INFECTION AND IMMUNITY, Aug. 1996, p. 3196–3200 Vol. 64, No. 8
0019-9567/96/$04.00+0

Polysaccharide Side Chains Are Not Required for Attaching And Effacing Adhesion of *Escherichia coli* O157:H7

F. COCKERILL III,[1]* G. BEEBAKHEE,[1] R. SONI,[2] AND P. SHERMAN[2,3,4]

The Samuel Lunenfeld Research Institute, Mount Sinai Hospital,[1] Division of Gastroenterology, Research Institute, The Hospital for Sick Children,[2] and Departments of Pediatrics[3] and Microbiology,[4] University of Toronto, Toronto, Ontario M5G 1X8, Canada

Received 1 March 1996/Returned for modification 18 April 1996/Accepted 22 April 1996

Escherichia coli **of the serotype O157:H7 is an enterohemorrhagic human pathogen which demonstrates attaching and effacing adhesion to colonocytes in vivo and to epithelial cells grown in tissue culture. Transposon Tn*phoA* mutants of *E. coli* O157:H7 strain CL-8 were produced. Two of 300 alkaline phosphatase positive mutants, designated JB6 and JB27, did not express O157 side chains as assessed by agglutination with specific polyclonal O157 antiserum, silver staining of lipopolysaccharide extracts separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and Western immunoblots with polyclonal O157-specific antiserum. Both O157-negative mutants and the parent strain demonstrated localized adherence to HEp-2 cells when examined by Giemsa staining and bright-field microscopy. Furthermore, both O157-negative mutants showed enhanced adherence to HEp-2 cells compared with the parent strain when assessed by quantification of adherent bacterial CFUs. The parent strain, CL-8, and both of the mutants produced fluorescent foci when adherent bacteria and HEp-2 cells were stained with fluorescein isothiocyanate-labelled phalloidin. Transmission electron microscopy confirmed attaching and effacing adherence of strain CL-8 and the O157-negative mutants to HEp-2 cells. These findings indicate that mutants deficient in O157 polysaccharide repeats exhibit adherence to tissue culture cells in vitro and that O157 polysaccharide repeats are not required to produce the attaching and effacing lesion.**

Enterohemorrhagic *Escherichia coli* (EHEC) of the serotype O157:H7 has been associated with a spectrum of illness in humans, including self-limited watery diarrhea, hemorrhagic colitis, and the hemolytic uremic syndrome (2). This serotype has been identified as a closely related clonal group which is well adapted to survival in many niches in the food chain. Furthermore, it is frequently associated with major point source outbreaks followed by significant person-to-person spread. On this basis it has been suggested that the infectious inoculum is significantly less than the 10^8 characteristic of enterotoxigenic *E. coli* and enteropathogenic *E. coli* (EPEC) (7). *E. coli* serogroup O157:H7 organisms produce verotoxins (Shiga-like toxins) which are thought to be of etiologic importance in the pathogenesis of hemolytic uremic syndrome (13). While verotoxins vts are the most likely causative agents of the vascular complications of EHEC infection, study of infection in animal models has shown that epithelial cell adherence and colonization of the distal ileum, cecum, and colon are both necessary and sufficient to cause diarrhea regardless of the production of verotoxin (33). The adherence to epithelial cells both in vitro (14, 27) and in vivo (33, 34) takes place by the attaching and effacing (AE) lesion. This is characterized by effacement of the microvilli, intimate adherence, and recruitment of polymerized actin to the underlying cytoplasm of the eukaryotic cell (14, 15, 26). Adherence by this mechanism proceeds by initial adherence, which is probably mediated by plasmid-encoded fimbriae (12, 31); this is followed by a signaling process and intimate adherence mediated by the *cfm* and *eae* genes, respectively (4, 17, 21). Because of the remarkable success of serogroup O157 organisms, we considered whether lipopolysaccharide (LPS) side chains might play an accessory role in adherence in vitro. We report the isolation of Tn*PhoA* mutants deficient in the production of O157 side chains. The mutants produce the AE lesion and exhibit increased binding to tissue culture cells in vitro.

