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Enterohemorrhagic *Escherichia coli* (EHEC) O157:H7 causes hemorrhagic colitis and hemolytic uremic syndrome (HUS) by colonizing the gut mucosa and producing Shiga toxins (Stx). The only factor clearly demonstrated to play a role in EHEC adherence to intestinal epithelial cells is intimin, which binds host cell integrins and nucleolin, as well as a receptor (Tir) that it injects into the host cell. Here we report that EHEC O157:H7 produces adhesive type IV pili, which we term *hemorrhagic coli pilus* (HCP), composed of a 19-kDa pilin subunit (HcpA) that is encoded by the *hcpA* chromosomal gene. HCP were observed as bundles of fibers greater than 10 µm in length that formed physical bridges between bacteria adhering to human and bovine host cells. Sera of HUS patients, but not healthy individuals, recognized HcpA, suggesting that the pili are produced in vivo during EHEC infections. Inactivation of the *hcpA* gene in EHEC EDL933 resulted in significantly reduced adherence to cultured human intestinal and bovine renal epithelial cells and to porcine and bovine gut explants. An *escN* mutant, which is unable to translocate Tir, adhered less than the *hcpA* mutant, suggesting that adherence mediated by […]

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Intestinal adherence associated with type IV pili of enterohemorrhagic

*Escherichia coli* O157:H7

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**Enterohemorrhagic *Escherichia coli* (EHEC) O157:H7 causes hemorrhagic colitis and hemolytic uremic syndrome (HUS) by colonizing the gut mucosa and producing Shiga toxins (Stx). The only factor clearly demonstrated to play a role in EHEC adherence to intestinal epithelial cells is intimin, which binds host cell integrins and nucleolin, as well as a receptor (Tir) that it injects into the host cell. Here we report that EHEC O157:H7 produces adhesive type IV pili, which we term *hemorrhagic coli pilus* (HCP), composed of a 19-kDa pilin subunit (HcpA) that is encoded by the *hcpA* chromosomal gene. HCP were observed as bundles of fibers greater than 10 μm in length that formed physical bridges between bacteria adhering to human and bovine host cells. Sera of HUS patients, but not healthy individuals, recognized HcpA, suggesting that the pili are produced in vivo during EHEC infections. Inactivation of the *hcpA* gene in EHEC EDL933 resulted in significantly reduced adherence to cultured human intestinal and bovine renal epithelial cells and to porcine and bovine gut explants. An *escN* mutant, which is unable to translocate Tir, adhered less than the *hcpA* mutant, suggesting that adherence mediated by intimin-Tir interactions is a prelude to HCP-mediated adherence. An *hcpA* and *stx1,2* double mutant and an *hcpA* mutant had similar levels of adherence to bovine and human epithelial cells while a *stx1,2* double mutant had only a minor defect in adherence, indicating that HCP-mediated adherence and cytotoxicity are independent events. Our data establish that EHEC O157:H7 HCP are intestinal colonization factors that are likely to contribute to the pathogenic potential of this food-borne pathogen.**

**Introduction** Since its first description in the early 1980s (1), enterohemorrhagic *Escherichia coli* (EHEC) of O157:H7 and other serotypes have emerged as a significant cause of serious human gastrointestinal disease worldwide (2–4). EHEC infections can result in diarrhea ranging from mild to bloody and can induce hemorrhagic colitis (2, 5), and some patients with hemorrhagic colitis develop a severe complication known as the hemolytic uremic syndrome (HUS). HUS is defined as a triad of clinical features that includes acute renal failure, thrombocytopenia, and microangiopathic hemolytic anemia, which commonly lead to death (6). This deadly infectious disease affects humans of all ages, but the young and old are the most susceptible to developing HUS (7). Adult cattle, other farm animals, and wild animals are common reservoirs of many EHEC serotypes (8, 9). Human infection occurs through acquisition of the bacteria via consumption of contaminated food (ground meat or vegetables), water, unpasteurized fruit juices, and milk (10, 11).

**Nonstandard abbreviations used:** AE, attaching and effacing; ECP, *Escherichia coli* common pilus (pili); EHEC, enterohemorrhagic *Escherichia coli*; HCP, hemorrhagic coli pilus (pili); HUS, hemolytic uremic syndrome; IFM, immunofluorescence microscopy; LB, Luria-Bertani; LEE, locus for enterocyte effacement; MDRK, Madin-Darby bovine kidney; SEM, scanning electron microscopy; STEC, Shiga-toxigenic *E. coli*; Stx, Shiga toxin(s); TEM, transmission electron microscopy; TTP, type IV pili (pili).

