

Localized Adherence by Enteropathogenic *Escherichia coli* Is an Inducible Phenotype Associated with the Expression of New Outer Membrane Proteins

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Summary

Enteropathogenic *Escherichia coli* grow as discrete colonies on the mucous membranes of the small intestine. A similar pattern can be demonstrated in vitro; termed localized adherence (LA), it is characterized by the presence of circumscribed clusters of bacteria attached to the surfaces of cultured epithelial cells. The LA phenotype was studied using B171, an O111:NM enteropathogenic *E. coli* (EPEC) strain, and HEP-2 cell monolayers. LA could be detected 30–60 min after exposure of HEP-2 cells to B171. However, bacteria transferred from infected HEP-2 cells to fresh monolayers exhibited LA within 15 min, indicating that LA is an inducible phenotype. Induction of the LA phenotype was found to be associated with de novo protein synthesis and changes in the outer membrane proteins, including the production of a new 18.5-kD polypeptide. A partial NH₂-terminal amino acid sequence of this polypeptide was obtained and showed it to be identical through residue 12 to the recently described bundle-forming pilus subunit of EPEC. Expression of the 18.5-kD polypeptide required the 57-megadalton enteropathogenic *E. coli* adherence plasmid previously shown to be required for the LA phenotype in vitro and full virulence in vivo. This observation, the correspondence of the 18.5-kD polypeptide to an EPEC-specific pilus protein, and the temporal correlation of its expression with the development of the LA phenotype suggest that it may contribute to the EPEC colonial mode of growth.

Enteropathogenic *Escherichia coli* (EPEC)¹ are a common cause of diarrhea, particularly for children living in developing countries (1–4). Small bowel biopsies of these children reveal discrete colonies of bacteria attached to mucous membranes (5–8). Infection of cultured epithelial cells by EPEC yields a similar pattern of adherence in vitro, which now is termed “localized adherence” (LA) and is characterized by the presence of circumscribed clusters of adherent bacteria (9–11). Beneath these bacterial clusters, cup-like projections of the epithelial cell plasma membrane are seen, and actin condensation is evident (7, 12, 13). Observations of this kind have led to the idea that EPEC pathogenesis proceeds in two steps, an early stage characterized by long-range, adhesin-mediated attachment of the bacteria to the host-cell surface, and a later stage in which the outer membrane of the organism and the plasma membrane of the cell are closely juxtaposed and actin condensation has occurred (12). However, at present, little is known about how EPEC adhere to epithelial cells or why they attach as clusters rather than as individual bacteria.

¹ Abbreviations used in this paper: BFP, bundle-forming pili; EAF, enteroadherent factor; EPEC, enteropathogenic *Escherichia coli*; L, Luria; LA, localized adherence; LB, Luria broth; Tcp, toxin-coregulated pilin.

Most LA-positive EPEC strains belong to a restricted number of *E. coli* serotypes (2, 14). These strains also harbor a ~ 50-megadalton (MD) plasmid that codes for a putative enteroadherent factor (EAF) (11, 15–18). Strains cured of the EAF plasmid no longer adhere to epithelial cells, and when tested in human volunteers, are found to be less virulent (18, 19). Thus, the EAF plasmid appears to be required for the LA phenotype in vitro and for infectivity in vivo. The experiments reported here were designed to learn more about the LA phenomenon, and in particular whether this unusual pattern of adherence is a constitutive or inducible property of EPEC. During the course of these studies, we discovered that new outer membrane proteins are expressed by EPEC during the process of forming a colony. Based on this observation and other data that we present below, we now believe that one of these proteins may participate directly in the capacity of EPEC to grow as colonies on mucosal surfaces.

Materials and Methods

Bacteria, Media, and Chemicals. Bacterial strains used in this study are shown in Table 1. They were stored in small volumes of skim milk (Difco Laboratories, Detroit, MI) at –70°C.

DME with 4,500 mg/ml glucose and L-glutamine and without

sodium pyruvate was purchased from JRH Biosciences (Lenexa, KS). MEM without cysteine and methionine, and FCS were obtained from Gibco Life Tech. Inc. (Grand Island, NY).

Premixed acrylamide/BIS, Ultrapure agarose and Protein Assay Reagent were purchased from Bio-Rad Laboratories (Richmond, CA). Penicillin streptomycin mixture was purchased from Whitaker Bioproducts, Inc. (Walkersville, MD). Analytical grade methanol and acetic acid were obtained from J.T. Baker (Phillipsburg, NJ). All other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

DNA Isolation. Plasmid DNA was extracted and precipitated from lysed cells essentially as described by Birnboim and Doly (24). The plasmid DNA was subjected to electrophoresis in 0.7% agarose gels and stained with ethidium bromide.

Hep-2 Adherence Assay. For standard adherence assays, a single bacterial colony was harvested from a Luria (L) plate (25) grown at 37°C, inoculated into Luria broth (LB) (25), and grown overnight to late-logarithmic growth phase at 37°C with shaking. For radiolabeling experiments, bacteria were similarly inoculated into M9 medium (23) and propagated to mid-logarithmic growth phase ($OD_{600nm} = 0.5$) at 37°C with shaking. The number of CFU of bacteria per milliliter of culture was determined by plating serial dilutions of bacteria on L plates.

The adherence assay was a modification of the method of Scaletsky et al. (10). HEP-2 cells, a human laryngeal epithelial carcinoma cell line, were obtained from American Type Culture Collection (Rockville, MD) and maintained in DME supplemented with 10% FCS (vol/vol), 100 U/ml penicillin, and 100 µg/ml streptomycin. They were grown at 37°C in a humidified atmosphere containing 5% CO₂. For the standard adhesion assay, HEP-2 cells were split 1/5 at 48 h before the assay, and seeded onto 4-well Permax Labtek tissue culture slides (Nunc Inc., Naperville, IL) 24 h before the assay in order to form a semi-confluent monolayer. Each well (~10⁶ cells) was infected with 10⁷ to 10⁹ CFU of bacteria in fresh DME with 10% FCS (vol/vol) (10% FCS/DME) and 0.5% D-mannose (wt/vol) to prevent type 1 fimbrial-mediated adherence. The course of the infection was monitored after washing the wells four times with PBS, pH 7.4, to remove unbound bacteria at 15, 60, and 120 min from the beginning of the assay. The wells were then fixed with 100% methanol for 20 min and stained with a 1:20 dilution of Giemsa stain for 20 min. The tissue culture slides were then air dried, mounted for microscopy, and examined using a 100× oil immersion objective. The adherence rate was expressed as the percentage of HEP-2 cells infected per at least 200 cells counted. Only cells containing clusters comprised of more than four bacteria were counted. Each experiment was repeated at least three times and was performed in triplicate.