* Corresponding author. Present address: Division of Clinical Microbiology, Department of Laboratory Medicine, Mayo Clinic, Hilton 470, 200 First St. S.W., Rochester, MN 55905.

MATERIALS AND METHODS

Bacteria and mating procedures. *E. coli* O157:H7 strain CL-8 was obtained from M. Karmali at the Hospital for Sick Children in Toronto, Canada. Strain CL-8 was originally isolated from a child with hemorrhagic colitis and the hemolytic uremic syndrome (13). Binding properties of strain CL-8, both in vitro and during infection in vivo, have been reported previously by us (26, 27) and by Junkins and Doyle (11). A spontaneous streptomycin-resistant mutant was selected on agar that was supplemented with streptomycin (100 μg/ml), and it is referred to as CL-8Sm. Transposon mutagenesis with Tn*phoA* was performed, as described by Taylor et al; the O157 mutants were obtained by using the plasmid pRT291 carrying Th*phoA* (30). *E. coli* SM10 (29), containing plasmid pRT291, was kindly provided by R. Taylor (Department of Microbiology and Immunology, University of Tennessee, Memphis). Briefly, plasmid pRT291 was transferred to CL-8Sm by plate mating, and transconjugants were selected on Luria agar supplemented with kanamycin (45 μg/ml) and streptomycin (100 μg/ml). Transconjugants were then plate mated with *E. coli* MM294 carrying the incompatible plasmid pPH1JI. Recipients were selected on Luria agar supplemented with gentamicin (30 μg/ml), kanamycin (45 μg/ml), streptomycin (100 μg/ml), and 40 μg of the p-toluidine salt of 5-bromo-4-chloro-3-indolylphosphate (XP; Sigma Chemical Co., St. Louis, Mo.) per ml. Blue colonies were picked and subcultured for purification. Each of the 300 Tn*phoA* mutants was screened for agglutination with polyclonal O157-specific antiserum (Difco Laboratories, Detroit, Mich.), by using methods described by the manufacturer.

In vitro adhesion assays. The protocol of Knutton et al. (14) was followed to assess the adherence of both CL-8Sm and the polysaccharide-deficient strains designated JB6 and JB27. Briefly, HEp-2 cells were obtained from the American Type Culture Collection (Rockville, Md.) and used in adhesion assays during passages 1 through 10. Subconfluent monolayers were grown on 13-mm sterile glass coverslips in multiwell tissue culture plates (Costar, Cambridge, Mass.). For binding assays, cells were maintained in minimal essential medium buffered in HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) and supplemented with 10% fetal calf serum (Bocknek, Rexdale, Canada), 0.5% D-mannose (Sigma), and the antibiotic combination of penicillin, streptomycin, and ampho-

tericin B. Following overnight static culture at 37°C in Pennassay broth, 20 ml of bacteria was added to 1 ml of medium without supplemental antibiotics and incubated with the HEp-2 cells for 6 h at 37°C. The culture medium was changed once after 3 h of incubation. Monolayers were then washed three times in phosphate-buffered saline (PBS) and fixed in formalin (3% in PBS) for 10 min. HEp-2 cells were permeabilized with Triton X-100 (Sigma) (0.1% in PBS) for up to 3 min, washed three times with PBS, and incubated with fluorescein isothiocyanate-conjugated phalloidin (Sigma) at a concentration of 5 mg/ml. After being washed three times with PBS, coverslips were mounted cell side downward onto glass microscope slides in a 1:1 mixture of glycerol and PBS. Identical fields were examined by both phase-contrast and fluorescence microscopy, as described previously (14). To determine inhibitory effects on the adhesion of strain CL-8Sm to HEp-2 cells, polyclonal O157-specific antiserum (Difco) was added in separate experiments to tissue culture wells at a dilution of 1 in 100. The procedures for the binding assay were then performed, as described above.

