The *lpf* Gene Cluster for Long Polar Fimbriae Is Not Involved in Adherence of Enteropathogenic *Escherichia coli* or Virulence of *Citrobacter rodentium*

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Using the enteropathogenic *Escherichia coli* (EPEC) genome sequence, we found that EPEC E2348/69 has an *lpfABCDE* gene cluster homologous (about 60% identical at the protein level) to the *Salmonella* long polar fimbria (LPF) operon. To determine whether this operon is essential for adherence, the *lpfABCDE23* genes were deleted from EPEC strain E2348/69 by allelic exchange. Analysis of the resulting EPECΔ*lpfABCDE23* strain showed no change in adherence to HeLa cells or to human intestinal biopsy cells in the in vitro organ culture (IVOC) system compared to the wild type. Sera from volunteers experimentally infected with E2348/69 showed no antibody response to the major subunit protein, LpfA. These results suggested that the *lpf* gene cluster is not necessary for EPEC adherence and attaching/effacing (A/E) lesion formation on human biopsy samples and is not expressed during human infection. We also identified an *lpf* gene cluster in *Citrobacter rodentium* strain ICC168 (*lpf_{cr})*. A Δ*lpfA_{cr}* mutant of ICC168 retained wild-type adherence and A/E lesion-forming activity on HeLa cells. C3H/HeJ mice were infected with a wild-type *C. rodentium* strain and its *lpfA_{cr}* isogenic mutant. Both strains were recovered at high levels in stools, and there were no significant differences between the groups both in terms of the number of CFU/organ (colon and cecum) and in terms of the amount of hyperplasia, as measured by weight. Similar results were observed in a second mouse strain, C57BL/6. These data suggest that in addition to playing no apparent role in EPEC pathogenesis, *lpf_{cr}*, is not required for *C. rodentium* virulence in either the C3H/HeJ or C57BL/6 mouse model.

Enterohemorrhagic *Escherichia coli* (EHEC), enteropathogenic *E. coli* (EPEC), rabbit-specific enteropathogenic *E. coli* (REPEC), and *Citrobacter rodentium* are attaching/effacing (*A/E*) bacterial pathogens that attach to host intestinal epithelium and efface brush border microvilli, forming pathogenic A/E lesions (6, 12). This characteristic adherence pattern is encoded by the locus of enterocyte effacement (LEE) pathogenicity island (23), which contains genes encoding the outer membrane protein intimin (18), its receptor Tir/EspE (7, 20), and a type III secretion system (17). EPEC, the first A/E pathogen to be described, also has a type IV pilus, bundle-forming pilus (BFP), as a potential adherence factor (13). While EPEC is a human pathogen, *C. rodentium* is a natural pathogen of mice and now is used as an animal model for studying virulence-associated factors of EPEC or EHEC (8, 31).

The long polar fimbriae (LPF), another potential adherence factor that was originally described in *Salmonella*, have been identified in other A/E bacteria, such as EHEC and REPEC. The LPF of *Salmonella enterica* serovar Typhimurium were shown to direct the attachment of bacteria to murine Peyer’s patches in vivo (4, 5). Two nonidentical *lpf* loci are present in EHEC O157:H7 (35, 36), and one or both of them were found to be involved in microcolony formation, increased adherence to HEP-2 cells when cloned into a nonfimbriated K-12 strain, and the colonization by and persistence of *E. coli* O157:H7 in swine and sheep (19). The *lpf* locus of REPEC O15:H- strain 83/39 appeared to play a role in the early stages of REPEC infection of rabbits and was essential for the development of severe diarrhea (26). Recently, sequencing of the EPEC O127:H6 strain E2348/69 and *C. rodentium* ICC168 genomes revealed *lpf* loci in each of those pathogens (http://www.sanger.ac.uk/Projects/Escherichia_Shirgella/ and http://www.sanger.ac.uk/Projects/C_rodontium, respectively), but it is unclear whether these loci are also functional in contributing to disease caused by these A/E pathogens. We therefore investigated the potential role of the EPEC and *C. rodentium* LPF in adherence and/or virulence.

