NOTES

Contribution of FliC to Epithelial Cell Invasion by Enterohemorrhagic
Escherichia coli O113:H21

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Enterohemorrhagic Escherichia coli (EHEC) O113:H21 can invade epithelial cells. In this study, we found that invasion but not adherence was inhibited by anti-FliC<sub>H21</sub> specific antibodies. In addition, deletion of fliC<sub>H21</sub> from EHEC O113:H21 resulted in an eightfold decrease in invasion that was restored upon transcomplementation with fliC<sub>H21</sub> but not with fliC<sub>H6</sub>. These results suggested that FliC plays an important role in the pathogenesis of infections caused by EHEC O113:H21 by allowing bacteria to penetrate the intestinal epithelium.

Shiga toxin-producing Escherichia coli (STEC) strains associated with hemorrhagic colitis or the hemolytic-uremic syndrome (HUS) in humans are commonly referred to as enterohemorrhagic E. coli (EHEC) (32). Most EHEC strains carry the locus for enterocyte effacement (LEE), which is essential for intestinal colonization (12, 14, 19, 27, 34). Nevertheless, strains of EHEC that do not carry LEE are regularly isolated from patients with severe disease, and these isolates have been termed eae- or LEE-negative EHEC or STEC (2, 4, 11, 13, 17, 20, 24, 25). We have shown previously that clinical isolates of LEE-negative EHEC have the ability to invade Chinese Hamster Ovary (CHO-K1) cells and the human colonic cell lines HCT-8 and Caco-2 (21). We speculate that this atypical subgroup of EHEC may use a mechanism of host cell invasion to colonize the intestinal mucosa.

Recently, Rogers et al. used the streptomycin-treated mouse model to show that LEE-negative EHEC O113:H21 strain 98NK2 was closely associated with the colonic mucosa during infection (30). In addition, bacteria were observed to penetrate the gut epithelium, and this close interaction was dependent on FliC. Although there was no difference in the fecal shedding of wild-type 98NK2 and a 98NK2 fliC<sup>−</sup> mutant, FliC was required for full lethality in mice mediated by Shiga toxin, suggesting that a close interaction of 98NK2 with the intestinal mucosa was important for virulence. In the present study, we have extended these findings to examine the contribution of FliC to invasion of HCT-8 epithelial cells by EHEC O113:H21.

FliC<sub>H21</sub>-mediated invasion of HCT-8 cells. Previous work has shown that FliC is more highly expressed at temperatures below 37°C in the invasive pathogens Yersinia enterocolitica and Listeria monocytogenes (3, 9, 18). We therefore examined FliC<sub>H21</sub> expression by wild-type STEC O113:H21 strain EH41 at two different growth temperatures. EH41 is a clinical isolate that has been described previously (10). Bacteria were grown in Luria-Bertani (LB) broth to the same optical density (i.e., an optical density at 600 nm of 0.8 [mid-log phase]) at 25 and 37°C, and whole-cell lysates were analyzed by immunoblotting with anti-H21 antibodies (Statens Serum Institut, Copenhagen, Denmark). The results showed that FliC<sub>H21</sub> production by EH41 was more abundant at 25°C than at 37°C (Fig. 1A). To determine whether increased expression of FliC<sub>H21</sub> correlated with increased invasion, we performed invasion assays with EH41 grown in LB broth overnight at 25°C and at 37°C as described previously (21, 28). Briefly, washed semiconfluent cell monolayers were infected in the presence of 0.5% mannose with ca. 10<sup>7</sup> CFU of different bacterial strains. After incubation for 3 h, some cell monolayers were washed three times with phosphate-buffered saline (PBS) and lysed in 0.1% (wt/vol) digitonin. After lysis, bacteria were resuspended in LB broth and quantified by plating serial dilutions. This represented the total adherent bacteria. To obtain the number of intracellular bacteria, a second set of infected wells was washed three times and incubated with 100 μg of gentamicin/ml for 60 min. After this incubation period, cells were washed three times with PBS, lysed with 0.1% (wt/vol) digitonin, and resuspended in LB broth for quantification by serial dilution. Assays were carried out in duplicate, and the results represented data obtained from at least three independent experiments. Invasion was expressed as the percentage of total adherent bacteria that resisted killing by gentamicin (mean ± the standard deviation), and adherence was expressed as the percentage of cell-associated bacteria from the original inoculum (mean ± the standard deviation) as described previously (21, 28). Differences in invasion and adherence were assessed for signifi-
The results showed that EH41 grown at 25°C was significantly more invasive for HCT-8 cells than bacteria grown at 37°C (Fig. 1B). In contrast, adherence to HCT-8 cells by EH41 was not affected by growth at a lower temperature (Fig. 1B). Invasion was therefore regulated by temperature, and this correlated with the production of FliC. Since we calculated invasion as the percentage of cell-associated bacteria that were internalized, only bacteria already in contact with host cells were counted. In addition, there was no difference in bacterial adherence, suggesting that these results were independent of FliC-mediated motility.