Quantitation of *E. coli* O157:H7 binding to HEp-2 cells was performed, as described previously (24, 25). Briefly, HEp-2 cells (American Type Culture Collection) were grown in 12-well cluster dishes (Costar) overnight at 37°C. After the cells were washed in antibiotic-free tissue culture medium, 10^8 bacteria were added in 2.0 ml of medium to the wells containing HEp-2 cells. Following incubation for 3 h at 37°C, the medium was removed and the wells were washed six times to remove loosely attached and nonadherent organisms. HEp-2 cells and adherent *E. coli* O157:H7 were then removed from the polystyrene surfaces by trypsinization. Viable bacterial CFUs in lysates of trypsin-removed extracts were then determined by serial spread plating onto bile salt agar plates.

Results were expressed as means ± standard errors. Differences between multiple groups were determined by one-way analysis of variance, and differences between two groups were determined by using the two-tailed, unpaired Student *t* test (23, 24).

LPS analysis. LPS was extracted from *E. coli* O157:H7 strains CL-8Sm, JB6, and JB27 with the proteinase K digestion procedure of Hitchcock and Brown (8). The LPS extract was analyzed by polyacrylamide gel electrophoresis (PAGE) in 1% sodium dodecyl sulfate (SDS) by using a 5% stacking gel and a 10% separating gel. LPS was visualized on the gels by the silver staining procedure of Tsai and Frasch (32). LPS separated by gel electrophoresis was then transferred to nitrocellulose paper, as described by Bradbury et al. (3). The blot was incubated sequentially with a 1 in 1,000 dilution of polyclonal O157-specific rabbit antiserum (Difco) and peroxidase conjugated to anti-rabbit immunoglobulin. Blots were then developed with 4-chloro-1-alpha naphthol.

Outer membrane preparations. Outer membrane protein extracts of strains CL-8Sm, JB6, and JB27 were prepared by precipitation of bacterial sonicates in *N*-lauroyl sarcosine, 1.7% wt/vol (Sarkosyl, Sigma), as described previously (25). Detergent-insoluble outer membranes were separated by electrophoresis in 12.5% polyacrylamide separating gels in the presence of 0.1% SDS and in parallel with proteins of known molecular weight used as reference standards (Bio-Rad, Richmond, Calif.). Gels were stained with Coomassie brilliant blue to visualize proteins (28).

Electron microscopy. Organisms were incubated in 25-cm^2 tissue culture flasks (Corning Glassworks Co., Corning, N.Y.) containing confluent HEp-2 cell monolayers. After 6 h, cells were washed to remove nonadherent and loosely attached bacteria. The HEp-2 cells were gently scraped off the polystyrene surface with a rubber policeman and then suspended in PBS. Cells were then pelleted and washed in distilled water, and the pellet was fixed with 2.5% glutaraldehyde for 15 min at 4°C, postfixed in 1% osmium tetroxide, and embedded in Epon (27). Specimens were mounted onto grids, stained with uranyl acetate and lead citrate, and examined in a Phillips 300 transmission electron microscope at an accelerating voltage of 80 kV.

RESULTS

Three hundred independent Tn*phoA* mutants were produced and screened for O157 polysaccharide by slide agglutination with polyclonal O157-specific antiserum. Two of the 300 mutants (0.7%) failed to agglutinate with O157 antiserum. These two strains, designated JB6 and JB27, were selected for further study. Both strain JB6 and strain JB27 produced cytotoxic effects on Vero cells at dilutions equal to that of the parent CL-8Sm strain. (Cytotoxicity assays were kindly performed by M. Karmali, Hospital for Sick Children, Toronto, Canada.) Vero cytotoxicities of the parent and both transposon derivatives were each partially neutralized by the coincubation of anti-Verocytotoxin-1 immune serum. The two Tn*phoA* derivatives also agglutinated with polyvalent H7 monospecific antiserum (Difco).