**MATERIALS AND METHODS**

**Strains and plasmids.** *E. coli* E2348/69 (O127:H6) is a prototypic EPEC strain proven to cause diarrhea in volunteers (22). JPN15 is a derivative of E2348/69 cured of the EPEC adherence factor plasmid. *E. coli* HB101(pCVD462) (also known as pLEE) contains the cloned LEE pathogenicity island and confers A/E on HEP-2 and Caco-2 cells (24). pMAR7 is a Tn3 insertional derivative of the EPEC adherence factor plasmid from EPEC strain E2348/69 (O127:H6) (1). *C. rodentium* ICC168 is a wild-type strain shown to cause colonic epithelial cell hyperplasia in mice (8, 31). A reference collection of representative diarrheae-
genic *E. coli* clones known as the diarrheapgenic *E. coli* (DEC) collection was screened for the presence of *lpf* genes. This collection is available from the STEC Center at Michigan State University (http://www.shigatox.net). Two of these strains, 572-56 and 3787-62, representative of the DEC1 and DEC2 clones, respectively, were further analyzed by mutating the *lpf* genes and testing for resulting changes in adherence as described below.

**Cloning of the EPEC *lpf* gene cluster.** The *lpf* gene cluster of EPEC strain E2348/69 was cloned by PCR with Easy-A high-fidelity PCR cloning enzyme (Stratagene) using specific primers *lpf*-Fto (5'-CTCGGTTTTGGATGATGATG-3') and *lpf*-Rto (5'-CGCATGCTGGGCGGCCAAAAC-3'). The 6.84-kb amplicon was cloned into pGEM-Easy (Promega) to yield pELP102, whose insert was sequenced. The Apal-Sac fragment containing the amplified insert DNA of pELP102 was subcloned into the low-copy-number vector pWK5130 (38) to yield pELP310. A 1.1-kb EcoRI fragment containing the *lpfA* gene of *lpf* was subcloned into plpUS18 (3) to yield plpA2348F and plpA2348R, in which the *lpf* gene is oriented in the same and opposite direction, respectively, as the *lacUV5* promoter.

**Bacterial adhesion to cultured epithelial cells.** Adherence of bacterial strains to cultured Hep-2, HeLa, and Caco-2 epithelial cells was evaluated as previously described (35). Briefly, bacteria were grown statically in Dulbecco's modified Eagle's medium (DMEM) for 3 h and then washed three times to remove any nonadherent bacteria and then pre-

**Organ culture assay.** In vitro organ culture (IOV) was performed as described previously (14), with ethical approval and informed consent, using small intestinal samples. Large-bowel mucosa was not used. The assay was terminated at 2 and 4 h on three occasions to determine "early" events. "Later" events were observed at 6 h on two occasions, and all other experiments were performed at 8 h. Tissue culture medium was changed every 2 h, although some medium plus bacterial inoculum would remain within the foam insert supporting the tissue sample and in association with the tissue by surface tension. Each bacterial strain was examined in IOV on at least three occasions with tissue from different children. Strain E2348/69 and an uninoculated specimen were included with each experimental culture to act as positive and negative controls, respectively.

**Tissue processing.** After the specified culture period, specimens were thoroughly washed three times to remove any nonadherent bacteria and then prepared for scanning electron microscopy (SEM) as described previously (14). Following fixation and dehydration, specimens were placed in 100% ethanol and critical point dried in liquid CO2 with an Emitech K850 critical point drying apparatus. Following fixation and dehydration, specimens were placed in 100% ethanol and critical point dried in liquid CO2 with an Emitech K850 critical point drying apparatus. Then, after critical point drying, samples were mounted on aluminum stubs and sputter coated with approximately 100 Å of gold-palladium using a Polaron sputter coating apparatus. The assays were performed with a JEOL JSM-5300 SEM at an accelerating voltage of 30 kV.

**Construction of *lpf* mutants.** In-frame *lpf* deletion mutants were constructed in three EPEC strains and one *C. rodentium* strain. The wild-type strains and the resulting mutant designations, in parentheses, were E2348/69 (Δlpf*ABCDe23*), 572-56 (Δlpf*ABCDge27*), 3787-62 (Δlpf*ABCDge73*), and *C. rodentium* (Δlpf*ABCD*). Bacteria were cultured at 30°C for all experiments. *A. sobria* gene was amplified by PCR using the in-frame *lpfABCDe23* deletion mutation and additional flanking regions from pELP102) by PCR. The corresponding oligonucleotides contained restriction sites for the endonuclease Kpn1 (shown in boldface in the primer sequences).