For some experiments, the bacteria were preincubated in various media before their use in the HEP-2 adherence assay. The preincubation of bacteria was performed by diluting the late-logarithmic, overnight, shaken LB bacterial culture (see above) 1:20 into 6.5-mm tissue culture wells (Costar, Cambridge, MA) containing a HEP-2 cell monolayer and 10% FCS/DME (0.25 ml of overnight culture to 4.75 ml of 10% FCS/DME). The preincubation procedure was performed at 37°C in a 5% CO₂ incubator without shaking for 60 min after which free, unbound, floating bacteria were collected from the medium above the HEP-2 cells. The preincubated bacteria (10⁸ CFU/ml) were then transferred to new four-well tissue culture slides containing HEP-2 cell monolayers and 10% FCS/DME with 10% mannose. Then, the expression of the LA phenotype was assayed as described above. To characterize the preincubation conditions required for induction of LA, bacterial prein-

cubation was also performed in fresh 10% FCS/DME, in DME alone and in fresh LB as a control.

The standard adherence assay was modified by the preincubation of bacteria in DME in order to detect the autoaggregation phenotype (see below). To determine the effect of de novo protein synthesis or bacterial replication on the LA phenotype, strain B171 was pretreated with rifampicin (32 µg/ml, 15 min, 20°C) or nalidixic acid (10 µg/ml, 15 min, 20°C) before the standard adherence assay. The same concentration of antibiotics was also present in the assay media.

Autoaggregation Phenotype. Bacteria were preincubated as described above in DME or LB for 120 min. Direct microscopy to detect the autoaggregation phenotype in various media was performed using an inverted or phase-contrast microscope.

Outer Membrane Proteins Expressed by EPEC. Outer membrane proteins from LB-grown or preincubated bacteria (see above) were prepared by first breaking the bacteria by sonication (twice for 20 s, on ice, microtip control setting 7; Branson Sonifier 450; Branson Inc., Danbury, CT) or with a French press (SLM Instruments Inc., Urbana, IL). The intact bacteria were removed by low speed centrifugation (1,000 g, 10 min). The membranes were harvested by high-speed centrifugation (237,363 g, 1.5 h). The membrane pellet was then resuspended in 10 mM Hepes buffer, pH 7.4, and subjected to a discontinuous sucrose gradient (2.02, 1.44, and 0.77 M sucrose in 10 mM Hepes, pH 7.4) to separate the cytoplasmic and outer membranes. The outer membranes were collected and then concentrated by high-speed centrifugation (237,363 g, 5 h), and the membrane pellets were resuspended in 10 mM Hepes buffer, pH 7.4. The protein concentration of these membrane preparations was determined by using the Bio-Rad Protein Assay Reagent (Bio-Rad Laboratories, Richmond, CA) with BSA as standard.

Radiolabeling of the Bacterial Membranes. M9-grown bacteria were diluted 1/20 in MEM without methionine or cysteine. ³⁵S-labeled methionine and cysteine (25 µCi/10⁸ CFU, sp act 1,136 mCi/mmol; Translat™; Amersham, Arlington Heights, IL) was added to the medium at 30 or 90 min after preincubation of the bacteria in the medium at 37°C. Bacteria were harvested from the medium after 30 min by centrifugation (1,000 g, 15 min), washed once with distilled H₂O, and the outer membranes were processed as described above. In experiments with HEP-2 cells, the protein synthesis of the host cells was blocked by cycloheximide pretreatment (15 µg/ml [wt/vol], for 3.5 h). At the termination of the preincubation phase of the experiment, the HEP-2 cell-bound bacteria were collected by first detaching the HEP-2 cells from the tissue culture flasks by trypsin-EDTA treatment, followed by sonication of the sample as described above. The bacterial membranes were then isolated by the same discontinuous sucrose gradient. A HEP-2 cell lysate was shown to migrate together with the cytoplasmic membrane at the interface of the 0.77 and 1.4 M sucrose layers. However, the outer membrane appeared as an opalescent band at the interface of the 1.4 and 2.2 M layers. The outer membrane band was collected and processed as above.

SDS-PAGE. Analysis of the membrane proteins was carried out by 15% SDS-PAGE essentially as described by Laemmli (26). The outer membrane proteins were first solubilized by boiling in sample buffer for 5 min. Electrophoresis was carried out at a constant current of 8 mA for 17 h and the gel stained with coomassie brilliant blue to visualize the proteins. In case of ³⁵S-radiolabeled proteins, the SDS-PAGE gel was first fixed in Amplify™ (Amersham Corp., Arlington Heights, IL), dried, and then subjected to autoradiography. Low molecular weight standards were purchased from Pharmacia LKB (Piscataway, NJ), and the ¹⁴C-labeled mo-

lecular weight standards from BRL Life Technologies, Inc. (Gaithersburg, MD).

Protein Sequencing. A 15% SDS-PAGE gel (gel size 150 × 150 mm, thickness 1.5 mm) was cast 24 h before the run, stored at room temperature, and pre-run at a constant current of 25 mA at room temperature until the stacking dye had reached the gel front. The protein sample was loaded and run at a constant current of 25 mA at +4°C. The proteins were transferred to an Immobilon™ (Millipore Continental Water Systems, Bedford, MA) PVDF membrane using a Trans-Blot cell (80 V, 2 h, +4°C) (Bio-Rad Laboratories). After transfer, the membrane was briefly stained with amido black to visualize the 18.5-kD protein band, the band was cut out of the membrane, and subjected to automated Edman degradation (27).

Statistical Analysis. Differences between groups were calculated with student's two-tailed *t* test using Stat View™ II (Abacus Concepts Inc., Berkeley, CA) data analysis program for Macintosh computers.

Results

Localized Adherence Is an Inducible Phenotype. B171 is a wild-type O111:NM EPEC strain that was isolated from a child with a diarrheal illness. To determine how B171 interacts with cultured epithelial cells, it was grown overnight in LB to early stationary phase and then added to a microtiter well containing an HEp-2 cell monolayer and DME supplemented with 10% FCS. 2 h later, microscopic examination of the

monolayer revealed circumscribed clusters of adherent bacteria typical of the LA pattern of attachment reported for other EPEC strains.