Figure 1 shows a photograph of a polyacrylamide gel after staining with silver and the corresponding Western immunoblot produced with O157-specific antiserum. Lane A shows

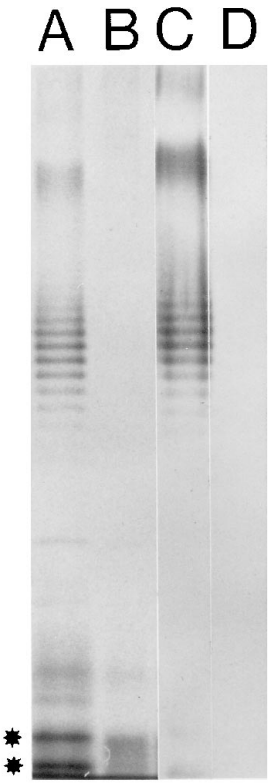

FIG. 1. Silver-stained polyacrylamide gel of LPS extracted from *E. coli* O157:H7 strain CL-8Sm (lane A) and an O157 slide agglutination-negative transposon mutant (lane C). Lanes B and D are immunoblots of CL-8Sm and the O157-negative Tn*phoA* mutant, respectively, stained with polyvalent O157 specific immune serum. Stars indicate bands corresponding to O157 polysaccharide.

LPS extracted from strain CL-8Sm, and lane C shows the immunoblot with extract from the same parent strain. A well-developed polysaccharide side chain ladder was visualized and these side chains bound to O157 antiserum. An LPS extract of the JB6 mutant showed no ladder pattern of polysaccharide, although some low-molecular-weight material ran within the region consistent with the LPS core (lane B). Material extracted from this mutant did not react with O157 antiserum in the immunoblot (lane D). Strain JB27 showed identical negative results (data not shown).

As we previously provided evidence showing that constituents of the outer membrane could serve as a bacterial adhesin for another *E. coli* O157:H7 isolate (24, 25), we also compared outer membrane profiles of strains CL-8, JB6, and JB27. The outer membranes were identical for each of the three isolates despite documented differences in polysaccharide composition (data not shown).

Strains CL-8Sm, JB6, and JB27 each produced fluorescent lesions when adherent bacteria and HEp-2 cells were stained with fluorescein isothiocyanate-labelled phalloidin. Figure 2 shows that like the parent strain CL-8Sm (Fig. 2A), the mutant JB6 strain adhered to HEp-2 cell monolayers (Fig. 2B) and resulted in fluorescent foci. This finding indicated that actin in the eukaryotic cells had polymerized at sites adjacent to foci of intimate bacterial attachment (14). Knutton et al. (14) showed that these fluorescent foci represent bacteria adhering by the attaching and effacing mechanism. Polyclonal O157-specific antiserum, employed at a dilution of 1 in 100, did not prevent the development of fluorescent foci on HEp-2 cells incubated with the parent CL-8Sm strain. Higher concentrations of an-

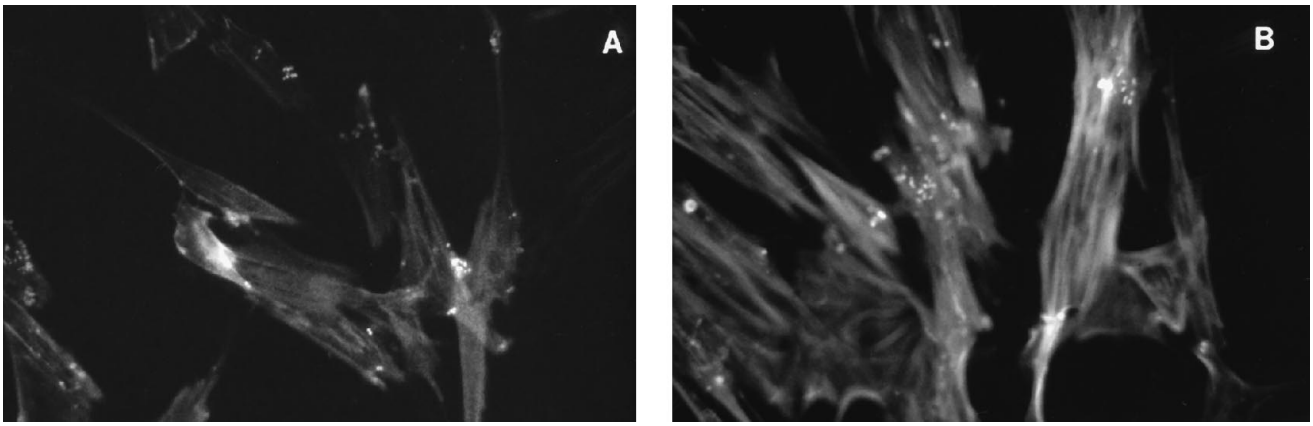

FIG. 2. Fluorescence micrograph of the same field showing bright spots of fluorescence corresponding to foci of adherent bacteria at sites of actin accumulation in the eukaryotic cell cytosol below regions of pedestal formation in the plasma membrane(s). (A) CL-8SM; (B) Tn*phoA* mutant JB6. Magnification, ×500 for panels A and B.