The production of invasins at temperatures below 37°C has been described previously for the enteropathogenic Yersinia species and Bartonella bacilliformis (5, 23, 26). Although temperature represses invasin expression in vitro, this may be overcome by exposing Y. enterocolitica or Y. pseudotuberculosis to conditions simulating pH and osmotic conditions in the gut (23, 26). The observed increase in production of FliC at 25°C could either reflect environment-to-host transmission of EHEC O113:H21 as for B. bacilliformis or, alternatively, temperature repression of FliC production in vitro may be overcome in vivo by changes in osmolarity and pH. Previous work on flagellin from enteropathogenic E. coli (EPEC) O127:H6 has suggested that an epithelial-cell-derived factor stimulates FliC production during infection of tissue culture cells (16). Therefore, although we used an ambient temperature as a trigger to increase invasion in vitro, it is possible that a separate unknown host signal is responsible for increasing flagellin expression in the gut.

To determine whether we could interfere with epithelial cell invasion with FliC-specific antibodies, we performed invasion assays in the presence of anti-H21 monovalent antiserum and anti-H6 as a control (Statens Serum Institut). In this experiment, wild-type STEC O113:H21 strain EH41 was grown at 25°C overnight, and bacteria were then incubated with antisera diluted 1:500 or 1:1,000 in PBS for 15 min at room temperature.
We examined the ability of EH41 and an isogenic fliC::cat mutant to invade HCT-8 cells. The fliC cassette was ligated into the internal Pst/EcoRI sites of fliC, and the mutation was amplified by using the primers described above and introduced into EH41 using the red recombinase system (7). The resulting mutant was nonmotile in semisolid agar (Fig. 3).

EH41/fliC::cat was complemented in trans by fliC122 encoding H21 flagellin inserted into the BamHI/SalI sites of pTrc99A. fliC122 was amplified with the primers 5'-AACGATCCCGGGATCCGCACCATGCG-3' and 5'-AAAGCTTCTCGAGCTCAGAGAGAC-3' (Fig. 3). In addition, EH41/fliC::cat was complemented in trans with fliC16 encoding H6 flagellin derived from EPEC strain 2348/69 inserted into the NcoI/BamHI sites of pTrc99A. fliC16 was amplified with the primers 5'-CA TGCCATGGCACAAGTCTTATACCC-3' and 5'-CGCGATATCCACCCGACGAGAC-3'. Complemented strains were tested for expression of FliC by immunofluorescence with anti-H21 or anti-H6 antibodies and then assessed for motility. As expected, EH41/fliC::cat (pTrc/fliC122) and EH41/fliC::cat (pTrc/fliC16) were motile and expressed FliC122 and FliC16, respectively (Fig. 3). EH41, EH41/fliC::cat, EH41/fliC::cat (pTrc/fliC16), and EH41/fliC::cat (pTrc/fliC122) were then grown overnight at 25°C and tested for their ability to adhere to and invade HCT-8 cells. The results for the mutant and complemented strains were expressed as a percentage of wild-type EH41 invasion and adherence (Table 1). The data showed that deletion of fliC from EH41 resulted in an eightfold decrease in invasion of HCT-8 cells (Table 1). Complementation of EH41/fliC with pTrc/fliC122 partially restored the ability of EH41/fliC::cat to invade HCT-8 cells, whereas complementation with pTrc/fliC16 had no effect on EH41/fliC::cat invasion (Table 1). Since complementation with either fliC16 or fliC122 restored the production of flagella and motility (Fig. 3), this suggested that invasion was directly related to flagellar type and not related to the motility of the strain. In addition, the deletion of fliC from EH41 had no affect on adherence to HCT-8 cells, and the apparent difference in adherence be-

