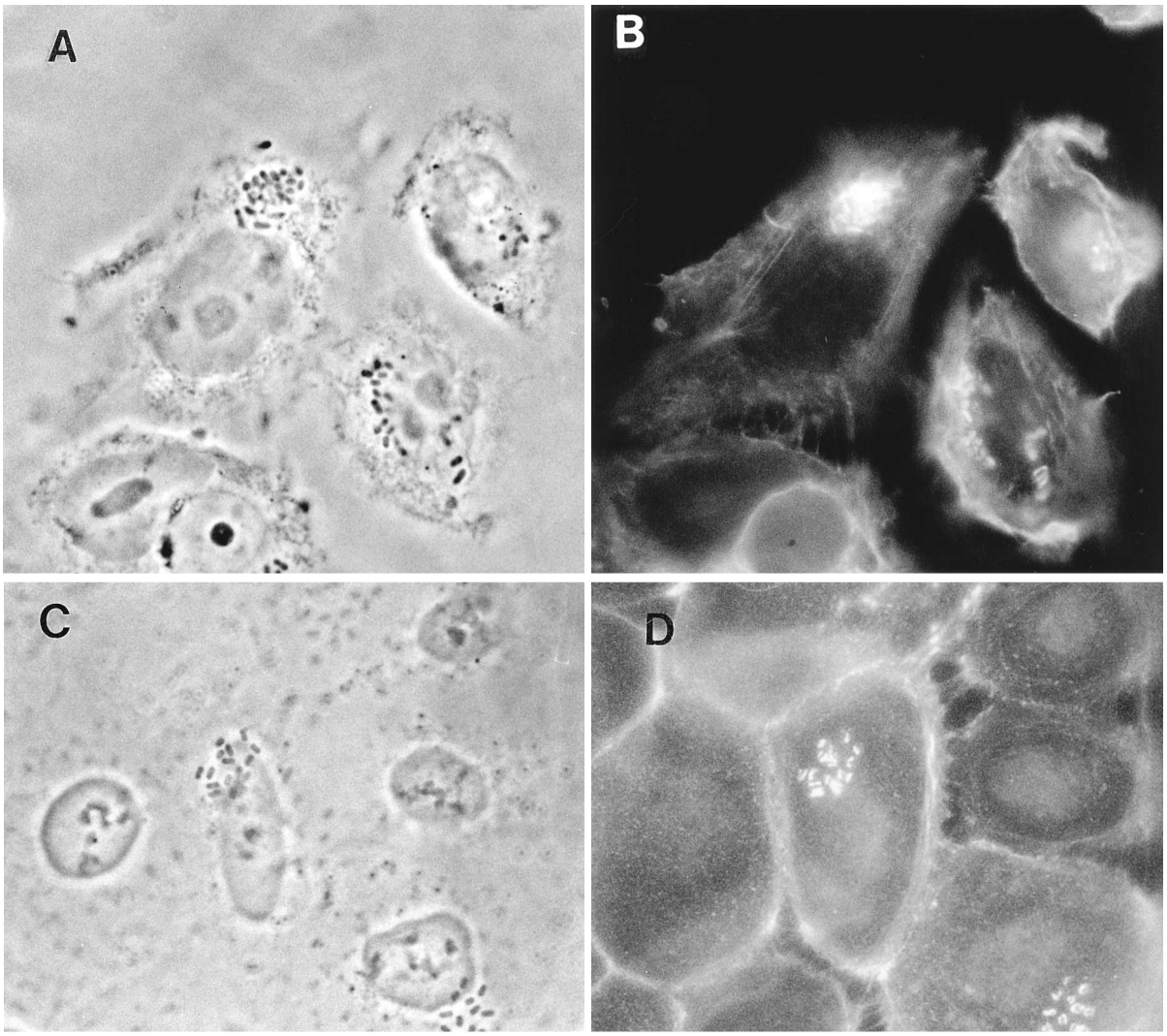


FIG. 1. Phase-contrast and fluorescence micrographs of HEp-2 (A and B) and HCT-8 (C and D) cells incubated for 5 h with EHEC strain 86-24 (O157:H7). Microcolonies of adherent 86-24 were seen on top of the cells in the phase-contrast micrographs (A and C). Bright fluorescence with the fluorescein isothiocyanate-phalloidin stain, indicating condensation of F-actin, was visualized under the microcolonies by fluorescence microscopy (B and D). The K-12 strain, DH5α, was unable to adhere to either HEp-2 or HCT-8 cells (data not shown).