**Conventional culture technique.** Conventional culture technique was used for the following experiment. The resulting purified LpfA E23 (Lampire Biological Laboratories Inc.) was included with each experimental culture to act as positive and negative controls, respectively.

**Tissue processing.** After the specified culture period, specimens were thoroughly washed three times to remove any nonadherent bacteria and then prepared for scanning electron microscopy (SEM) as described previously (14). Following fixation and dehydration, specimens were placed in 100% ethanol and critical point dried in liquid CO2 with an Emitech K850 critical point drying apparatus. Then, after critical point drying, samples were mounted on aluminum stubs and sputter coated with approximately 100 Å of gold-palladium using a Polaron sputter coating apparatus. The assays were performed with a JEOL JSM-5300 SEM at an accelerating voltage of 30 kV.

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The percent G
predicted EPEC LPF proteins to LPF proteins of other pathogens was introduced by horizontal gene transfer. The lpf E. coli of the inserted between the of EPEC to human small intestinal tissue, an EPEC

Human immune responses. A serological response against Lpf during the course of human infection was evaluated using convalescent human serum obtained from volunteers experimentally infected with E2348/69 in previous studies (9, 22, 32). The GST-LpfE23 fusion protein was prepared and transferred to polyvinylidene difluoride membrane as described above, and immunoblots were performed as previously described (22) using horse-radish peroxidase-conjugated goat antibody to human immunoglobulin G.

Mouse experiments. Male, specific-pathogen-free C3H/HeJ mice (6 to 8 weeks old) were purchased from Harlan Olac (Bicester, United Kingdom), and female, specific-pathogen-free C57BL/6 (6 to 8 weeks old) were purchased from B and K (Hull, United Kingdom). Mice were housed in individual ventilated cages with free access to food and water. Unanesthetized mice were orally gavaged with 200 μl of bacterial suspension using a gavage needle. The viable count of the inoculum was determined by retrospective plating on LB agar containing nalidixic acid to select for nalidixic acid-resistant C. rodentium.

In the infection experiments, bacteria were grown to stationary phase in LB broth plus nalidixic acid. For the C3H/HeJ mice, the bacteria were pelleted by centrifugation, washed, and then diluted 1:10 with PBS and gavaged into mice (approximately 1 × 10^7 CFU per mouse). The C57BL/6 mice required a higher inoculum, so 10 ml of stationary-phase bacteria was pelleted by centrifugation and resuspended in 1 ml of PBS and then gavaged into mice (approximately 1 × 10^8 CFU per mouse). Each strain was tested in groups of at least four mice per bacterial strain. Stool samples were recovered aseptically at various time points after inoculation, and the numbers of viable bacteria per gram of stool were determined by plating onto LB agar containing appropriate antibiotics. Mice were killed at either the peak of infection, 11 days postchallenge (C3H/HeJ mice), or once the infection had been cleared, 28 days postchallenge (C57BL/6 mice). In all cases, the distal 8 cm of colon was aseptically removed and weighed after removal of fecal pellets. Colon sections were then homogenized mechanically using a Seward 80 stomacher (London, United Kingdom), and the numbers of viable bacteria per colon were determined by plating onto LB agar containing appropriate antibiotics.

RESULTS

Identification and cloning of EPEC lpfE23 genes. The complete genome sequence of E2348/69 available at http://www.sanger.ac.uk/Projects/Escherichia_Shigella/ revealed a locus whose predicted protein products showed homology to the LPF proteins of S. enterica serovar Typhimurium. The organization of the EPEC lpfE23 locus and the similarity of the predicted EPEC LPF proteins to LPF proteins of other pathogens are shown in Fig. 1 and Table 1. The percent G+C composition of the EPEC lpf locus is 43.1, substantially lower than that of the E. coli K-12 genome (50.8%), suggesting that this locus was introduced by horizontal gene transfer. The lpf locus was inserted between the yhiW and yhdI genes corresponding to minute 79.9 of the E. coli K-12 chromosome (http://www.uni-giessen.de/~gstx1052/ECDC/ecdce/emap75.htm). The same insertion site is seen with the lpf operons of S. enterica serovar Typhimurium, EHEC O157:H7 lpfI, and REPEC (26, 35). The EPEC lpfE23 gene cluster was amplified by PCR, and the amplicon was cloned to yield pELP102 (see Materials and Methods). The resulting cloned amplicon was sequenced, and the insert DNA sequence was identical to that determined in the genomic sequencing.