prior to inoculation of HCT-8 cell monolayers. Although preincubation of EH41 with anti-H21 antibodies significantly reduced the level of invasion, preincubation with anti-H6 antibodies had no affect on EH41 uptake into HCT-8 cells (Fig. 2A). Neither anti-H21 nor anti-H6 antibodies had any affect on EH41 adherence to HCT-8 cells (Fig. 2B). No reactivity was observed between the ΔfliC mutant and anti-H2 antibodies (Fig. 3) or anti-H6 antibodies (data not shown), indicating that the inhibition of invasion was not due to nonspecific serum factors. These results supported the hypothesis that FliC122 was necessary for full invasion of EHEC O113:H21 into epithelial cells.

To test the contribution of fliC122 to epithelial cell invasion, we examined the ability of EH41 and an isogenic fliC mutant to invade HCT-8 cells. EH41/fliC::cat was constructed from EH41 by interruption of fliC with a chloramphenicol resistance (cat) gene derived from Tn4451 (1). fliC122 was amplified by using the primers 5'-TCTCAGTCTTCTCTGAAGTTCG-3' and 5'-ATGGAATATTTGCAGCGGCT-3' and cloned into pCR-Script digested with KpnI and Smal. The cat cassette was ligated into the internal Pst/EcoRI sites of fliC, and the mutation was amplified by using the primers described above and introduced into EH41 using the red recombinase system (7). The resulting mutant was nonmotile in semisolid agar (Fig. 3). EH41/fliC::cat was complemented in trans by fliC122 encoding H21 flagellin inserted into the BamHI/SalI sites of pTrc99A. fliC122 was amplified with the primers 5'-AACGATCCCGGGATCCGCACCATGCG-3' and 5'-AAAGCTTCTCGAGCTCAGAGAGAC-3'. In addition, EH41/fliC::cat was complemented in trans with fliC16 encoding H6 flagellin derived from EPEC strain 2348/69 inserted into the NcoI/BamHI sites of pTrc99A. fliC16 was amplified with the primers 5'-CA TGCCATGGCACAAGTCTTATACCC-3' and 5'-CGCGATATCCACCCGACGAGAC-3'. Complemented strains were tested for expression of FliC by immunofluorescence with anti-H21 or anti-H6 antibodies and then assessed for motility. As expected, EH41/fliC::cat (pTrc/fliC122) and EH41/fliC::cat (pTrc/fliC16) were motile and expressed FliC122 and FliC16, respectively (Fig. 3). EH41, EH41/fliC::cat, EH41/fliC::cat (pTrc/fliC16), and EH41/fliC::cat (pTrc/fliC122) were then grown overnight at 25°C and tested for their ability to adhere to and invade HCT-8 cells. The results for the mutant and complemented strains were expressed as a percentage of wild-type EH41 invasion and adherence (Table 1). The data showed that deletion of fliC from EH41 resulted in an eightfold decrease in invasion of HCT-8 cells (Table 1). Complementation of EH41/fliC with pTrc/fliC122 partially restored the ability of EH41/fliC::cat to invade HCT-8 cells, whereas complementation with pTrc/fliC16 had no effect on EH41/fliC::cat invasion (Table 1). Since complementation with either fliC16 or fliC122 restored the production of flagella and motility (Fig. 3), this suggested that invasion was directly related to flagellar type and not related to the motility of the strain. In addition, the deletion of fliC from EH41 had no affect on adherence to HCT-8 cells, and the apparent difference in adherence be-