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host epithelial cells, suggesting that the bacteria produce other yet-unidentified adhesins. Other, less well–characterized surface proteins have been proposed to be associated with adherence properties (13, 15, 16, 25). However, some of these putative adhesins are found only in certain STEC strains, and more studies are required to clarify their role in cell adherence.

The genome of *E. coli* O157:H7 contains 16 loci encoding genes putatively involved in fimbriae or pilus biosynthesis (26–28), but it is unknown how many of them are functional in vivo. Several fimbriae have been identified in EHEC strains, including the sorbitol-fermenting EHEC O157 fimbiae plasmid-encoded (SFP) (29), 2 loci-encoding long polar fimbriae (30), curli (31), F9 (a type I pilus homolog) (32), and a type IV pilus (TFP) in LEE-negative non-O157:H7 STEC (33). The role of most of these pili in host colonization is unknown. The long polar fimbiae of EHEC O157:H7 appear to play a role in pig and sheep colonization (34). Intriguingly, EHEC mutants in the F9 pilus showed more adherence to epithelial cells than the wild-type strain (32). Recently, we reported that EHEC strains produce an adhesion appendage called *E. coli* common pilus or ECP, which is also produced by other pathogenic and nonpathogenic *E. coli* (35). EHEC and normal flora *E. coli* unable to produce ECP were hampered in their ability to adhere to cultured epithelial cells. Nevertheless, it remains to be elucidated whether pili play a biologically significant role in EHEC colonization of the intestine of their natural bovine or accidental human hosts.

TFP are important virulence factors in many pathogenic Gram-negative bacteria, as they mediate adherence to eukaryotic cells and host colonization and are associated with other phenomena related to pathogenicity, including bacterial aggregation, biofilm formation, DNA binding and uptake, and twitching motility (36–41). So far, TFP have not been observed in AE-producing EHEC O157:H7. In this study, we demonstrate the expression and assembly of TFP in EHEC O157:H7 and provide data showing that these pili are involved in adherence to cultured human colonic epithelial cells and to porcine and bovine intestinal explants and that the pili induce an antibody immune response in HUS patients. These observations contribute to our knowledge of the mechanisms underlying the interaction of EHEC with host cells.

### Results

**Visualization of pilus structures produced by EHEC O157:H7.** For the last 20 years, attempts to reproducibly express and characterize pili produced by EHEC strains have been unsuccessful. Analysis of the EHEC EDL933’s genome sequence reveals 16 loci with potential to encode pili proteins; however, it is still an enigma what role, if any, pili play in host colonization. We were interested in determining whether, in addition to ECP, EHEC O157:H7 could express and produce any additional pili structures when interacting with host cells.

Ultrastructural studies by negative staining and transmission electron microscopy (TEM) were performed to investigate the presence of pili on 3 prototypic, well-characterized EHEC O157:H7 strains (EDL933, 86-24, and 85-170) cultivated at 37°C on bacteriological media such as Luria-Bertani (LB), Mueller-Hinton, CFA agar, or blood agar. No pili were observed on these strains under these growth conditions (Figure 1A and data not shown). These observations indicate that the expression of pili depends on the composition of the growth medium and is under a strict regulatory and environmental control. Since EHEC is a commensal of cattle, we decided to use minimal casein (Minca) medium, as this medium was used in the past to successfully induce expression of fimbriae in bovine enterotoxigenic *E. coli* (ETEC) (42). After growth at 37°C on Minca agar, we were able to see long (>20 μm), thin, rod-like pili (Figure 1, B and C) that associated laterally into bundles,
morphologically reminiscent of the ETEC Longus TFP (43). Similar to the production of pili in other E. coli pathogroups (43, 44), the degree of production of the novel EHEC pili varied among the 3 EHEC strains tested.

Biochemical characterization of novel pili. To determine the nature of these novel structures, EHEC EDL933 growing on Minca agar at 37°C with 5% CO2 atmosphere was harvested and the pili purified by mechanical shearing and differential centrifugation (45). The purified pili (Figure 2A) were dissociated into pilin monomers of 19 kDa in 16% SDS-PAGE gels (Figure 2B). This protein was subjected to mass spectrometry analysis after trypsin digestion. The peptides obtained showed amino acid sequences that corresponded to the predicted protein PpdD, which is encoded by the pilin gene, hereinafter called hcpA (pilin gene), hofB ( usher gene, herein called hcpB), and hofC (chaperone gene, herein called hcpC). These genes are located immediately downstream from hcpA and share similarity to accessory genes pilB and pilC, respectively, involved in the biogenesis of TFP in P. aeruginosa (Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI30727DS1).