To examine how rapidly the LA phenomenon develops and whether it is preceded by a latent period, the LA assay was conducted in a quantitative manner and for variable incubation times. Small clusters of bacteria began to appear on the surfaces of the HEp-2 cells within 30–60 min (Table 2). Thereafter, the number of individual clusters per HEp-2 cell and the size of the clusters (i.e., the number of bacteria per cluster) were found to increase over time so that by 120 min, 90% of the HEp-2 cells were infected by at least one bacterial cluster, and each cluster was comprised of an average of 20 bacteria (Table 2).

The data depicted in Table 2 indicate that after bacteria are added to the monolayers, ~30 min must elapse before the adherence of bacteria to HEp-2 cells is evident. The existence of a 30-min latent period led us to determine if the LA phenotype might be induced by the epithelial cell or its products. This possibility was examined experimentally by determining whether the LA phenotype could be accelerated by first incubating the bacteria in a tissue culture medium that had been "conditioned" by growing epithelial cells. In these experiments, the standard bacterial adherence assay was conducted for 60 min. Then, unbound, free-floating bacteria were collected from the medium above the HEp-2 cell monolayer and transferred to new wells containing uninfected

Table 1. *E. coli* Strains Used in this Study

Strain	Description	Reference	Source	Plasmids	Phenotype		
					LA	Autoaggregation in DME	Expression of the 18.5-kD polypeptide
B171	EPEC O111:NM	20	I. K. Wachmuth	60,56*,3	+	+	+
B171-5	Spontaneous, plasmid-minus mutant of B171	This study		60	–	–	–
E2348/69	EPEC O127:H7	21	S. Falkow	60*,5	+	+	+
JPN15	Plasmid-minus mutant of E2348/69	18	S. Falkow	5	–	–	–
E6	EPEC O111:B4	None	S. Giono	56,40	+	+	+
IH3080	O18ac:K1 isolate from neonatal meningitis	22	P. H. Mäkelä	ND	–	ND	–
HB101	(F ⁻ <i>hsdS20</i> [r _k m _k] <i>recA13 ara-14 proA2 lacxY1 galK2 rpsL20</i> [Sm ^r] <i>xyl-5 ml-1 supE44</i>)	23	S. Falkow	None	–	ND	–

* EAF plasmid.

Table 2. Induction of LA by Preincubation of EPEC Strain B171 and its Plasmid-minus Derivative in Conditioned Tissue Culture Media

Strain	Percent of HEp-2 cells with LA*					
	Noninduced [†]			Induced [§]		
	15 min	60 min	120 min	15 min	60 min	120 min
B171	0.0	7.3 ± 1.0	89.8 ± 3.0	74.4 ± 4.0	96.3 ± 0.5	ND
B171-5	0.0	0.0	0.0	0.0	0.0	0.0

* Mean percent of HEp-2 cells with at least one adherent bacterial cluster ± SEM. *p* values were calculated with two-tailed *t* test by comparing the LA percentages of corresponding noninduced and induced groups at each of the indicated time points.

† The noninduced bacteria were grown overnight in LB broth, adjusted to the inoculum size (10^8 CFU/ 10^6 HEp-2 cells), and then dispensed into wells containing HEp-2 cell monolayers as described in Materials and Methods.

§ The induced bacteria were pre-incubated in wells containing DME supplemented with 10% FCS and HEp-2 cell monolayers. After 60 min, unattached bacteria were removed, adjusted to the inoculum size (10^8 CFU/ 1×10^6 HEp-2 cells), and then dispensed into wells containing fresh DME, 10% FCS, and HEp-2 cell monolayers. LA was then monitored by light microscopy as described in Materials and Methods at 15, 60, and 120 min after their addition to the second well. All time points were conducted in quadruplicate.

|| B171-5 is a derivative of strain B171 that spontaneously lost the 56-MD EAF plasmid, but that retains the 60-MD antibiotic resistance plasmid (Table 1).

HEp-2 cells. Large clusters of bacteria now formed on the HEp-2 cells within 15 min (Table 2 and Fig. 1 *b*), indicating that accelerated binding had occurred (Table 2; $p < 0.0003$).

The 50–60-MD EAF plasmid is required for the expression of the LA phenotype by many of the classic EPEC serotypes (11, 18, 28, 29, 30). A spontaneous EAF plasmid-minus mutant of B171, designated B171-5, was examined in the adherence assay described above in order to determine if the EAF plasmid is required for the induction of the LA phenotype. B171-5 did not exhibit the LA phenotype when pre-incubated with conditioned tissue culture media (Table 2). Thus, the LA phenotype per se, as well as its induction by conditioned tissue culture media, requires the EAF plasmid.

Expression of the LA Phenotype Can Be Induced by the Transfer of Bacteria from a Complex to a Defined Medium. Heretofore, induction of the LA phenotype had been studied using bacteria grown overnight in LB, a medium rich in nutrients. These bacteria were first diluted in DME and then incubated in a tissue culture medium that had been conditioned by a viable HEp-2 cell monolayer. The conditioned medium also contained 10% FCS (DME/10% FCS). To determine if the LA phenotype could be induced by transfer of the bacteria from LB to DME, in the absence of the HEp-2 cell or its products, LB grown bacteria were pre-incubated in fresh DME or fresh DME containing 10% FCS and then tested using the LA assay. Pre-incubation of EPEC strain B171 with DME