Adherence phenotype of an EPEC lpf mutant to IVOC. In order to assess whether LpfE23 is associated with adherence of EPEC to human small intestinal tissue, an EPECΔlpfABCDE23 mutant was constructed and tested in IVOC. As shown in Fig. 2, an EPECΔlpfABCDE23 mutant strain adhered to IVOC in a manner similar to that seen with the wild type, indicating that EPEC LpfE23 is not essential for adherence to human intestinal tissue in the IVOC system.

Effect of lpfE23 on bacterial adherence to cultured epithelial cells. EPEC LpfE23 was also characterized for adherence to cultured epithelial cell lines. When EPECΔlpfABCDE23 was investigated for the ability to adhere to Caco-2 cells, it showed an adherence phenotype similar to that of parental wild-type EPEC (data not shown). Similar results were also seen in HeP-2 and T-84 cells under various experimental conditions, including different growth phase (log or stationary), static or shaking culture conditions, and growth in LB and DMEM (data not shown). To address the possibility that the function of LpfE23 is masked by other EPEC adherence factors, we investigated whether the cloned lpfE23 confers adherence to E. coli K-12. When the lpfE23 gene cluster was cloned in a high-copy vector, HB101(pELP102) did not show increased adherence to T-84 cells (4.7% ± 2.5%, CFU of output/input) compared to the parental HB101 strain containing the control vector only, which demonstrates minimal adherence (7.5% ± 3.8%). As previously observed, HB101(pMAR7) expressing BFP showed increased adherence to cultured epithelial cell lines (20.5% ± 6.0%). EPECΔlpfABCDE23 and E. coli K-12 containing the cloned lpfE23 genes on high-copy-number
(pELP102) or low-copy-number (pELP130) cloning vectors were further tested using combinations of the following experimental conditions: different epithelial cells (HEp-2, Caco-2, T-84, and HeLa), different hosts for cloned \(lpfE23\) (DH5\(\alpha\) and HB101(pCVD462)), different multiplicities of infection (1 to 200), different bacterial culture conditions (growth phase with log or stationary, shaking versus static, DMEM versus LB), and different infection times (2 to 5 h). Regardless of the conditions tested, no increased adherence activity was detected due to the presence of the \(lpfE23\) locus (data not shown).

Because no apparent role was observed for LPF E23 in the adherence of EPEC strain E2348/69 to four epithelial cell lines or human intestinal tissue, we mutated the \(lpfE23\) genes in two other EPEC strains to determine whether E2348/69 was unusual in this regard. We first examined the presence of \(lpfE23\) genes in the DEC collection of diarrheagenic \(E.\) coli clones and found \(lpfE23\) sequences in representatives of the EPEC1, EHEC1, and EHEC2 groups (Table 2). The amplified fragments in these clones were the same size, 6.5 kb, as that seen with E2348/69. Strains of the EPEC2 group gave a mixed pattern, with two representative strains of DEC11 clones yielding a 6.5-kb fragment and two strains of the DEC12 group yielding a 3.0-kb fragment. We generated \(lpf\) mutants of two additional strains from the EPEC1 group, \(lpfABCD_{527}\) and \(lpfABCD_{3787}\), and tested them for adherence to HeLa cells. No differences were seen in the adherence of these mutants from that of the parent strains (data not shown).

Expression of \(lpfE23\) genes in vitro. To investigate why the adherence phenotype associated with other LPF was not detected with EPEC LPF E23, the expression of the \(lpfE23\) genes was analyzed by electron microscopy. LPF from three enteric pathogens, \(S.\) enterica serovar Typhimurium, EHEC, and REPEC, have previously been visualized using negative staining and electron microscopy (4, 26, 36). In each of these cases, visualizing LPF was much easier with the cloned \(lpf\) genes expressed in a nonfimbriated K-12 strain than in the wild-type strain. We used these same techniques to examine wild-type E2348/69 for LPF without success. When pELP102 containing the EPEC E2348/69 \(lpfE23\) genes was transformed into nonfimbriated \(E.\) coli strain ORN172, we also failed to observe fimbrial structures (data not shown). We examined five different EPEC strains containing \(lpfE23\) genes grown under a variety of different growth conditions but still did not observe fimbrial structures (data not shown).