Reactivity of HCP with sera from HUS patients. The presence of antibodies in convalescent patients is a biological marker of the production of an antigen in vivo. We sought to determine whether patients who suffered HUS developed antibodies against HCP. A pool of 5 sera from patients with HUS was reacted with purified HCP by Western blotting (Figure 2B). A pool of normal human sera was used as negative control. The 19-kDa pilin subunit was recognized by the HUS sera but not by normal human sera, strongly suggesting that HCP might be produced in vivo during EHEC infection.

Demonstration of HCP on EHEC adhering to host cells. We then investigated the presence of pilike structures on EHEC bacteria adhering onto cultured HT-29 colonic epithelial cells by high-resolution scanning electron microscopy (SEM). We saw that EDL933 attaching to these human intestinal epithelial cells produced wick-like structures (approximately 20-nm wide) protruding from the bacterial surface (Figure 3A). Similar structures were also seen in EHEC adhering to human colonic cell lines (T84 and Caco2), nonintestinal cell lines (HeLa and HEp-2), and bovine kidney (Madin-Darby bovine kidney [MDBK]) epithelial cells (data not shown). These structures appeared to tether bacteria to each other within the bacterial cluster and to mediate direct binding of the bacteria to the mammalian cell membrane (Figure 3B). We hypothesized that these structures could be HCP and so the identity of these structures was investigated by immunofluorescence microscopy (IFM). EDL933-infected HT-29 cells incubated with anti-HCP antibodies revealed the presence of a specific fluorescence profile consistent with the presence of pili (Figure 4A). Similar fluorescent structures were also seen on EHEC incubated with other cell lines (data not shown).

Disruption of hcpA reduces HCP adherence to epithelial cells. To provide genetic evidence regarding the possible role of HCP in EHEC adherence to epithelial cells, we produced an in-frame deletion in the hcpA gene of EHEC O157:H7 strains EDL933 and 85-170 (Stx1,2–), generating mutants EDL933ΔhcpA and 85-170ΔhcpA (triple Δstx1,2ΔhcpA mutant), respectively. Flow cytometry, IFM, and Western blotting (of whole bacterial cell extracts) analyses demonstrated the lack of HCP production in these mutants (Figure 4 and Supplemental Figure 2A). No fluorescent structures were seen on the isogenic hcpA mutant with or without pBAD-TOPO vector in the presence of epithelial cells (Figure 4, B and E). Infection of HT-29 cells with EDL933ΔhcpA complemented with plasmids pJX12 (carrying hcpA) or pJX22 (carrying hcpABC) caused the appearance of an abundant fluorescent meshwork of large bundles of long filaments protruding from the bacteria and extending throughout the obser-
vation fields (Figure 4, C and D). These data indicated that overexpression of hcpA alone or together with hcpB and hcpC was sufficient for assembly of HCP but also that the addition of HcpB and HcpC assembly factors in the hcpA mutant was sufficient to increase the amount of HCP produced. The long bundles of pili produced by EDL933ΔhcpA complemented with pJX12 or pJX22 were decorated with anti-HCP antibodies and secondary antibodies conjugated to 30-nm gold particles, as determined by immuno-SEM (Figure 4F). No gold particles were observed in the control with preimmune serum (data not shown). The specificity of the anti-HCP antiserum was further demonstrated with whole-cell extracts of EDL933 (Figure 4I), in which only the reactive HcpA protein was detected.

To judge the significance of the loss of HCP, EDL933ΔhcpA was used to measure adherence to human colonic cells (T84, HT-29, and Caco2), nonintestinal (Hep-2 and HeLa) cell lines, and bovine (MDBK) cultured epithelial cells. The 85-170ΔhcpA strain was tested only for adherence to MDBK and HT-29 cells. For these infection experiments, we propagated all the strains overnight in Minca medium at 37°C in order to activate them for the production of HCP. The light microscopy images of infected, Giemsa-stained cells show obvious impairment of adherence of the hcpA mutant as compared with the wild-type and the complemented strains (Figure 5).