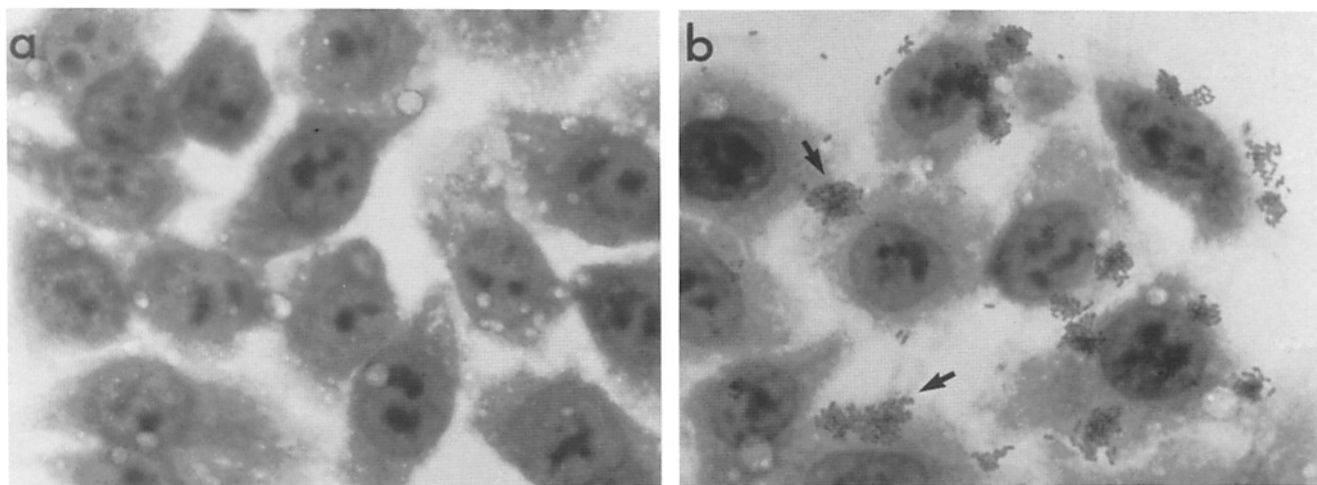


Figure 1. Development of the LA phenotype by noninduced and induced EPEC B171. HEp-2 monolayers were first infected with an overnight LB culture of B171 (10^8 CFU/ 10^6 HEp-2 cells), and the presence of bacterial clusters was monitored at 15 min (*a*). At 60 min, unattached, floating bacteria (10^8 CFU/ 10^6 HEp-2 cells) from the medium bathing this monolayer were then transferred to new wells containing a fresh HEp-2 cell monolayer, and the LA phenotype was assayed as before (*b*). Large clusters of EPEC B171 are now detected on HEp-2 cells 15 min after the addition of the induced bacteria, as shown by the arrow ($\times 5,000$).

alone, DME/10% FCS, or DME conditioned by a HEp-2 cell monolayer all caused induction of the LA phenotype, so that by 15 min, each of the pre-incubation conditions resulted in the presence of bacterial clusters on between 10% and 14% of the HEp-2 cells (Table 3). Other kinds of tissue culture media, including MEM and RPMI also induced the LA phenotype in the absence of epithelial cell products. In contrast, pre-incubation of the bacteria in fresh, static LB had no such effect (Table 3).

To examine the possibility that differences in the induction capacity of DME and static LB might be due to differences in their effect on the bacterial growth rate of B171, the growth phase of this strain was determined for each of the pre-incubation conditions just before the use of the bacteria in the LA assay. For each of conditions shown in Table 3, including the noninducing effect of growing the bacteria in static LB, B171 was found to be in early log-phase growth (data not shown). Thus, induction of the LA phenotype is not due to a change in bacterial growth phase nor does it require the presence of HEp-2 cells. Rather, the induction phenomenon is associated with transfer of the bacteria from a rich, complex medium to a medium that is relatively simple and defined.

Induction of the LA Phenotype Is Associated with the Formation of Bacterial Aggregates. The LA phenotype is characterized by the formation of bacterial aggregates attached to the epithelial cell surface (Fig. 1 b). Scanning electronmicrographs indicate that the bacteria within these clusters not only interact with the host cells to which they are bound, but also with each other (30). To determine if this tendency to form aggregates also occurs in the absence of epithelial cells, EPEC strain B171 was incubated under conditions known to in-

duce the LA phenotype, but in the absence of a HEp-2 cell monolayer. The presence of bacterial aggregates was then monitored using phase-contrast microscopy.

When grown overnight in LB, B171 was found to be disposed as well-separated individual organisms (Fig. 2 a). However, upon transfer to DME, small aggregates of bacteria begin to form within 30 min, and after 2 h of growth in DME, large bacterial clusters could be detected floating in the medium (Fig. 2 b). In contrast, similar large aggregates were never detected when B171 was transferred from an overnight culture of LB to fresh, static LB and then incubated 2 h.

Direct, continuous examination by phase-contrast microscopy of induced bacteria suspended in DME showed that the size of a bacterial aggregate increases with time through the addition of individual bacteria to the cluster. According to these phase-contrast images, autoaggregation seems to occur when randomly moving, individual bacteria at the periphery of a growing cluster collide with the cluster to which they then become attached. Autoaggregation was not prevented by the addition of D-mannose (0.5% [wt/vol]) to the medium, indicating that the expression of type 1 pili was probably not responsible for the phenomenon.

The EAF plasmid-minus derivative of B171, B171-5, was similarly tested. It was disposed only as free-floating individual organisms, even when cultivated for 2 h in DME (Fig. 2 c). Two other EPEC strains, namely E2348/69 and its EAF plasmid-minus mutant JPN15, were also tested for autoaggregation. Like B171, induction of the autoaggregation phenotype by E2348/69 occurred during growth in DME (Fig. 2 d), whereas JPN15 grew as individual bacteria in the same medium (data not shown). Taken together, these results indicate that tissue culture medium induces both the LA and the autoaggregation phenotypes and that both phenotypes require the EAF plasmid.

Induction of the LA and the Autoaggregation Phenotypes Is Associated with New Protein Synthesis. To determine if the LA and autoaggregation phenotypes are associated with de novo protein synthesis by the bacteria during the induction process, B171 was pretreated with rifampicin before the LA assay. Rifampicin is a bacteriostatic agent that blocks protein synthesis by binding RNA polymerase. As a control, bacteria were also pretreated with nalidixic acid, which prevents bacterial multiplication (but not protein synthesis) by inhibiting DNA gyrase. Both antibiotics were used in concentrations that prevented bacterial growth, but that were not bactericidal (Table 4). LA was completely prevented when B171 was treated with 32 µg/ml of rifampicin before and during the course of the standard adherence assay (Table 4). Similarly, addition of rifampicin to the medium prevented the autoaggregation of B171, the bacteria remaining as individual organisms throughout the 2-h incubation period (data not shown).