tiserum resulted in cytotoxicity of HEp-2 cells to such an extent that fluorescent patterns were not interpretable.

Quantitation of bacterial binding to HEp-2 cells showed that the two Tn*phoA* mutants were not less efficient in their attachment to eukaryotic cells. In fact, calculated as a percentage of the bacterial inoculum, both strain JB-6 (64% ± 27%, mean ± standard error) and strain JB27 (70% ± 23%) adhered to a greater degree than the parent CL-8Sm strain (18% ± 2%) ($P < 0.05$). We confirmed by transmission electron microscopy that the O157 side chain deficient mutants exhibited intimate, attaching and effacing adherence to plasma membranes of eukaryotic cells (Fig. 3).

DISCUSSION

Adherence of *E. coli* enteric pathogens to intestinal epithelium plays a crucial role in the pathogenesis of diarrheal diseases because it promotes bacterial colonization and enhances delivery of bacterial exoproducts such as toxins to the epithelium. It is also a prerequisite for cellular invasion in those strains that have invasive properties. In animal models, Vero-cytotoxin-producing *E. coli*, including those of serotype O157:H7, have been shown to adhere to the apical surface of enterocytes by the AE lesion (18). This type of adherence is necessary and sufficient to produce a diarrheal response in gnotobiotic piglets independent of verotoxin production (33). Knutton and associates showed that the AE lesion produced by both EPEC and EHEC is associated with actin polymerization in the cytosol immediately beneath the area of bacterial attachment (14). AE lesion formation is thought to be a multistage process, requiring multiple bacterial products. For EPEC strains, pilus-mediated initial adherence is followed by signaling which induces tyrosine kinase and protein kinase activity in the eukaryotic cell (6, 21). We have provided initial evidence that this may not prove to be the case for EHEC serotype O157:H7 strains (9). A 94-kDa outer membrane protein, intimin, which is the product of the *eaeA* locus, is required for intimate adherence and recruitment of filamentous actin to the subjacent cytoplasm (4, 10, 14, 15, 21). A similar mechanism for AE lesion formation for EHEC strains is probable (14), although further studies are required to substantiate this point. Karch and colleagues demonstrated that expression of a fimbrial antigen was associated with EHEC adhesion to epithelial cells (12). An *eaeA* gene homolog in *E. coli* serotype O157:H7 has been cloned and sequenced (17), and EHEC strains have been reported to express Eae proteins which share significant homology with EPEC Eae factors (4).

Because of the remarkable ecological success of *E. coli* serotype O157:H7, we were interested in determining whether these LPS side chains play some role in promoting adherence. Finlay et al. showed that LPS mutants of *Salmonella cholerae-*