![FIG. 3. (A) Immunofluorescence microscopy of wild-type EH41 incubated with anti-H21 antibodies (i). EH41/fliC::cat incubated with anti-H21 antibodies (ii). EH41/fliC::cat (pTrc/fliC122) incubated with anti-H21 antibodies (iii), and EH41/fliC::cat (pTrc/fliC16) incubated with anti-H6 antibodies (iv). All fields were also incubated with anti-rabbit fluorescein isothiocyanate-conjugated secondary antibodies, and bacterial nucleic acid was stained with DAPI (4',6'-diamidino-2-phenylindole). (B) Motility of wild-type EH41, EH41/fliC::cat, EH41/fliC::cat (pTrc/fliC122), and EH41/fliC::cat (pTrc/fliC16) were motile and expressed FliC122 and FliC16, respectively (Fig. 3). EH41, EH41/fliC::cat, EH41/fliC::cat (pTrc/fliC16), and EH41/fliC::cat (pTrc/fliC122) were then grown overnight at 25°C and tested for their ability to adhere to and invade HCT-8 cells. The results for the mutant and complemented strains were expressed as a percentage of wild-type EH41 invasion and adherence (Table 1). The data showed that deletion of fliC from EH41 resulted in an eightfold decrease in invasion of HCT-8 cells (Table 1). Complementation of EH41/fliC with pTrc/fliC122 partially restored the ability of EH41/fliC::cat to invade HCT-8 cells, whereas complementation with pTrc/fliC16 had no effect on EH41/fliC::cat invasion (Table 1). Since complementation with either fliC16 or fliC122 restored the production of flagella and motility (Fig. 3), this suggested that invasion was directly related to flagellar type and not related to the motility of the strain. In addition, the deletion of fliC from EH41 had no affect on adherence to HCT-8 cells, and the apparent difference in adherence be-

![TABLE 1. Relative invasion of and adherence to HCT-8 cells of derivatives of EHEC O113:H21 strains EH41 and 98NK2](http://iai.asm.org/Downloaded from)

| Strain | Invasion | Adherence |
|--------|----------|-----------|
| EH41 | 100 ± 82.6 | 100 ± 60.7 |
| EH41/fliC::cat | 12.63 ± 5.1 | 121.7 ± 29.2 |
| EH41/fliC::cat (pTrc/fliC122) | 43.38 ± 7.8 | 208.5 ± 122.2 |
| EH41/fliC::cat (pTrc/fliC16) | 15.42 ± 3.4 | 120.6 ± 26.5 |
| 98NK2 | 100 ± 43.4 | 100 ± 30.9 |
| 98NK2/fliC | 17.4 ± 7.0 | 47.9 ± 10.7 |

* Results are expressed as a percentage of wild-type EH41 invasion (mean ± standard deviation of at least three independent experiments in duplicate wells). * Significantly less than EH41/fliC::cat (pTrc/fliC122) (P < 0.05; unpaired, two-tailed t test). ! Significantly less than 98NK2 (P < 0.05; unpaired, two-tailed t test).

* Results are expressed as a percentage of wild-type EH41 adherence (mean ± standard deviation of at least three independent experiments in duplicate wells).
FIG. 4. CLUSTAL W alignment of FliC proteins from *E. coli* O127:H6 (Scaffold57_cons ORF_36 from EPEC E2348/69 genome sequence at www.sanger.ac.uk/Projects/Escherichia_Shigella), *E. coli* O142:H6 (AAD28526), *E. coli* O55:H6 (AAD28519), *E. coli* O113:H21 (DQ862122), *E. coli* O8:H21 (AAP13312), and *E. coli* O91:H21 (ABE69176). Shading indicates shared amino acid sequence identity and similarity.
between EH41 and EH41/fliC::cat (pTrcfliC\textsubscript{H21}) was not significant (Table 1, P = 0.29, unpaired two-tailed \(t\) test). FliC\textsubscript{H12} and FliC\textsubscript{H6} share 48.6% amino acid sequence identity, and an alignment between the H6 and H21 flagellins from several organisms showed that this homology occurs in the N- and C-terminal regions of the proteins, whereas the middle region is quite divergent (Fig. 4). We assume that this amino acid sequence variation is responsible for the observed differences in phenotype between the two flagellin types.