In contrast, nalidixic acid (10 µg/ml) did not prevent or delay the LA phenotype. However, at 120 min, the number of bacteria per cluster was slightly diminished (data not shown), indicating that the size of an adherent cluster is probably a function of both bacterial aggregation and mul-

Table 3. Effect of Preincubation Conditions on LA

Preincubation conditions	LA at 15 min	LA at 60 min
DME + 10% FCS	13.75 (± 4.9)* (<i>p</i> = 0.07) [†]	74.0 (± 1.3)
DME alone	9.7 (± 2.4) (<i>p</i> = 0.03)	45.5 (± 1.8)
DME + 10% FCS + HEp-2 cells	10.2 (± 0.2) (<i>p</i> = 0.0003)	62.5 (± 4.7)
LB	0.0	ND

EPEC B171 were preincubated in wells containing DME supplemented with 10% FCS; with DME alone; with DME supplemented with 10% FCS and HEp-2 cell monolayers; or fresh LB after 60 min. Unattached bacteria were removed and dispensed into wells containing fresh DME, 10% FCS, and HEp-2 cell monolayers. LA was then monitored by light microscopy as described in Materials and Methods at 15 and 60 min after the addition of the bacteria to the second well.

* Mean percent of HEp-2 cells with at least one adherent bacterial cluster ± SEM.

[†] *p* values refer to differences between the LA percentages of the corresponding DME and LB preincubated groups.

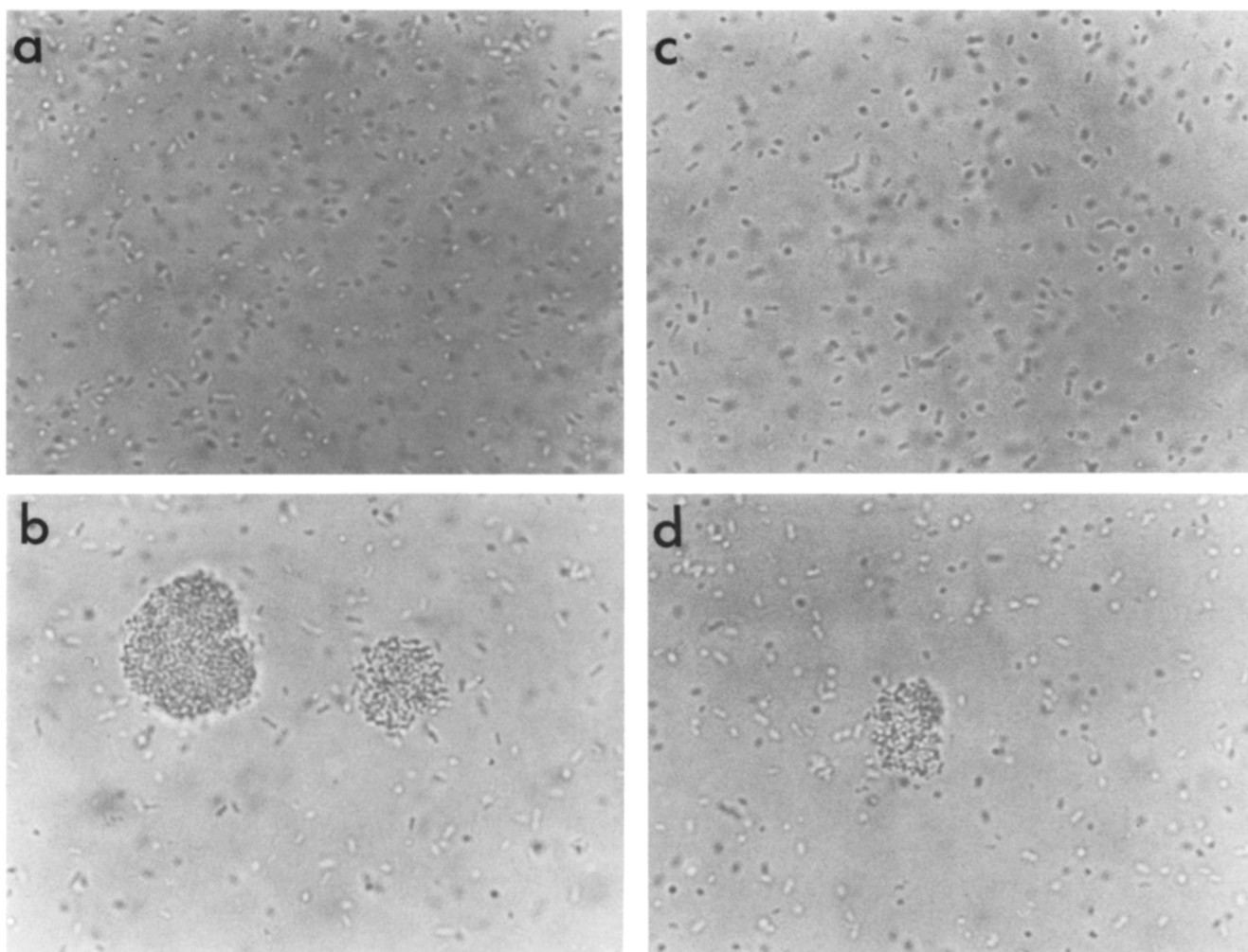


Figure 2. Induction of the autoaggregation phenotype by EPEC strains. The bacteria were preincubated in DME as described in the text, and the presence of aggregates was then monitored by phase-contrast microscopy after 2 h. (a) A nonpreincubated, overnight LB culture of EPEC B171 showing individual bacteria. (b) EPEC B171 preincubated in DME exhibited the autoaggregation phenotype, as indicated by the formation of bacterial clusters in the medium. (c) EPEC B171-5, lacking the EAF plasmid, grew as individual bacteria when preincubated in DME. (d) E2348/69, an O127:H7 EPEC strain, also exhibited the autoaggregation phenotype when preincubated in DME ($\times 5,000$).

tiplication. Nalidixic acid-treated bacteria were also able to autoaggregate normally, providing further evidence that this phenotype is not primarily due to bacterial division in situ.

Thus, the LA and the autoaggregation phenotypes occur only when bacterial protein synthesis is intact and neither is strictly associated with bacterial replication.

New Outer Membrane Proteins Are Expressed during Growth of EPEC on Tissue Culture Cell Monolayers. The data presented in the preceding section indicate that the induction of the LA and autoaggregating phenotypes is associated with de novo protein synthesis. To determine if the induction process is also associated with the production of new outer membrane proteins, the outer membrane of strain B171 was isolated from induced and noninduced bacteria, their proteins were separated by SDS-PAGE and the gel was stained with coomassie blue. Increased expression of three proteins of ~ 95 – 100 kD, a protein of ~ 32 kD, and a polypeptide of

~ 18.5 kD (Fig. 3) was noted in the outer membranes of bacteria grown under inducing conditions in DME. Decreased expression of a 67-kD and a 24-kD protein was also noted in the outer membranes of induced bacteria.