The level of impairment in adherence of EDL933ΔhcpA was determined by counting bacteria adhering to several human colonic cell lines after extensive washing and cell lysis. Depending on the cell line employed, mutation of the hcpA gene resulted in a 3- to 5-fold decrease in adherence relative to EDL933 (P < 0.0001) (Figure 6). Complementation of the hcpA mutant with pJX22 increased adherence (P < 0.0001) to a level slightly higher than that seen in the parental strain. The increase in adherence might correlate with the abundant HCP produced by this strain (Figure 4D). The hcpA mutant grew normally in LB broth, ruling out a growth defect as the cause of the reduced adherence. Adherence of EHEC was greatly, although not completely, reduced by the hcpA deletion, indicating that other colonization factors (e.g., intimin-Tir
and ECP) contribute to EHEC adherence to different cell lines. We also observed reduction of adherence by EDL933ΔhpcA to other human nonintestinal (HeLa and HEp-2) and bovine (MBDK) cell lines tested (data not shown). Taken together, the data strongly indicate that HCP mediates adherence to various epithelial cell lines, perhaps by recognition of a common surface receptor.

**Adherence to pig and cow intestinal explants.** Intestinal explants obtained from a 3-week-old healthy pig (ileum and colon) and from cow colon were used in quantitative experiments to compare the ability of HCP-producing strains and the hpcA mutant to adhere. In agreement with the results obtained with the tissue culture adherence assays, the hpcA mutant was also impaired in adherence to the animal intestinal tissue in comparison with the HCP-producing strains (Figure 7). Taken together, the adherence data strongly suggest that HCP are intestinal colonization factors with affinity for human, bovine, and porcine epithelial cells. We sought to determine whether EHEC would produce HCP while adhering to animal intestinal explants. Thin sections of infected pig tissues were reacted with anti-HCP antibodies by IFM, and we found that HCP are produced by EDL933 during interaction with pig intestinal tissue and not by the hpcA mutant (Figure 5, B and C, and Figure 6). Last, the mutation of stx1,2 genes in EDL933 had a negative effect in adherence but not as significant as the mutation in escN, hpcA, or hpcAstrx1,2 (Figures 5 and 6). EHEC strain 85-170, which lacks Stx production, remains capable of adhering to host cells, further indicating that cytotoxicity is not required for HCP-mediated adherence (Supplemental Figure 3), suggesting that the hpcA mutant remains capable of recruiting actin and inducing the formation of AE lesions. This notion was confirmed by the fluorescent actin-staining assay, which allowed detection of actin condensation in host cells beneath adhering bacteria (data not shown). Second, we found that EDL933 mutated in escN (a T3SS-associated ATPase gene) was significantly reduced in adherence to colonic cell lines (P < 0.0001), even more than the hpcA mutant (Figure 5, B and C, and Figure 6).

**Relationship between the intimin-Tir interaction, HCP-mediated adherence, and cytotoxicity.** EHEC causes AE lesions via the intimin-Tir interaction, which is strictly dependent on the function of the T3SS. In some pathogenic bacteria such as *P. aeruginosa*, TFP are not specifically required for contact-dependent T3SS-translocation of exoenzymes (50). In enteropathogenic *E. coli* (EPEC), initial attachment to host cells is thought to be mediated by TFP, and T3SS is required in the formation of AE lesions (39, 51, 52). Here, we explored the contribution of T3SS (intimin-Tir interaction), HCP, and cytotoxicity on adherence to host cells. We set out to investigate whether HCP-mediated adherence was a prerequisite for intimate attachment to host cells or whether T3SS-dependent adherence led to activation of HCP production. To test this hypothesis, we employed HCP and T3SS mutants in comparative and quantitative adherence assays. In addition, we included double stx1,2 and triple hpcAstrx1,2 mutants to determine how Stx might influence the outcome of the interaction of EHEC with host cells. First, we tested the EDL933 hpcA mutant for production of T3-secreted proteins EspA, B, and D and found that this strain was able to produce EspA at the same level as the wild-type strain (Supplemental Figure 4). Next, we tested the EDL933 ΔhpcA; hpcAstrx1,2; stx1,2 mutant and its complemented strain for 6 hours as described in Methods.

![Figure 5](http://www.jci.org)  
**Figure 5**  
Qualitative comparison of adherence of wild-type EDL933 and derivative mutants to HT-29 cells. (A) EDL933; (B) EDL933ΔescN (T3SS-ATPase mutant); (C) EDL933ΔhpcA; (D) EDL933ΔhpcA Δstx1,2; (E) EDL933ΔhpcA Δstx1,2; and (F) EDL933ΔhpcA(pJX22). HT-29 cell monolayers were incubated with these strains for 6 hours as described in Methods.