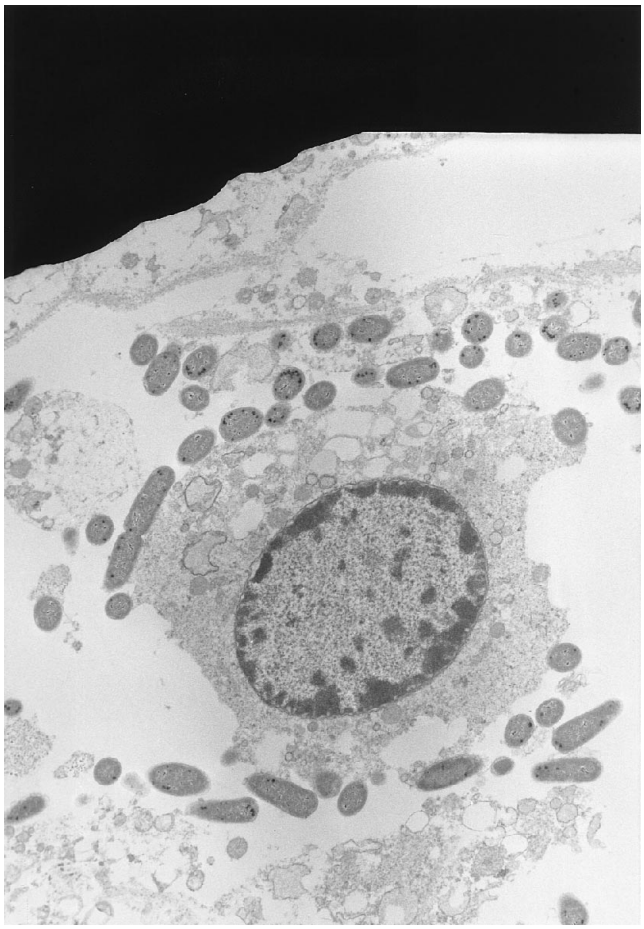

FIG. 3. Transmission electron photomicrograph of O157 side chain-negative Tn*phoA* mutant (JB6) adherent to the apical plasma membrane of an HEp-2 cell.

suis are not able to cross polarized epithelial cell monolayers by transcytosis (5). LPS side chain determinants have been shown to be associated with *Shigella* virulence plasmids. For example, Kopecko and colleagues (16) showed that a 120-megadalton plasmid necessary for HeLa cell invasion encodes the expression of O side chain antigens in *Shigella sonnei*. The LPS side chain in this case, however, is not necessary for cell invasion but is necessary to produce conjunctivitis in guinea pigs (positive Sereny test). Thus, in this case, the side chains may be important to allow the organism to produce disease in the whole animal. For one *Shigella flexneri* LPS mutant (N1308), which has a short O side chain, Rajakumar and associates showed that although invasion occurs in epithelial cells, there is a decreased ability for the microorganisms to spread from cell to cell (20). In agreement with this finding, Sandlin and colleagues showed, for the same mutant, aberrant surface localization of IcsA, a protein essential for intracellular motility and intercellular spread (22). Finally, Pierson showed that *Yersinia enterocolitica* Tn*5B50* mutants which lacked LPS (no O side chains were detectable) had enhanced adherence and entry into mammalian cells. Pierson speculated that as a result of a change in the LPS on the surface of these mutants, adherence and invasion proteins also located on the surface could more easily interact with mammalian cell surfaces (19). The ability of these *Y. enterocolitica* LPS mutants to produce disease requires further study.

We previously showed that LPS purified from *E. coli* O157:H7 strain CL-56 failed to competitively inhibit adherence of the homologous strain to HEp-2 cells in tissue culture. Furthermore, antiserum raised against purified LPS failed to inhibit the binding of whole organisms (24, 25). In the present study we ablated production of O157 group-specific polysaccharide side chains by Tn*phoA* mutagenesis. The exact nature of the remaining LPS is not clear. Although on SDS-PAGE it is at a position consistent with core LPS, additional analytic studies will be required to determine its precise structure. The fluorescein isothiocyanate-labelled phalloidin and electron microscopic studies both showed that mutants JB6 and JB27 still produced the AE lesion. Indeed, the mutant strains showed increased adherence to HEp-2 cells in vitro. This observation has also been reported recently by Bilge and colleagues (1), and a possible explanation may be the same as that proposed by Pierson as reviewed above for *Y. enterocolitica* LPS mutants. That is, the absence of LPS may allow for increased contact of the outer membrane Eae proteins in *E. coli* O157:H7 and the surface of HEp-2 cells.

In conclusion, our findings suggest that O157 polysaccharide side chains are not necessary for attaching and effacing adherence to epithelial cells in vitro. The ecological advantage which might be conferred by LPS side chains for survival of EHEC O157 strains in the environment and the role of LPS in human disease require further study.

ACKNOWLEDGMENTS

This work was supported by grants from the Medical Research Council of Canada. P.S. is the recipient of a Career Scientist Award from the Ontario Ministry of Health.