To determine if the expression of new outer membrane proteins also occurs in situ for bacteria growing as clusters on HEp-2 cells, strain B171 was metabolically ^{35}S pulse-labeled during the LA assay as described in Materials and Methods. At the conclusion of the experiment, the outer membranes were isolated from bacteria attached to the HEp-2 cell surface, and the protein content of these membranes was analyzed by SDS-PAGE and autoradiography. When compared to the other induced proteins shown in Fig. 3, the 18.5-kD polypeptide became the most prominently labeled protein showing inducible expression in the outer membranes of bacteria bound to the HEp-2 cell after 90 min of incubation (Fig. 4, lane b). The 18.5-kD polypeptide was also strongly in-

Table 4. Effect of Treatment with Rifampicin and Nalidixic Acid on the LA Phenotype with Noninduced and Induced EPEC B171

Treatment*	LA at 120 min	
	Noninduced [†]	Induced [§]
Rifampicin	0.0	0.0
Nalidixic acid	37.6 (± 8.9) (<i>p</i> = 0.38) [†]	36.3 (± 4.6) (<i>p</i> = 0.01)
No treatment	52.0 (± 5.8)	77.9 (± 5.3)

* Rifampicin was used at a concentration of 32 µg/ml. The minimal inhibitory concentration of rifampicin for B171 is 16 µg/ml. Nalidixic acid was used at a concentration of 10 µg/ml. The minimum inhibitory concentration of nalidixic acid for B171 is 1 µg/ml. Use of the antibiotics at the indicated concentrations prevented bacterial replication. However, when the bacteria were removed from the antibiotic-containing media and plated onto L agar plates (without antibiotics), bacterial replication resumed, indicating that the bacteria remained viable during the LA assay.

[†] Noninduced, overnight LB-grown bacteria were pretreated with either rifampicin or nalidixic acid at room temperature for 15 min and then added to wells containing DME, 10% FCS, and HEp-2 cell monolayers. LA was monitored at 120 min from the beginning of the infection by light microscopy as described in Materials and Methods. As a control, noninduced B171 without antibiotic pretreatment was assayed.

[§] Induced B171 bacteria were first pretreated with the appropriate antibiotic at room temperature for 15 min and then inoculated in a dilution of 1:20 to DME with the same antibiotics. After 60 min, bacteria were removed and dispensed into wells containing DME, 10% FCS, and HEp-2 cell monolayers. LA was monitored at 120 min as described in Materials and Methods.

^{||} Mean percent of HEp-2 cells with at least one adherent bacterial cluster ± SEM.

[†] *p* values refer to differences between the LA results for pretreated and nonpretreated groups.

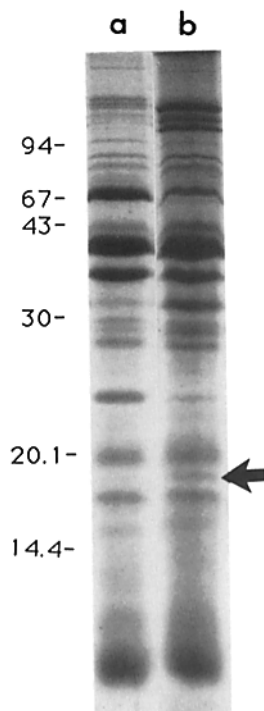


Figure 3. Induced expression of an 18.5-kD polypeptide in the outer membrane of EPEC strain B171. B171 was grown in LB (lane *a*) or preincubated under inducing conditions in DME (lane *b*), and their respective outer membranes were obtained and analyzed using coomassie blue-stained SDS-PAGE as described in Materials and Methods. An 18.5-kD polypeptide is detected in the outer membrane of bacteria grown in DME (arrow). Each lane was loaded with 10 µg of protein. Migration of molecular mass standards (kD) is indicated on the left margin.

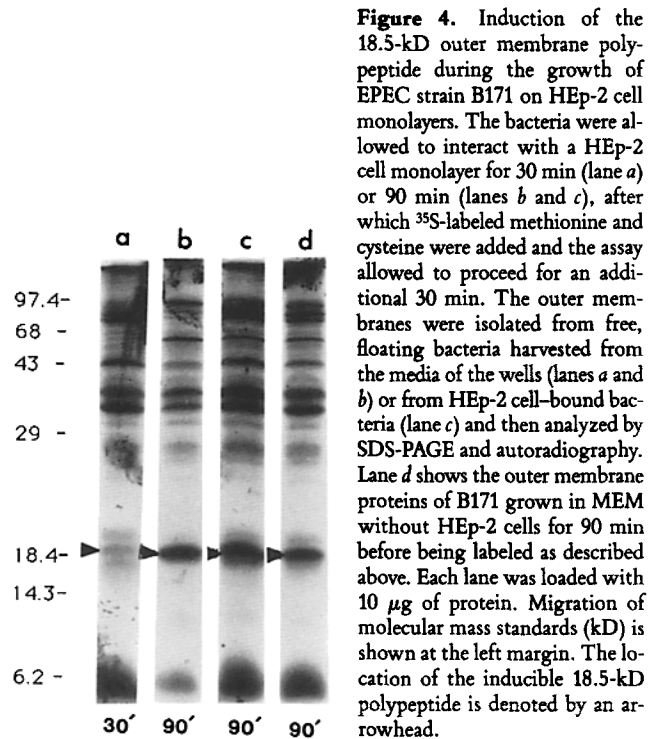


Figure 4. Induction of the 18.5-kD outer membrane polypeptide during the growth of EPEC strain B171 on HEp-2 cell monolayers. The bacteria were allowed to interact with a HEp-2 cell monolayer for 30 min (lane *a*) or 90 min (lanes *b* and *c*), after which ³⁵S-labeled methionine and cysteine were added and the assay allowed to proceed for an additional 30 min. The outer membranes were isolated from free, floating bacteria harvested from the media of the wells (lanes *a* and *b*) or from HEp-2 cell-bound bacteria (lane *c*) and then analyzed by SDS-PAGE and autoradiography. Lane *d* shows the outer membrane proteins of B171 grown in MEM without HEp-2 cells for 90 min before being labeled as described above. Each lane was loaded with 10 µg of protein. Migration of molecular mass standards (kD) is shown at the left margin. The location of the inducible 18.5-kD polypeptide is denoted by an arrowhead.

duced by unattached bacteria in the same well (Fig. 4, lane *c*) and by bacteria cultivated under inducing conditions in DME without HEp-2 cells (Fig. 4, lane *d*). Thus, the same conditions that induce the LA and autoaggregating phenotypes also induce de novo synthesis of this polypeptide. In contrast, the 18.5-kD polypeptide could not be detected in the outer membrane of the nonadhering, nonaggregating, plasmid-cured variant B171-5, even when this mutant was grown under inducing conditions (Fig. 5, lane *b*).