EHEC strain 86-24 (O157:H7) associated with HEp-2 cells in a localized manner and induced actin polymerization at the site of adherence (Fig. 1A and B). Microcolonies of bacteria were observed on a majority of cells and corresponded to FAS-positive areas below the microcolonies. Heat-killed bacteria were unable to adhere to HEp-2 cells (data not shown). The size of the EHEC microcolony at 5 h was comparable to the magnitude of the EPEC microcolony (the positive control for the LA/FAS phenotype) after 3 h of incubation with the cells (data not shown).

Because EHEC bacteria colonize the colon, a region contiguous with the cecum, we hypothesized that EHEC strains might adhere better or differently to HCT-8 cells, which are derived from the human ileocecum, than the less physiologically relevant, but more commonly employed, HEp-2 cell line. We observed that EHEC 86-24 adhered to and formed microcolonies on HCT-8 cells (Fig. 1C). Actin rearrangement occurred at the site of microcolony formation in a manner similar to that seen with HEp-2 cells (Fig. 1D). Other EHEC strains that were poorly adherent to HEp-2 cells gave a clearer LA/FAS phenotype on HCT-8 cells (data not shown). An additional pattern of adherence of EHEC to HCT-8 cells, but not HEp-2 cells, was noted (Fig. 2); bacteria appeared to be adherent to and lined up at the junctions between the HCT-8 cells, but no organisms were seen sticking to the plastic slide (Fig. 2A). We called this phenotype log jam adherence because the bacteria resembled a mass of logs crowded together floating down a river. This attachment phenotype was FAS negative (Fig. 2B) and limited to the HCT-8 cells. We sought to determine whether the log jam pattern of adherence to HCT-8 cells was specific for EHEC or whether other *E. coli* strains exhibited this phenotype. Therefore, we examined the interaction of both pathogenic and nonpathogenic *E. coli* isolates with HCT-8 cells. The log jam adherence pattern was observed among intestinally derived pathogenic and nonpathogenic *E. coli* strains (Table 1 and Fig. 2C and D). Even normal flora *E. coli* isolates formed log jams on the HCT-8 cells, but neither of the two