The failure to visualize EPEC LPF E23 is consistent with our inability to detect a difference in adherence to epithelial cell

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**TABLE 1. Predicted proteins encoded by the \(lpfE23\) genes of EPEC E2348/69**

<table>
<thead>
<tr>
<th>Protein</th>
<th>Putative function</th>
<th>No. of amino acids</th>
<th>(S.) enterica serovar Typhimurium</th>
<th>(O157-1)</th>
<th>(O157-2)</th>
<th>(O113)</th>
<th>REPEC</th>
<th>C. rodentium</th>
</tr>
</thead>
<tbody>
<tr>
<td>LpfA</td>
<td>Major fimbrial subunit</td>
<td>178</td>
<td>75</td>
<td>89</td>
<td>32</td>
<td>35</td>
<td>67</td>
<td>32</td>
</tr>
<tr>
<td>LpfB</td>
<td>Chaperone</td>
<td>217</td>
<td>65</td>
<td>79</td>
<td>41</td>
<td>36</td>
<td>64</td>
<td>40</td>
</tr>
<tr>
<td>LpfC</td>
<td>Usher</td>
<td>859</td>
<td>69</td>
<td>74</td>
<td>38</td>
<td>38</td>
<td>68</td>
<td>41</td>
</tr>
<tr>
<td>LpfD</td>
<td>Minor fimbrial component</td>
<td>351</td>
<td>33</td>
<td>46</td>
<td>27(^{a})</td>
<td>26</td>
<td>38</td>
<td>NH(^{d})</td>
</tr>
<tr>
<td>LpfE</td>
<td>Adhesin</td>
<td>176</td>
<td>43</td>
<td>52</td>
<td>NA(^{c})</td>
<td>NA</td>
<td>NH</td>
<td>NH</td>
</tr>
</tbody>
</table>

\(^{a}\) Properties and predicted function of putative gene products are based on their homology to related gene products.

\(^{b}\) The GenBank accession numbers for \(S.\) enterica serovar Typhimurium \(lpf\), EHEC \(O157:H7\) \(lpf1\) (\(O157-1\)), \(O157:H7\) \(lpf2\) (\(O157-2\)), \(O113:H12\) \(lpf\) (\(O113\)), and REPEC (\(O157-\), strain 63/39) \(lpf\) are AE008868, AE005581, AE005604, AY057066, and AY156523, respectively.

\(^{c}\) \(lpfD\) is duplicated in \(O157\) \(lpf2\) (\(lpfD2\) and \(lpfD\)/\(H11032\)2).

\(^{d}\) NH, no homologue found.

\(^{e}\) NA, not applicable; \(O157\) \(lpf2\) and \(O113:H12\) lack \(lpfE\) gene.

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**FIG. 2.** Adherence of wild-type EPEC (left panel; magnification, \(\times2,400\)) and an isogenic \(lpfABCD_{E23}\) mutant (right panel; magnification, \(\times3,100\)) to human duodenum tissue in IVOC. The white bar is 5 \(\mu\)m. Arrowheads indicate adherent bacteria. WT, wild type.
lines or to IVOC. We therefore examined expression of LPF by protein and mRNA analysis. To generate antisera against LPF E23, the lpfAE23 gene was cloned into the expression vector pGEX-2T, which creates a GST-LpfA E23 fusion under the transcriptional control of the lacUV5 promoter contained in this vector. The addition of IPTG induced the expression of a protein with the expected size of GST-LpfA E23, and digestion of this protein with thrombin protease resulted in a protein with the expected size of LpfAE23 (data not shown). This protein was purified and used to raise rabbit antisera against LPF E23 fusion protein in immunoblots. None of the volunteers showed an immune response to LpfAE23 in the convalescent-phase sera obtained 28 days after infection other than a faint, nonspecific band that was unchanged in intensity between preinfection and postinfection sera (data not shown).