The 18.5-kD polypeptide was sought in other *E. coli*, in-

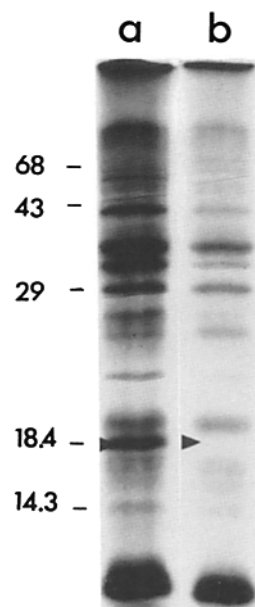


Figure 5. Induction of the 18.5-kD outer membrane polypeptide by EPEC strain B171 and its plasmid-cured derivative, B171-5. Bacteria were grown in M9 medium to mid-logarithmic growth phase, diluted 1:20 in methionine and cysteine-free MEM, and grown for 90 min. Then, the ³⁵S-labeled amino acids were added and the bacteria were incubated for an additional 30 min. The bacteria were then collected and the outer membranes isolated, and their protein content was determined by SDS-PAGE followed by autoradiography. Lanes *a* and *b* are autoradiographs of the outer membrane proteins of B171 and B171-5, respectively. The location of the 18.5-kD polypeptide, as indicated by an arrowhead, can be detected in lane *a*, but not in lane *b*. Each lane was loaded with 10 µg of protein. Migration of molecular mass standards (kD) is indicated at the left margin.

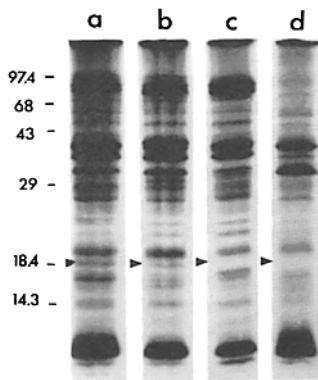


Figure 6. Induction of the 18.5-kD polypeptide by heterogenous EPEC and non-EPEC strains. Bacteria were grown, labeled with ^{35}S methionine and cysteine, and their outer membranes prepared and analyzed as described in Fig. 5. Autoradiographs of outer membranes from four strains are shown in the following lanes: (a) O111 EPEC, strain 6; (b) O127:H6 EPEC, strain E2348/69; (c) O18:K1 non-EPEC meningitis strain, IH3080; and (d) a K-12 derivative non-EPEC strain, HB101. The 18.5-kD polypeptide, as indicated by an arrow, can be detected in lanes a and b, but not in lanes c and d. Each lane was loaded with 10 μg of protein. Migration of molecular mass standards (kD) is indicated at the left margin.

cluding two more EPEC strains that exhibit the LA phenotype. In addition to B171, a second O111 EPEC strain (Fig. 6, lane a) and an O127:H6 EPEC strain (Fig. 6, lane b) were each found to produce an 18.5-kD outer membrane polypeptide under similar inducing conditions. Two non-EPEC strains were also evaluated. Neither an O118:K1 strain from a child with neonatal meningitis (Fig. 6, lane c), nor a nonadherent K-12 derivative strain, HB 101 (Fig. 6, lane d), expressed the 18.5-kD polypeptide, indicating that its expression may be restricted to EPEC strains that contain the EAF plasmid and exhibit the LA and autoaggregating phenotypes.

The Inducible 18.5-kD Polypeptide Is a Membrane-bound Subunit of the Bundle-forming Pili (BFP) of EPEC. To learn more about the identity of the 18.5-kD protein, outer membranes were prepared from strain B171 after it had been grown in DME under inducing conditions, and the outer membrane proteins separated by SDS-PAGE and then transferred to a PVDF membrane. The 18.5-kD polypeptide was excised from the membrane and subjected to automated Edman degradation. A partial NH_2 -terminal amino acid sequence was obtained

through residue 12 (Table 5). However, at positions 1,2,6,8, and 11, the identity of the amino acid could not be determined with certainty owing to the small quantity of protein available for Edman degradation. Of the remaining seven residues, all were found to be identical to the corresponding residues of the major, repeating subunit of a BFP recently isolated from the same EPEC strain (30). Moreover, the inducible 18.5 polypeptide was found to comigrate with the BFP subunit and to crossreact with a BFP-specific antiserum when analyzed by immunoblotting (data not shown). Examination of the 18.5-kD polypeptide/BFP subunit consensus sequence also revealed that it is partially homologous with the corresponding region of the toxin-coregulated pilin (Tcp) sequence of *Vibrio cholerae* (31), 8 of the 12 NH_2 -terminal residues being either identical or highly conserved (Table 5).

Discussion

EPEC are found in the small intestine of infected children as colonies of bacteria attached to the surfaces of epithelial cells. This pattern of adherence, including the underlying changes in the cytoskeleton of the host cell, appears to be faithfully reproduced in vitro by the LA phenotype. Hence, in the studies reported here, we have used the LA assay to discover what transpires within these bacteria during the process of establishing an adherent colony.

LA was found to begin after a latent period of 30–60 min. In contrast, bacteria preincubated in a tissue culture medium exhibited the LA phenotype within 15 min, indicating that it is an inducible property of these strains. In the absence of epithelial cells, autoaggregation of the bacteria was also observed and this now appears to be a second inducible phenotype of EPEC. Moreover, LA and autoaggregation were found to develop at the same rates, to be induced by the same growth conditions, and to each require the presence of the EAF plasmid.