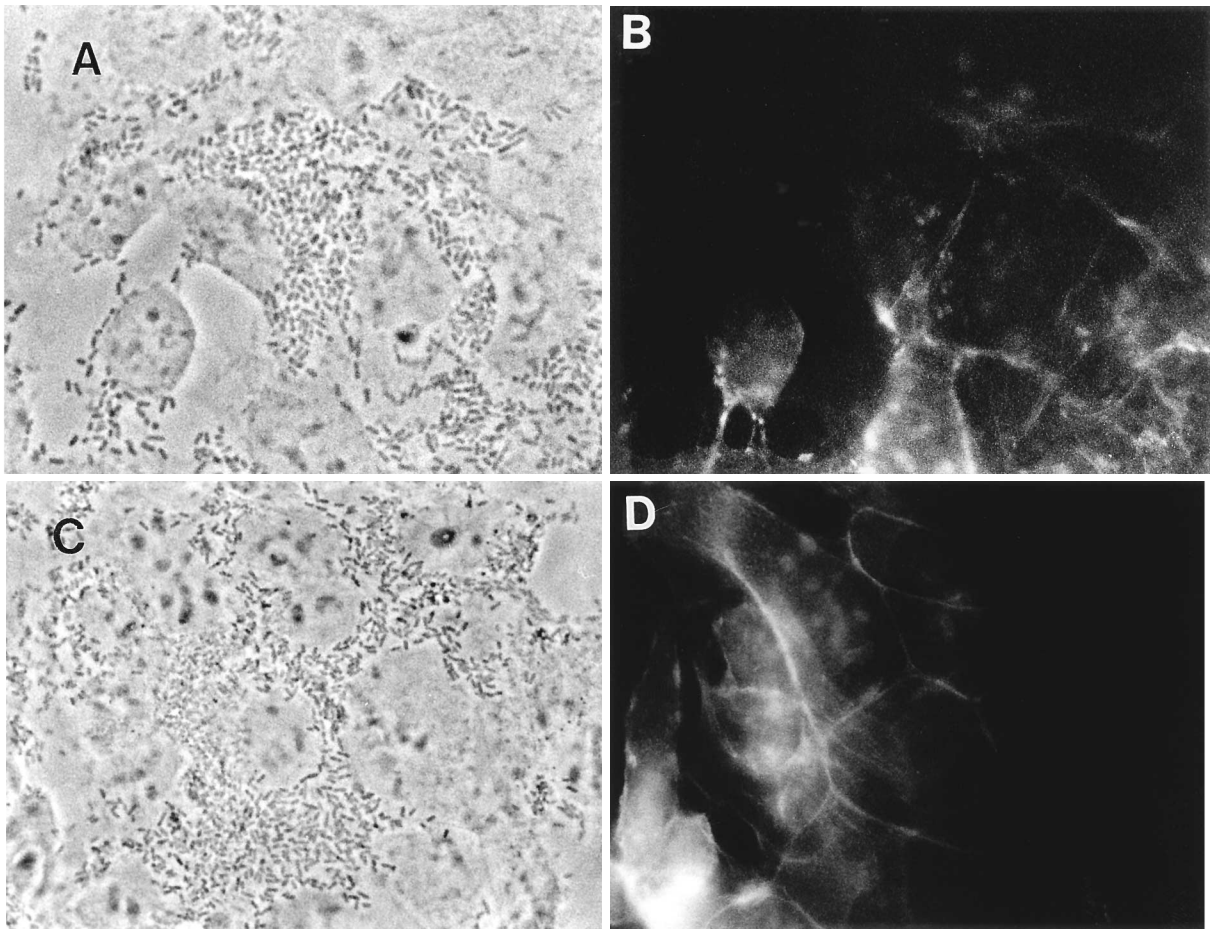


FIG. 2. Photomicrographs of fluorescein isothiocyanate-phalloidin-stained HCT-8 cells infected with EHEC 86-24 O157:H7 (A and B) or ETEC E2531 O25:H98:NM (C and D) showing the log jam phenotype. The bacteria adhered in the crevices at the junctions of the cells as seen by phase-contrast microscopy (A and C). This mechanism of adherence was FAS negative (B and D). FAS-positive microcolonies were also present in the EHEC-infected monolayer but were in a different plane of focus.

laboratory K-12 strains, DH5α and HB101, did so. All of the EHEC O157:H7 strains and the one nonmotile O157 strain tested were both LA/FAS and log jam positive. The wild-type EPEC strains formed large FAS-positive microcolonies only. A derivative of EPEC E2348/69 mutated at the bundle-forming pilus (*bfp*) locus [mutant designation 10-1-1(1), reference 3] was able to form log jams. From this observation, we concluded that the tight interbacterial association among the EPEC organisms masked the log jam pattern. Enteroaggregative *E. coli* adhered to the top of the HCT-8 cells in such large numbers that the junctions of the HCT-8 cells, where log jams typically were observed, could not be seen. Thus, the aggregative or stacked-brick pattern of enteroaggregative *E. coli* (19) may have obscured any low-level or background adherence such as the log jam. Enterotoxigenic *E. coli* strains either adhered diffusely or formed microcolonies on the HCT-8 cells in addition to the log jam pattern (Fig. 2C and D). The enteroinvasive *E. coli* strain tested was diffusely adherent and log jam positive. Only the *E. coli* strains carrying the *eaeA* locus (i.e., EHEC and EPEC) were FAS positive. An in-frame deletion mutant in *eaeA* of 86-24 (13) was LA/FAS negative but remained log jam positive. A derivative of O157:H7 strain 933 cured of the 60-Mda plasmid, 933cu (10), adhered to the HCT-8 cells by both the LA/FAS and log jam mechanisms; therefore, the capacity of EHEC to form log jams is not mediated by the 60-MDa plasmid present in EHEC strains (12).

The log jam pattern does not appear to be lipopolysaccharide (LPS) type specific, because we observed the phenotype across a range of LPS serogroups. Further, an LPS rough *E. coli* strain and its isogenic smooth derivative (serotype O8) (14, 15) were able to adhere to HCT-8 cells in the log jam manner. Since all adherence assays were carried out in the presence of 1% mannose, we also concluded that the log jam phenotype was mannose resistant.