Identification of C. rodentium lpf genes. Another lpf operon of an A/E organism was identified in the genome sequence of C. rodentium strain ICC168 (http://www.sanger.ac.uk/Projects/C_rodentium/). The predicted LpfAcr, LpfBcr, and LpfCcr proteins have amino acid similarities ranging from 32 to 46% to Lpf proteins in EPEC, REPEC, E. coli O157, and Salmonella (Fig. 1B, Table 1). No protein with striking homology to LpfE was detected in the C terminus of LpfDcr using the “Conserved Domain Search” program (http://www.ncbi.nlm.nih.gov). LpfEcr also did not show high homology with other LpfE proteins (with a maximum of 22% identity seen with LpfE of E. coli O157 and Salmonella). However, this is consistent with LpfD and LpfE being poorly conserved among the lpf genes in vivo.

Expression of lpfE23 genes in vivo. To investigate whether there was a serological response against LPF E23 during the course of human infection, convalescent-phase sera of 10 volunteers previously infected with E. coli strain E2348/69 (9, 22, 32) were tested against GST-LpfA E23 fusion protein in immuno-blots. None of the volunteers showed an immune response to LpfAE23 in the convalescent-phase sera obtained 28 days after infection other than a faint, nonspecific band that was unchanged in intensity between preinfection and postinfection sera (data not shown).

Virulence of the C. rodentium lpfAcr mutant in the C3H/HeJ mouse model. To address whether LPF is associated with the

### Table 2. Predicted distribution of lpf loci among different DEC strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Clonal group</th>
<th>Predominant serotype</th>
<th>Host</th>
<th>lpf</th>
</tr>
</thead>
<tbody>
<tr>
<td>DEC1a</td>
<td>EPEC1</td>
<td>O55:H6</td>
<td>Human</td>
<td>+</td>
</tr>
<tr>
<td>DEC1b</td>
<td>EPEC1</td>
<td>O55:H6</td>
<td>Human</td>
<td>+</td>
</tr>
<tr>
<td>DEC2a</td>
<td>EPEC1</td>
<td>O55:H6</td>
<td>Human</td>
<td>+</td>
</tr>
<tr>
<td>DEC2b</td>
<td>EPEC1</td>
<td>O55:NM</td>
<td>Human</td>
<td>+</td>
</tr>
<tr>
<td>DEC3a</td>
<td>EHEC1</td>
<td>O157:H7</td>
<td>Human</td>
<td>+</td>
</tr>
<tr>
<td>DEC3b</td>
<td>EHEC1</td>
<td>O157:H7</td>
<td>Human</td>
<td>+</td>
</tr>
<tr>
<td>DEC4a</td>
<td>EHEC1</td>
<td>O157:H7</td>
<td>Calf</td>
<td>+</td>
</tr>
<tr>
<td>DEC4b</td>
<td>EHEC1</td>
<td>O157:H7</td>
<td>Human</td>
<td>+</td>
</tr>
<tr>
<td>DEC5d</td>
<td>EHEC1</td>
<td>O55:H7</td>
<td>Human</td>
<td>+</td>
</tr>
<tr>
<td>DEC6a</td>
<td>A</td>
<td>O111:H21</td>
<td>Human</td>
<td>(0.7)</td>
</tr>
<tr>
<td>DEC6c</td>
<td>A</td>
<td>O111:H12</td>
<td>Human</td>
<td>(0.7)</td>
</tr>
<tr>
<td>DEC8a</td>
<td>EHEC2</td>
<td>O111:NM</td>
<td>Human</td>
<td>+</td>
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<tr>
<td>DEC8b</td>
<td>EHEC2</td>
<td>O111:H8</td>
<td>Human</td>
<td>+</td>
</tr>
<tr>
<td>DEC9a</td>
<td>EHEC2</td>
<td>O26:H11</td>
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<td>EHEC2</td>
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<td>Human</td>
<td>+</td>
</tr>
<tr>
<td>DEC11a</td>
<td>EPEC2</td>
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<td>DEC12a</td>
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<td>O128:H21</td>
<td>Human</td>
<td>+</td>
</tr>
<tr>
<td>DEC15a</td>
<td>B1</td>
<td>O111:H21</td>
<td>Human</td>
<td>(0.7)</td>
</tr>
<tr>
<td>DEC15b</td>
<td>B1</td>
<td>O111:H21</td>
<td>Human</td>
<td>(0.7)</td>
</tr>
</tbody>
</table>

a The primers lpf-Fto and lpf-Rto (see Fig. 1A) were used for PCR analysis. + indicates the presence of a 6.5-kb fragment, the same size obtained with E2348/69; the 6.5-kb fragments were confirmed by sequencing to contain a lpfA gene. − indicates that a 6.5-kb fragment was not seen but that a 0.7-kb (the same size predicted for E. coli K-12) or a 3.0-kb fragment was amplified.

b Strain B171 is a prototype EPEC2 strain (28).