We had expected that induction of the LA phenotype would require contact of the bacteria with the HEP-2 cell surface or with soluble products of the monolayer. However, the experiments depicted in Table 3 clearly show that induction can occur to the same degree and at the same rate upon transfer

Table 5. NH_2 -terminal Amino Acid sequence of the 18.5-kD EPEC Outer Membrane Protein, the EPEC BFP, and their comparison to the *Vibrio cholerae* TcpA Pilin Sequence

	Residue no.											
	1	2	3	4	5	6	7	8	9	10	11	12
EPEC 18.5-kD outer membrane protein	-	-	Leu	<u>Ile</u>	Glu	-	Ala	-	Val	Leu	-	<u>Leu</u>
EPEC BFP (30)	Met	Ser	Leu	<u>Ile</u>	Glu	Ser	Ala	Met	Val	Leu	Ala	<u>Leu</u>
<i>Vibrio cholerae</i> TcpA (31)	modMet	Thr	Leu	<u>Leu</u>	Glu	Val	Ile	Ile	Val	Leu	Gly	<u>Ile</u>

Bold-face denotes residues of the EPEC proteins that are identical to the TcpA protein. Underlined residues are conservative substitutions. (-) Unidentified residues of the EPEC 18.5-kD outer membrane protein due to the small amount of the protein available for analysis.

of the bacteria from an overnight culture of LB to a fresh tissue culture medium devoid of epithelial cells. Moreover, the induction process could not be attributed to shifting the bacteria from the stationary phase of growth to the exponential phase of growth. Instead, the LA and autoaggregating phenotypes seem to have been induced by the transfer per se of the bacteria from a complex, rich medium to a medium that by comparison is relatively simple and defined.

The induction of virulence determinants by specific growth conditions has now been demonstrated for an increasing number of pathogenic bacteria and seems to allow them to adapt to a variety of environmental habitats. Examples include the osmotic regulation of Tcp and enterotoxin expression by *V. cholerae* (31, 32); induction of adherence and invasiveness by low oxygen tension for the *Salmonella* (33); the expression of virulence-associated proteins by *Shigella flexneri* growing in media having ion concentrations and pH values that simulate an intracellular or endosomal environment (34); and the expression of the flexible pili of *Aeromonas hydrophila* by low available concentrations of iron (35). It now appears that the induction of LA and autoaggregation by EPEC is another example of this kind, in which as yet unidentified physiochemical differences between LB and tissue culture media trigger the expression of these phenotypes.

Induction of the LA and autoaggregating phenotypes led us to search for changes of the bacterial surface that might cause the bacteria to coalesce into the clusters shown in Figs. 1 and 2. An 18-kD polypeptide was identified in the outer membrane of induced bacteria. We now believe that the expression of this polypeptide may be responsible for these phenotypes. Evidence in support of this hypothesis comes from the following observations. First, the 18.5-kD polypeptide is expressed under the same growth conditions and at the same time as the LA and autoaggregating phenotypes. Second, expression of the 18.5-kD polypeptide, like the LA and autoaggregating phenotypes, requires the EAF plasmid. Moreover, two other EPEC strains that manifest the LA phenotype also express an inducible 18.5-kD polypeptide that requires an EAF plasmid. Third, the amino acid sequence of the 18.5-kD polypeptide was found to be homologous with the corresponding region of a newly recognized, EPEC-specific pilus protein (30). Further evidence that the 18.5-kD polypeptide and the pilus subunit are identical comes from the observation that they comigrate when analyzed by SDS-PAGE, and from Western blotting experiments showing that an antiserum to the pilus protein crossreacts with the 18.5-kD polypeptide. Transmission electron micrographs of negatively stained EPEC show that this pilus type is deployed as many fine fibers that are gathered together into large bundles, thus creating a meshwork within which are embedded individual bacteria. Examination of these bundles by scanning electron microscopy reveals that they are present within colonies of EPEC growing on epithelial cells, where they appear to form multiple, interbacterial linkages (30). Taken together, these results indicate that the 18.5-kD outer membrane polypeptide is a

membrane-bound form of a pilin that, when assembled as a surface appendage of the organism, could be directly responsible for the LA and autoaggregating phenotypes. However, conclusive proof of this idea will need to come from the construction and study of isogenic strains that differ only in their capacity to express this polypeptide.

Previous studies by other workers have identified two additional EPEC-associated outer membrane proteins. Human volunteers infected with an O127 EPEC strain were shown to develop antibodies to a 94-kD outer membrane protein that also requires the EAF plasmid for expression (15, 18, 36). We could detect such a protein in our outer membrane preparations, particularly when a 10% polyacrylamide gel was used, and based on the increased expression of a group of three 95–100-kD proteins (Fig. 3 b), its expression might also be induced by growth in tissue culture media. If so, it might act coordinately with the 18.5-kD polypeptide during the infection of an epithelial cell by EPEC. More recently, Scaletsky et al. (37) identified a 32-kD, *N*-acetylgalactosamine-binding protein in the outer membrane of an O111:H⁻ EPEC strain. Because of its lectin-like properties, this protein was proposed as an adhesin that might mediate the attachment of EPEC to epithelial cell surfaces. However, Chart and Rowe (38) showed that the expression of this protein was not associated with the LA phenotype. Moreover, because it was found to be peptidoglycan associated and heat modifiable, it has now been identified as the porin protein Omp F, and thus is not likely to be a unique EPEC product.

The current model of EPEC pathogenesis proposes a sequence of events in which long-range adherence of a bacterium to an epithelial cell surface is mediated by the adhesins of the organism. This in turn is followed by the close juxtaposition of the bacterial and epithelial cell surfaces and by localized condensation of actin. This model, however, does not explain the striking tendency of EPEC to grow as adherent colonies on mucous membranes. The data presented in this report address this aspect of EPEC pathogenesis by showing that autoaggregation and LA are inducible phenotypes and that their induction is associated with changes in the outer membrane proteins of the organism. Moreover, the demonstration in Fig. 2 that the formation of bacterial aggregates can occur in tissue culture media even before bacterial adherence to an epithelial cell surface has taken place suggests that EPEC may not infect epithelial cells as single, independent organisms. Rather, through the autoaggregating effect described above, EPEC may preferentially attack host cell surfaces as infectious units comprised of several to many organisms. Then, once attached to the cell surface, these clusters may continue grow, not only by bacterial division, but also by a process through which unattached bacteria are added to existing colonies owing to the same autoaggregating effect. The resulting colony could contribute to EPEC's capacity to survive on mucosal surfaces and thus may be an essential aspect of its pathogenic program.

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