To our knowledge, there have been no reports to date of EHEC found in tissue samples from biopsies of hemorrhagic colitis patients. Recently, there has been one report that EHEC strains can invade HCT-8 cells in a microfilament-dependent manner (18). To test the possibility that the log jam phenotype represented intracellular bacteria, we examined O157:H7 strains for the capacity to enter the HCT-8 cells. Invasion of HCT-8 cells was assessed according to the method of Elsinghorst and Kopecko (6). Stationary-phase bacteria in adherence medium were allowed to interact with the HCT-8 monolayer for 3 h at a multiplicity of infection of 100:1; then the bacterium–HCT-8 mixture was washed with sterile 10 mM PBS, pH 7.4. Half of the cell samples were then solubilized in 0.1% Triton X-100 (Sigma) and processed to determine the total number of bacteria associated with the cells before gen-

TABLE 1. Adherence of pathogenic and nonpathogenic *E. coli* to HCT-8 cells

| *E. coli* type (no. of strains tested)[a] | Adherence phenotype[b] | *eae* genotype[c] |
|---|---|---|
| EHEC (5) | MC/FAS$^+$; log jam | + |
| EHEC cured[d] | MC/FAS$^+$; log jam | + |
| EHEC*eae*Δ*10*[e] | Log jam | In-frame deletion |
| EPEC (3) | Large MC/FAS$^+$ | + |
| EPEC*bfp*[f] | Log jam; FAS$^{+/-}$ | + |
| ETEC (3) | MC/FAS$^-$ or DA/FAS$^-$; log jam | − |
| EIEC (1) | DA/FAS$^-$; log jam | − |
| EAggEC (2) | Aggregative/stacked brick; FAS$^-$ | − |
| Normal flora (1) | Log jam | − |
| O8$^{+g}$ | DA/FAS$^-$; log jam | − |
| O8$^{-g}$ | DA/FAS$^-$; log jam | − |
| K-12 | Nonadherent | − |

[a] EHEC serotypes O157:H7 and O157:H$^-$, EPEC serotypes O127:H6, O111:NM, and O114:H2, enterotoxigenic *E. coli* (ETEC) serotypes O78:H11, O7:NM, and O25:NM, enteroinvasive *E. coli* (EIEC) serotype O143:ND, enteroaggregative *E. coli* (EAggEC) serotypes O44:H18 and O3:H2, and K-12 strains DH5α and HB101 were tested.

[b] MC, microcolony; FAS, fluorescence actin staining by fluorescein isothiocyanate-phalloidin; DA, diffuse adherence; log jam, bacteria adherent at the junctions between HCT-8 cells.

[c] The *eae* genotype was determined by DNA dot blot hybridization with an internal *eae* probe derived from a subclone of 86-24 *eaeA*.

[d] Strain 933cu (10).

[e] This EHEC *eae* mutant is an in-frame deletion mutant in the *eaeA* locus of strain 86-24 (13).

[f] The EPEC*bfp* (bundle-forming pilus) mutant is a Tn*phoA* insertional mutant of strain E2348/69 that no longer produces bundle-forming pili (3).

[g] The O8$^+$ and O8$^-$ strains represent an isogenic set of *rfb* mutants, strains 2443 and AB1133, respectively (14, 15).

tamicin treatment. The other half of the samples was overlaid for an additional hour with fresh medium containing 100 μg of gentamicin per ml. The infected monolayer was washed extensively with sterile PBS and solubilized with 0.1% Triton X-100. This solution was serially diluted and plated on MacConkey agar (Difco Laboratories, Detroit, Mich.), and colonies were counted to determine the number of CFU per milliliter associated with the HCT-8 cells after gentamicin treatment. Adherence and invasion of each strain were assessed in triplicate in each of three assays. We assessed two EHEC strains for the capacity to invade the HCT-8 cells as determined by survival after gentamicin treatment (Table 2). Both 86-24 and 933 adhered better than the normal flora *E. coli* isolate, HS (17.9 and 36.8%, respectively, versus 13.0% for HS). The K-12 strain, DH5α, exhibited minimal adherence (Table 2) which was not visible by light microscopy. When compared with the normal flora values, invasion percentages for the two EHEC strains tested were indistinguishable (86-24, 0.16%; 933, 0.17%; and HS, 0.22%). Further, the percentage of bacteria that survived gentamicin treatment (Table 2) was substantially below that of EPEC or *Shigella flexneri* 2457T, 2.6 and 1.21%, respectively. The difference between the invasive capacity of *S. flexneri* and that of the EHEC strains was even more marked when the percent invasive bacteria was calculated on the basis of total bacteria associated with the monolayer at the time of gentamicin treatment (Table 2). Both EHEC strains gave invasion values within the range of the normal flora isolate regardless of the method used to calculate invasion. Thus, we concluded from our data that EHEC strains do not invade HCT-8 cells to any significant extent. Rather, we believe that HCT-8 cells may nonspecifically take up a small portion of the EHEC or the normal flora control strains to which the cells are exposed. It should be noted that the invasion percentages we calculated for EHEC are similar to those reported by Oelschlaeger et al. (18). However, no normal flora control was included in that study. Additionally, electron microscopic examination of the intestines of gnotobiotic pigs infected with EHEC 86-24 revealed that the organism attached to but did not invade the mucosal surface (13).