![Figure 6](http://www.jci.org)  
**Figure 6**  
Quantification of bacterial adherence to human intestinal cells. The indicated strains were incubated with human colonic Caco-2, T84, and HT-29 cells for 6 hours and the adherent bacteria expressed as CFUs after plating serial dilutions. The data are representative of at least 3 experiments performed in triplicate. Note the significant reduction in adherence in the EDL933ΔhpcA and EDL933ΔescN strains compared with the wild type to all the cell lines tested.
Adherence of EHEC strains to pig and cow intestinal explants. Pig (ileum and colon) and cow (colon) gut tissues were cut into squares of 8 × 8 mm and 0.8 g of weight, washed, and incubated for 6 hours in DMEM with cultures of EDL933, EDL933ΔhcpA, EDL933ΔhcpA(pJX22), and EDL933ΔhcpA(pBAD-TOPO) obtained in Minca broth. Shown is the average of 3 experiments performed in triplicate. Note the reduction in adherence to animal intestinal sections of the hcpA mutant.

Discipline
The epidemiological renaissance and clinical significance of EHEC food-borne infections has stimulated increased interest in the understanding of the pathogenic mechanisms of this deadly organism (7). While it is well established that EHEC colonizes the human colonic mucosa and the terminal rectum of bovines (20), it is not clear what role pili play in host colonization. TFP have not been previously observed in EHEC O157:H7. In this context, HCP might play an accessory role in the colonization of the bovine host in association with other LEE- or non-LEE–encoded adhesins. Gnotobiotic pigs have been used as a model to study events of EHEC pathogenesis. In fact, the role of intimin as an adhesin in vivo was demonstrated in a porcine model (21). We employed porcine and bovine intestinal explants to study the role of HCP as a colonization factor in vitro. As predicted, the hcpA mutant was impaired in adherence to animal tissue as compared with HCP-producing strains. Interestingly, we showed that HCP are produced by EDL933 infecting pig intestinal tissue, as thin sections of infected pig explants reacted with anti-HCP antibody by IFM. Considerable tissue damage was observed by the wild type, the Stx mutant, and the EDL933ΔhcpA(pJX22) while the explants incubated with...
Colonization factor. The results of the ultrastructural and IFM intimin-Tir interaction and HCP-mediated adherence were strongly suggest that HCP functions as an important accessory adherence is independent of cytotoxicity and that Stx-mediated adherence are central to EHEC interaction with host cells and these data are compelling evidence that support our hypothesis that HCP are intestinal colonization factors.

The intimin-Tir interaction, which is dependent on the presence and function of the T3SS, is crucial for the development of intimate attachment and effacing of the enterocyte membrane, although not enough to mediate attachment in the absence of HCP. In addition to their central role in cytotoxicity and the development of HUS, Stx have been reported to induce the expression of surface-exposed nucleolin on mammalian cells (24), which acts as an alternative receptor for intimin. We explored whether T3SS-dependent or Stx-induced adherence was a requirement or a consequence of the adherence mediated by HCP to cultured epithelial cells. To this aim, we analyzed in comparative and quantitative assays the ability of escN, stx1,2, hcpA, and hcpA/stx1,2 mutants to adhere to various human intestinal colonic cell lines. Under our experimental conditions, EHEC strain 85-170, which does not produce Stx, adhered to colonic cells as efficiently as Shiga-toxigenic EDL933. When the stx1,2, and hcpA genes were interrupted in EDL933 and 85-170 and mutated in hcpA, we found that both mutants showed levels of adherence similar to those of the single EDL933 hcpA mutant. These results suggest that HCP-mediated adherence is independent of cytotoxicity and that Stx-mediated adherence does not contribute to the function of HCP. When the intimin-Tir interaction and HCP-mediated adherence were compared, we observed that the level of adherence of the escN mutant (unable to translocate Tir) and the hcpA mutant were 6- to 27-fold and 3- to 5-fold (depending on the cell line employed), respectively, in comparison with the wild-type strain. These results further support that the functional T3SS and the intimin-Tir-mediated adherence are central to EHEC interaction with host cells and strongly suggest that HCP functions as an important accessory colonization factor. The results of the ultrastructural and IFM studies obtained here strongly suggest that HCP contribute to consolidate the formation of infectious units by bridging bacteria. However, we cannot exclude the possibility that HCP also mediate direct contact of the bacteria to a particular receptor, which is apparently widely distributed among intestinal and nonintestinal cells. For P. aeruginosa, the contact-dependent translocation of exoenzymes and cytotoxins via T3SS occurs independently of TFP (50). Whether HCP acts at early or late stages of the colonization process requires further investigation.