FIG. 3. Transcription of the lpfA_E23 gene by RT-PCR. RT indicates the presence (+) or absence (−) of reverse transcriptase. M, 1-kb DNA ladder. D, chromosomal DNA of E2348/69. Transcription of tir is included as a positive control.
virulence of \textit{C. rodentium} in mice, a ICC168\textit{lpfA}_{cr} mutant was constructed. In initial in vitro studies, \textit{\Delta lpfA}_{cr} had no effect on the ability of ICC168 to adhere to HeLa cells or the ability to produce a positive fluorescent actin staining (FAS) test indicative of A/E lesion formation (data not shown). Thus, \textit{LpfA}_{cr} is not required for in vitro adherence and A/E lesion formation on HeLa cells. To examine potential effects of \textit{lpfA}_{cr} on virulence, groups of four mice were inoculated with the wild-type \textit{C. rodentium} strain and the \textit{lpfA}_{cr} mutant. The \textit{lpfA}_{cr} mutant behaved like the wild type. Both strains were recovered at high levels in stools collected at days 1, 4, 6, and 8 (Fig. 4A). At day 10, two mice infected with the wild-type strain and two mice infected with the \textit{lpfA}_{cr} mutant died. The remaining two mice in each group were sacrificed on day 11, and the colon and cecum were removed. The numbers of viable cells in the colon were similar in both groups, as was the extent of hyperplasia as measured by weight (data not shown).

\textbf{Virulence of the \textit{C. rodentium} \textit{lpf}_{cr} mutant in the C57BL/6 mouse model.} The experimental infection was repeated in C57BL/6 mice, which are less susceptible to infection than C3H/HeJ mice when infected with \textit{C. rodentium}. The milder response of BL/6 allows the length of infection to be extended 28 days. Colonos were taken at day 28, and at this time point all the crotobacteria had been cleared from both the colon and the cecum. The average weights of colons \pm standard errors are the following: wild-type-infected mice, 0.224 \pm 0.014 g; \textit{\Delta lpfA}_{cr}-infected mice, 0.242 \pm 0.009 g; and uninfected mice, 0.147 \pm 0.008 g. The C57BL/6 mice do not exhibit as much hyperplasia as the C3H/HeJ mice in response to infection; however, there was significant hyperplasia in the distal 1 to 2 cm of the colon in mice infected with both the wild type and the \textit{lpfA}_{cr} mutant. Collectively, these data suggest that \textit{lpf}_{cr} is not required for virulence in our mouse models.

\textbf{Expression of \textit{lpf}_{cr} genes in vitro.} The expression of \textit{lpf}_{cr} was examined to confirm that these genes were expressed. When the \textit{C. rodentium} wild-type strain was incubated at 37°C in DMEM, no expression of \textit{LpfA}_{cr} protein was detected in whole-cell extracts by immunoblotting with anti-EPEC LpfA antibody. Since the proteins share low similarity, it is possible that the anti-EPEC LpfA sera did not detect \textit{LpfA}_{cr}. Nevertheless, \textit{lpfABC}_{cr} mRNA was detected by RT-PCR using primers specific to \textit{lpfA}_{cr} and \textit{lpfC} (Fig. 5A) but not when the 5’ primer was outside the \textit{lpfA}_{cr} coding region (Fig. 5B). Thus, \textit{lpf}_{cr} appears to be at least transcribed under these conditions. Furthermore, this result suggests that \textit{lpfABC}_{cr} comprises an operon.