We thank M. Karmali for performing the cytotoxicity assays on Vero cells, R. Taylor for providing the vectors for Tn*phoA*, J. Brunton for help in experimental design, and R. Kondert for help in preparing the manuscript.

REFERENCES

1. **Bilge, S. S., J. C. Vary, K. Potter, S. F. Dowell, and P. I. Tarr.** 1995. *Escherichia coli* O157:H7 lipopolysaccharide mutants are hyperadherent, abstr. B-7, p. 166. *In* Abstracts of the 95th General Meeting of the American Society for Microbiology 1995. American Society for Microbiology, Washington, D.C.
2. **Boyce, T. G., D. L. Swerdlow, and P. M. Griffin.** 1995. Escherichia coli O157:H7 and the hemolytic-uremic syndrome. N. Engl. J. Med. **333:**364–368.
3. **Bradbury, W., S. D. Mills, M. A. Preston, L. J. Barton and J. L. Penner.** 1984. Detection of lipopolysaccharides in polyacrylamide gels by transfer to nitrocellulose followed by immunoautoradiography with antibody and 125-I protein A: "LPS blotting." Anal. Biochem. **137:**129–133.
4. **Donnenberg, M. S., and J. B. Kaper.** 1992. Enteropathogenic *Escherichia coli*. Infect. Immun. **60:**3953–3961.
5. **Finlay, 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* that are unable to pass through a polarized MDCK epithelial cell monolayer. Mol. Microbiol. **2:**757–766.
6. **Girón, J., A. S. Y. Ho, and G. K. Schoolnik.** 1991. An inducible bundle-forming pilus of enteropathogenic *Escherichia coli*. Science **254:**710–713.
7. **Griffin, P., and R. Tauxe.** 1991. The epidemiology of infections caused by *Escherichia coli* O157:H7, other enterohemorrhagic *E. coli* and the associated hemolytic uremic syndrome. Epidemiol. Rev. **13:**60–98.
8. **Hitchcock, P. J., and T. M. Brown.** 1983. Morphological heterogeneity among *Salmonella* lipopolysaccharide chemotypes in silver-stained polyacrylamide gels. J. Bacteriol. **154:**269–277.
9. **Ismaili, A., D. J. Philpott, M. T. Dytoc, and P. M. Sherman.** 1995. Signal transduction responses following adhesion of verocytotoxin-producing *Escherichia coli*. Infect. Immun. **63:**3316–3326.
10. **Jerse, A. E., J. Yu, B. D. Tall, and J. B. Kaper.** 1990. A genetic locus of enteropathogenic *Escherichia coli* necessary for the production of attaching and effacing lesions on tissue culture cells. Proc. Natl. Acad. Sci. USA **87:**7839–7843.
11. **Junkins, A. D., and M. P. Doyle.** 1989. Comparison of adherence properties of *Escherichia coli* O157:H7 and a 60-megadalton plasmid-cured derivative. Curr. Microbiol. **19:**213a–227a.
12. **Karch, H., J. Heesemann, R. Laufe, A. D. O'Brien, C. O. Tacket, and M. M. Levine.** 1987. A plasmid of enterohemorrhagic *Escherichia coli* O157:H7 is required for expression of a new fimbrial antigen and for adhesion to epithelial cells. Infect. Immun. **55:**455–461.
13. **Karmali, M. A., M. Petric, C. Lim, P. C. Fleming, G. S. Arbus, and H. Lior.** 1985. The association between idiopathic hemolytic uremic syndrome and infection by verotoxin-producing *Escherichia coli*. J. Infect. Dis. **151:**775–782.
14. **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.
15. **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:**69–77.
16. **Kopecko D. J., O. Washington, and S. B. Formal.** 1980. Genetic and physical evidence for plasmid control of *Shigella sonnei* form I cell surface antigen. Infect. Immun. **29:**207–214.
17. **Louie, M., J. C. S. de Azavedo, M. Y. C. Handelsman, C. G. Clark, B. Ally, M. Dytoc, P. Sherman, and J. Brunton.** 1993. Expression and characterization of the *eaeA* gene product of *Escherichia coli* serotype O157:H7. Infect. Immun. **61:**4085–4092.
18. **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.
19. **Pierson, D. E.** 1994. Mutations affecting lipopolysaccharide enhance Ail-mediated entry of *Yersinia enterocolitica* into mammalian cells. J. Bacteriol. **176:**4043–4051.
20. **Rajakumar, K., B. H. Jost, C. Sasakawa, N. Okada, M. Yoshikawa, and B. Adler.** 1994. Nucleotide sequence of the rhamnose biosynthetic operon of *Shigella flexneri* and role of lipopolysaccharide in virulence. J. Bacteriol. **176:**2362–2373.
21. **Rosenshine, I., M. Donnenberg, J. Kaper, and B. B. Finlay.** 1992. Signal transduction between enteropathogenic *Escherichia coli* (EPEC) and epithelial cells: EPEC induces tyrosine phosphorylation of host cell proteins to initiate cytoskeletal rearrangement and bacterial uptake. EMBO J. **11:**3551–3560.
22. **Sandlin, R. C., K. A. Lampel, S. P. Keasler, M. B. Goldberg, A. L. Stolzer, and A. T. Maurelli.** 1995. Avirulence of rough mutants of *Shigella flexneri*: requirement of O antigen for correct unipolar localization of IcsA in the bacterial outer membrane. Infect. Immun. **63:**229–237.
23. **Shefler, W. C.** 1984. Statistics for health professionals. Addison-Wesley, Inc., Reading, Mass.
24. **Sherman, P., F. Cockerill III, R. Soni, and J. Brunton.** 1991. Outer membranes are competitive inhibitors of *Escherichia coli* O157:H7 adherence to epithelial cells. Infect. Immun. **59:**890–899.
25. **Sherman, P., and R. Soni.** 1988. Adherence of Vero cytotoxin-producing *Escherichia coli* of serotype O157:H7 to human epithelial cells in tissue culture: role of outer membranes as bacterial adhesins. J. Med. Microbiol. **26:**1367–1372.
26. **Sherman, P., R. Soni, and M. Karmali.** 1988. Attaching and effacing adherence of Vero cytotoxin-producing *Escherichia coli* to rabbit intestinal epi-