To ensure that FliC\textsubscript{H12}-mediated invasion was not strain specific, another clinical isolate of EHEC O113:H21, 98NK2, and its isogenic fliC mutant were grown overnight at 25°C and assessed for their ability to invade HCT-8 cells. EHEC O113: H21 strains 98NK2 and 98NK2\textsubscript{fliC} have been described previously (29, 30). The results reflected those obtained for EH41 and showed that there was a 5.8-fold reduction in the ability of a 98NK2\textsubscript{fliC} mutant to invade HCT-8 cells compared to wild-type 98NK2 (Table 1). Although there was also an apparent decrease in adherence of 98NK2\textsubscript{fliC} to HCT-8 cells, this was not significant (Table 1, P = 0.06, unpaired two-tailed \(t\) test).

Concluding remarks. In the absence of the LEE pathogenicity island, the mechanisms by which LEE-negative EHEC colonize the gut are unknown. Recently, we reported that clinical isolates of LEE-negative EHEC, in particular strains of EHEC O113:H21, were invasive for epithelial cells (21), and we speculated that host cell invasion was important for virulence and colonization of the host intestine. Recently, Rogers et al. showed that fliC\textsubscript{H12} encoding flagellin of EHEC O113: H21 was necessary for lethality in a streptomycin-treated mouse model of disease (30). This role in virulence was not associated with the ability of FliC\textsubscript{H12} to stimulate the production of interleukin-8 and recruit polymorphonuclear leukocytes to the intestinal epithelium (29, 30). Instead, FliC appeared to contribute to the ability of EHEC O113:H21 to associate with and penetrate the gut epithelium of streptomycin-treated mice.

Flagellin has been implicated in the invasion of host cells by several pathogens, including L. monocytogenes, Legionella pneumophila, and Burkholderia cepacia (8, 9, 33). In L. monocytogenes, which also invades human colonic cells, deletion of the gene encoding flagellin, flaA, resulted in an 4-fold reduction in invasion of Caco-2 cells (9). Flagellin is also an important factor in host-pathogen interactions in organisms more closely related to EHEC O113:H21 such as Salmonella enterica serovar Typhimurium, EHEC O157:H7, and EPEC. A recent study of fliC expression in serovar Typhimurium showed that fliC was transcribed by bacteria in Peyer’s patches but not in the mesenteric lymph nodes or spleen, indicating that flagellin may contribute to early stages of host infection (6). Serovar Typhimurium can also translocate FliC across the intestinal epithelium in SPI-1-induced vesicles, where it stimulates a potent inflammatory response via Toll-like receptor 5 (TLR5) signaling (15). Proinflammatory chemokine production is also upregulated by FliC through TLR5 signaling during EHEC O157:H7 and EPEC O127:H6 infection of intestinal tissue (22, 31, 35). In addition to its involvement in inflammation, EPEC O127:H6 flagellin contributes to the adherence of bacteria to epithelial cells (16). Although all of these effects can be attributed to the presence of FliC, they do not rely on bacterial motility. Therefore, flagellin may play a role in vivo by variously promoting motility in the gut, stimulating host inflammatory pathways and/or by promoting a direct host-pathogen interaction.

In the present study, we found that the inactivation of fliC encoding flagellin in EHEC O113:H21 strain EH41 had no effect on bacterial adherence to HCT-8 cells, which confirmed observations made by Rogers et al. using strain 98NK2 (30). In contrast, however, the deletion of fliC in both EH41 and 98NK2 strains of EHEC O113:H21 resulted in a significant reduction in invasion of HCT-8 cells. This invasion defect was complemented by fliC\textsubscript{H12} but not by fliC\textsubscript{H6}. Furthermore, we showed that invasion but not adherence could be inhibited by anti-H21 antibodies but not anti-H6 antibodies, providing further evidence that FliC\textsubscript{H12} plays a specific role in the interaction of EHEC O113:H21 with host cells. Overall, these results strongly suggest that FliC\textsubscript{H12} contributes to the pathogenesis of EHEC O113:H21 infections by promoting invasion of the intestinal epithelium.

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