Our investigation of the in vitro conditions for pilus production in wild-type EHEC O157:H7 strains, using different growth environments and culture media, revealed that EHEC produces HCP after growth in Minca medium (42) and in contact with host cells. The data would suggest that, like TFP production in other pathogens (53, 54), the production of HCP in EHEC O157:H7 is probably tightly regulated. It is possible that growth of the bacteria in Minca relieves the negative regulation exerted in other growth conditions by an unknown repressor or that signals present in Minca induce the expression of a specific activator. We speculate that the environmental and nutritional signals that trigger HCP production must resemble those found in vivo, perhaps during colonization of the large intestine of animals and humans, and efforts are underway to identify these precise signals.

Earlier studies showed that laboratory or pathogenic E. coli strains were unable to produce HcpA-containing (PpD-containing) pili, even when expression levels of both hcpA/ pPd and assembly genes (hcpB/hofB and hcpC/hofC) were increased. Production of PpD pili was only observed in a heterologous host such as P. aeruginosa lacking endogenous TFP (49) or in E. coli producing the pullulanase type II secretor of Klebsiella oxytoca (55). The fact that HCP are produced in EHEC O157:H7 strains suggests the presence of a TFP biogenesis machinery and regulatory genes that are absent or are defective in E. coli K-12 (45, 49). Assembly of TFP requires, depending on the organism, from 16 to 40 biogenesis genes (37). Genetic complementation studies involving the transfer of hcpABC genes on a plasmid expressed from an inducible promoter to the EHEC HCP mutant yielded a strain that was able to hyperproduce HCP. This would suggest that the induction of

Figure 9
Pig ileum infected with EHEC strains, sliced, and stained with H&E. (A) EDL933; (B) EDL933ΔhcpA; (C) EDL933ΔhcppA(pJX22); (D) EDL933ΔescN; (E) EDL933Δstx1,2; (F) EDL933ΔhcppAΔstx1,2; and (G) mock infected. See text for description.

Figure 10
Inhibition of EHEC adherence by anti-HCP antibodies. EDL933 was preincubated with 1:10, 1:50, and 1:100 dilutions of anti-HCP antibody for 30 minutes before our standard 6-hour adherence assay. Adherence (number of bacteria per cell) was recorded visually under a light microscope. The experiments were repeated at least 3 times in triplicate. Note the dose-response inhibition seen with high concentration of antibodies.
only these 3 genes are sufficient for HCP production. We cannot rule out that other genes present in the chromosome of EHEC are important for HCP biogenesis. The hcpABC genes are remarkably well conserved in E. coli, Shigella, and several other enterobacterial species, suggesting that under certain environmental conditions, HCP might be produced by these organisms and play an important role in adaptation and survival.

The elucidation of the detailed mechanisms of colonization of human and bovine hosts will be important to further our understanding of EHEC pathogenesis and in the development of strategies to prevent EHEC infections. In this context, HCP should be considered an accessory colonization factor that together with intimin-Tir and ECP contributes to the colonization of the gastrointestinal tract of humans and bovines.

Methods

**Bacterial strains and culture conditions.** Bacterial strains and plasmids used in this study are listed in Table 1. Bacteria were routinely cultured in LB broth at 37°C with shaking, unless otherwise stated. When required, antibiotics kanamycin (100 μg/ml) and ampicillin (100 μg/ml) were added to the media. Minca minimal medium (1.36 g KH₂PO₄, 10.1 g Na₂HPO₄, 2H₂O, 1 g glucose, 1 g casaminoacids, 10 g MgSO₄·7H₂O, 1 g MnCl₂·4H₂O, 0.135 g FeCl₃·6H₂O, and 0.4 g CaCl₂·2H₂O per liter adjusted to pH 7.5) (42) was used for production of HCP and for preparing bacterial cultures for comparative adherence assays. Arabinose was used at 100 mM in LB broth to induce λ red recombinase from plasmid pKD46 (56). For induction of the pBAD-TOPO inducible promoter, 0.8% arabinose was added to the medium.

**DNA manipulations.** Agarose gel electrophoresis, transformation, PCR, restriction enzyme digestions, ligation, and other routine DNA procedures were carried out as previously described (57).