thelium in vivo. Infect. Immun. **56:**756–761.
27. **Sherman, P., R. Soni, M. Petric, and M. Karmali.** 1987. Surface properties of the Vero cytotoxin-producing *Escherichia coli* O157:H7. Infect. Immun. **55:**1824–1829.
28. **Sherman, P., R. Soni, and H. Yeger.** 1988. Characterization of flagella purified from enterohemorrhagic, Vero cytotoxin-producing *Escherichia coli* serotype O157:H7. J. Clin. Microbiol. **26:**1367–1372.
29. **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.
30. **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.
31. **Toth, I., M. L. Cohen, H. S. Rumschlag, L. W. Riley, E. H. White, J. H. Carr, W. W. Bond, and I. K. Wachsmuth.** 1990. Influence of the 60-megadalton plasmid on adherence of *Escherichia coli* O157:H7 and genetic derivatives. Infect. Immun. **58:**1223–1231.
32. **Tsai, C. M., and C. E. Frasch.** 1982. A sensitive silver stain for detecting lipopolysaccharides in polyacrylamide gels. Anal. Biochem. **119:**115–119.
33. **Tzipori, S., H. Karch, I. K. Wachsmuth, R. M. Robins-Browne, A. D. O'Brien, H. Lior, M. L. Cohen, J. Smithers, and M. M. Levine.** 1987. The role of a 60-megadalton plasmid and Shiga-like toxins in the pathogenesis of infection caused by enterohemorrhagic *Escherichia coli* O157:H7 in gnotobiotic piglets. Infect. Immun. **55:**3117–3125.
34. **Wadolkowski, E. A., J. A. Barris, and A. D. O'Brien.** 1990. Mouse model for colonization and disease caused by enterohemorrhagic *Escherichia coli* O157:H7. Infect. Immun. **68:**2438–2445.

Editor: P. E. Orndorff