Light microscopy was used to examine infected HCT-8 cell monolayers both before and after gentamicin treatment (data not shown). For all strains tested except DH5α, bacteria were associated with the cells after the samples were treated with gentamicin. For EHEC and HS, the remaining bacteria were not exclusively those bacteria in the log jams. By light microscopy, we were unable to determine whether the bacteria which survived gentamicin treatment were intracellular or extracellular. The EPEC strain, E2348/69, was still contained within formed microcolonies, but the clusters were smaller than those microcolonies observed without gentamicin treatment. The EPEC survivors may have been protected from gentamicin by the close interbacterial association or the intimate association of the bacterium with the HCT-8 cell membrane.

In summary, we confirmed the findings of Knutton et al. (11) that EHEC cells adhere to HEp-2 cells in a localized, FAS-positive manner and have extended these observations to HCT-8 cells. We found that an increased incubation time and the use of the ileocecally derived epithelial cells resulted in a greater number of LA/FAS-positive EHEC strains. We also described a novel pattern of EHEC attachment, termed log jam adherence, that was clearly visible only on HCT-8 cells. Because the log jam phenotype was shared by a variety of intestinally derived *E. coli* strains of both pathogenic and non-

TABLE 2. Invasion of HCT-8 cells

| Bacteria | % Adherence (range)[a] | % Invasion (range) | |
|---|---|---|---|
| | | Method I[b] | Method II[c] |
| EHEC O157:H7 86-24 | 17.9 (10.1–32.2) | 0.16 (0.15–0.19) | 1.1 (0.5–1.85) |
| EHEC O157:H7 933 | 36.8 (14.3–47.8) | 0.17 (0.12–0.20) | 0.49 (0.25–1.38) |
| Normal flora *E. coli*, strain HS | 13.0 (11.0–14.4) | 0.22 (0.22–0.22) | 1.7 (1.5–1.98) |
| *E. coli* K-12 DH5α | 2.2 (1.4–3.1) | 0.02 (0.002–0.025) | 0.7 (0.08–1.7) |
| EPEC E2348/69 | 16.8 (14.6–19.1) | 2.6 (2.3–2.9) | 13.5 (10.9–16.5) |
| *S. flexneri* 2457T | 4.4 (2.3–7.8) | 1.21 (0.9–1.5) | 21.4 (10.7–34.8) |

[a] Percent adherence = {[CFU associated with the HCT-8 cells at 3 h minus CFU surviving gentamicin treatment (100 μg/ml) for 1 h]/CFU inoculated} × 100%.

[b] Percent invasion was calculated on the basis of the starting inoculum: (CFU surviving gentamicin treatment/CFU inoculated) × 100%.

[c] Percent invasion was calculated on the basis of the total bacteria associated with HCT-8 cells after 3 h: (CFU surviving gentamicin treatment/CFU associated with the HCT-8 cells after 3 h) × 100%.

pathogenic types, this phenotype does not appear to be associated with virulence. That the log jam pattern was observed only on the intestinally derived epithelial cells suggests that this phenotype may represent a basal adherence mechanism that allows a variety of *E. coli* bacteria to bind to and colonize the human intestine whether or not the organism expresses additional specific adhesive factors.

We thank M. Donnenberg for graciously providing the EPEC*bfp* mutant, S. Savarino for the enteroaggregative strains, T. Maurelli for *S. flexneri* 2457T, and the P. Rick lab for the O8 isogenic strains. We also thank C. K. Schmitt for critical reading of the manuscript and A. R. Melton-Celsa for her thoughtful discussions.

This study was supported by National Institutes of Health grant AI20148-12.

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