**Construction of EHEC pili mutants.** The λ red recombinase system was used to construct hcpA nonpolar deletion mutants of EHEC O157:H7 strains EDL933 and 85-170 (56). G68 and G69 primers, homologous to sequences within the 5′ and 3′ ends of the hcpA gene, were used to replace this gene with a kanamycin-resistance cassette derived from the template plasmid pKD4 (Table 1) (56). Primers G336 and G337 were used for mutagenesis of stx1,2. Primers flanking the hcpA (G98 and G99) or to stx1,2 (G338 y G339) genes as well as primers inside the kanamycin resistance gene (K1 and K2) were used to confirm the required gene replacement by PCR. The resulting hcpA mutants in EDL933 and 85-170 were named EDL933ΔhcpA and 85-170ΔhcpA, respectively. To complement hcpA mutants, hcpA alone or together with the hcpBC genes was amplified from EDL933 using the primers listed in Table 2 and cloned into the Ndel site and thymidine overhang of pBAD-TOPO. The resulting plasmids (pJX12 and pJX22) were obtained in HB101 and then electroporated into the hcpA mutants to yield EDL933ΔhcpA(pJX12) and EDL933ΔhcpA(pJX22).

Expression of genes under araBAD promoter control was induced with 0.8% arabinose.

**Antisera.** Rabbit antibody against an E. coli K-12 PppD-GST fusion was used after 4x adsorption with the EDL933 hcpA mutant to remove non-specific antibodies. This antibody is referred to as anti-HCP in the text. Antisera against EspA, EspB, and EspD were available from previous studies

**Electron microscopy and immunogold labeling.** For TEM, a drop (10 μl) of bacterial culture or pili sample was placed on a Formvar carbon–coated grid for 3 minutes, negatively stained with 1% sodium phosphotungstic acid (pH 7.2) for 1 minute, and examined under a Philips transmission electron microscope. For immunogold EM, bacteria were mixed with anti-HCP sera diluted 1:10 in PBS for 1 hour and washed with PBS-BSA. Goat anti-rabbit immunoglobulin labeled with 10-nm gold particles (BB International) diluted 1:10 in PBS-BSA was used to detect antibodies bound to pili and then negatively stained as above (58). For SEM, infected cells were fixed with 2% formalin in PBS, postfixed with 1% osmium tetroxide, dehydrated by sequential ethanol concentrations, dried to critical point, and coated with a mixture of gold and palladium. Specimens were examined in a high-resolution Leo scanning electron microscope.

**Pilus purification.** HCP produced by EDL933 were detached from the bacteria cultivated in 40 plates (150 x 15 mm) of Minca agar supplemented with 0.8% arabinose by vigorous shaking. Pilus purification was monitored by TEM and SDS-PAGE, as previously described (39). In brief, bacteria were separated by centrifugation at 10,000 g for 30 minutes, and the supernatant was centrifuged at 18,000 g for 30 minutes to remove flagella, outer membranes, and bacterial debris. The pili-containing supernatant was centrifuged at 78,000 g for 2 hours and the pellet dissolved in distilled water and then loaded onto a cesium chloride/1% Saran gradient (density 1.2 g/ml) to obtain relatively pure pili (58). The gradient was centrifuged at 50,000 g for 24 hours at 18°C, after which an opaque thick band was seen in the middle of the gradient. The material in this band was recovered by lateral puncture, dialyzed against distilled water, and observed by TEM.

**SDS-PAGE and Western blotting analyses.** Pili preparations from the purification steps and normalized whole-cell lysates were separated by SDS-PAGE on 16% acrylamide gels (60). Western blotting was carried out using

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**Table 1.** *E. coli* strains and plasmids used

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<thead>
<tr>
<th>Strains or plasmids</th>
<th>Notes</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>EDL933 (O157:H7)</td>
<td>Wild type</td>
<td>(1)</td>
</tr>
<tr>
<td>EDL933ΔhcpA</td>
<td>EDL933ΔhcpAkm</td>
<td>This study</td>
</tr>
<tr>
<td>EDL933Δstx1,2</td>
<td>EDL933Δstx1,2, cm</td>
<td>This study</td>
</tr>
<tr>
<td>EDL933ΔescN</td>
<td>EDL933ΔescN Km</td>
<td>This study</td>
</tr>
<tr>
<td>EDL933ΔhcpA(pJX12)</td>
<td>hcpA mutant complemented with pJX12</td>
<td>This study</td>
</tr>
<tr>
<td>EDL933ΔhcpA(pJX22)</td>
<td>hcpA mutant complemented with pJX22</td>
<td>This study</td>
</tr>
<tr>
<td>EDL933ΔhcpABC(pBAD-TOPO)</td>
<td>hcpABC mutant transformed with pBAD-TOPO</td>
<td>This study</td>
</tr>
<tr>
<td>85-170 (O157:H7)</td>
<td>Wild type, Stx²</td>
<td>(63)</td>
</tr>
<tr>
<td>85-170ΔhcpA</td>
<td>85-170ΔhcpAkm</td>
<td>This study</td>
</tr>
<tr>
<td>HB101</td>
<td>E. coli B/K-12 hybrid</td>
<td>(64)</td>
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<tr>
<td>HB101(pBAD-TOPO)</td>
<td>HB101 transformed with pBAD-TOPO</td>
<td>This study</td>
</tr>
<tr>
<td>HB101(pJX22)</td>
<td>HB101 complemented with pJX22</td>
<td>This study</td>
</tr>
<tr>
<td>HB101(pJX12)</td>
<td>HB101 complemented with pJX12</td>
<td>This study</td>
</tr>
<tr>
<td>pBAD-TOPO</td>
<td>Cloning expression vector, Ap′</td>
<td>This study</td>
</tr>
<tr>
<td>pJX12</td>
<td>EHEC hcpA cloned in pBAD-TOPO</td>
<td>This study</td>
</tr>
<tr>
<td>pJX22</td>
<td>EHEC hcpABC cloned in pBAD-TOPO</td>
<td>This study</td>
</tr>
<tr>
<td>pKD46</td>
<td>Plasmid containing the red recombinase</td>
<td>(56)</td>
</tr>
<tr>
<td>pKD4</td>
<td>Km cassette</td>
<td>(56)</td>
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</table>
anti-HCP antibody at a dilution of 1:2,000 and a horseradish peroxidase conjugate HRP antibody detection reagent (Denville Scientific Inc.).

**Mass spectrometry.** A protein band of 19 kDa was excised from polyacrylamide gels and subjected to mass spectrometry analysis after digestion with trypsin at the Proteomics Core Facility at the College of Pharmacy, University of Arizona.

**Flow cytometry.** Flow cytometry was used to detect the expression of HCP produced by EHEC O157:H7 strains grown overnight at 37°C in Minca media. The cultures were adjusted to an optical density (OD_{600}) of 1.1, and 45-μl aliquots were incubated for 1 hour on ice with 25 μl of anti-HCP using 10-fold dilutions of the bacterial suspension (wild type versus mutants) to test their ability to block adherence by 3 EHEC O157:H7 strains, namely EDL933, 86-24, and 85-170. Before the 6-hour HT-29 cell adherence assay to induce activation of HCP. We employed EDL933, EDL933(pJX22), and EDL933ΔhcpA(pJX22), and EDL933ΔhcpA(pBAD-TOPO) strains. After infection, unbound bacteria were removed by washing with HEPES-Hanks buffer and the attached bacteria were detached by vortexing for 10 minutes with glass beads and then serially diluted and plated on MacConkey sorbitol agar to obtain CFUs. The results shown are the mean of 3 experiments performed in triplicate. The significance level was 5% in all tests. The SPSS statistical package was used.

**Table 2**

<table>
<thead>
<tr>
<th>Name</th>
<th>Gene</th>
<th>Sequence (5’ to 3’)</th>
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</thead>
<tbody>
<tr>
<td>G68</td>
<td>hcpA-P1</td>
<td>AAATCAAGGAGCGAAACAGATGGACGAAGAAGCGGTGTATCTAGTAGCTGGCTTTTGGCTTTCG</td>
</tr>
<tr>
<td>G69</td>
<td>hcpA-P2</td>
<td>AAATTCATGTGCGCCCTTTAGTGTTGGCTCATCAAGCGGAACTATGGAATATCCTCCTTAG</td>
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<tr>
<td>G80</td>
<td>hcpAFnocoll</td>
<td>ACATGTAACAGAAGCGGTGTT</td>
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14. Kenny, B., and Finlay, B.B. 1997. Intimin-dependent binding of enteropathogenic Escherichia coli to host cells triggers novel signaling events, including tyrosine phosphorylation of phospholipase C-
35. Sundin, C., and Hultberg, S., and Fritze-Lindsten, E. 2002. Type IV pilin are not

Characterization of a novel type IV pilus locus encoded on the large plasmid of locus of enterocyte effacement-negative Shiga-toxigenic Escherichia coli strains that are virulent for humans. Infect. Immun. 70:3094–3100.

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