Our in vitro studies did not indicate a role for LPF\textit{E23} in adherence or A/E lesion formation by EPEC. Similarly, mutation of \textit{C. rodentium} \textit{lpf}_{cr} did not alter the in vitro adherence of this organism to cultured epithelial cells. In addition, we examined expression of Lpf\textit{A}_{E23} using immunoblots of E2348/69 and K-12 containing cloned \textit{lpf}_{E23} genes grown under a variety of cultural conditions and found no evidence of expression using antisera raised against the cloned EPEC \textit{lpfA}_{E23} gene product. There are at least two possible reasons why LPF\textit{E23} is apparently not expressed in EPEC. The first possibility is that some of the Lpf\textit{ABCDE}_{E23} proteins are inactive and therefore cannot assemble into mature fimbriae. EHEC O157:H7 contains two \textit{lpf} loci, \textit{lpf1} and \textit{lpf2}, whose predicted protein products share sequence identities ranging from 24 to 37% (36). However, neither of these operons corresponds exactly with the intact \textit{Salmonella} \textit{lpf} operon. The EHEC \textit{lpf1} operon has two smaller \textit{lpfC} open reading frames (ORFs) compared to one large \textit{lpfC} ORF in \textit{Salmonella}, and the \textit{lpf2} operon has two smaller \textit{lpfB} ORFs compared to one large ORF in \textit{Salmonella} and lacks the \textit{lpfE} gene. Despite the apparent lack of intact \textit{lpf} operons, the cloned EHEC \textit{lpf1} and \textit{lpf2} loci still produced fimbriae in K-12, although the morphologies more closely resembled the \textit{E. coli} type 1 fimbriae (\textit{lpf1}) and a thin fibria-like structure (\textit{lpf2}) rather than the long polar fimbriae of \textit{Salmonella} (35, 36). Perhaps chaperone/usher proteins encoded in K-12 were utilized in assembly of these fimbriae. In contrast, the EPEC \textit{lpf}_{E23} operon appears to be intact and is predicted to encode five proteins very similar in size to the \textit{Salmonella} Lpf proteins (Table 1). The numbers of amino acid residues in the five predicted EPEC Lpf\textit{E23} subunits are 178, 217, 859, 351, and 176 for Lpf\textit{A}_{E23} through Lpf\textit{E}_{E23} compared to 178, 232,
842, 355, and 175 residues for the Salmonella Lpf proteins. A phylogenetic analysis using CLUSTAL (15) of Lpf proteins described to date indicates that the EPEC proteins are more closely related to the Salmonella proteins than are most E. coli Lpf proteins (data not shown). No structural information is available on any of the Lpf proteins, so it is impossible to predict whether key residues essential for assembly and function are altered in the EPEC proteins. A second possibility for our failure to detect a phenotype for the EPEC lpfE locus is poor transcription or translation of the operon. Using standard prediction tools (2, 29, 30), more than one potential σ70 promoter plus a reasonable Shine-Dalgarno sequence appear to be located upstream of the lpfA23 gene. However, RT-PCR analysis of transcripts extracted from wild-type EPEC grown under in vitro conditions suitable for demonstrating adherence to epithelial cell lines showed only a very weak lpfA23 transcript (Fig. 3). Expression of Lpf was not improved by growing the organism under a variety of conditions. Meanwhile, in vitro characterization of lpf genes in other A/E pathogenic E. coli strains has presented a mixed picture. As noted above, both EHEC O157:H7 lpf operons produced fimbriae in K-12. Mutation of lpfJ in the native EHEC O157:H7 resulted in slight, nonsignificant reductions in adherence to HeLa cells along with decreased microcolony formation (35). Mutation of the lpfJ locus in O157:H7 caused no detectable effect in adherence to HeLa cells but did slightly diminish adherence to Caco-2 cells at early time points (36). The REPEC strain 83/39 also contains two lpf loci, but mutation of one or both loci caused no alteration in adherence to CHO-K1 cells (26).

In any in vitro system used to study the contribution of potential adherence factors, the inability to detect a phenotype with an isogenic mutant or with the cloned adhesin genes in K-12 can always be attributed to cultural conditions or tissue culture cell lines that do not accurately reproduce the relevant in vivo situation. Since C. rodentium is a natural mouse pathogen, characterization of lpf in this model should provide further insight into the function of this putative adhesin. However, when tested in two different mouse strains, C3H/HeJ and C57Bl/6, the lpfA1 mutant was indistinguishable from the wild-type parent with regard to fecal shedding, colonic hyperplasia, or numbers of organisms associated with intestinal tissue. As with in vitro adherence studies, the contribution of lpf genes in other A/E pathogenic E. coli strains in animal models is variable. REPEC strain 83/39 is a natural pathogen of rabbits, and Newton et al. (26) tested mutants in one or both lpf operons in this host species and found no significant differences in body weight or mortality rate between the wild type and mutants. However, animals receiving the wild type had more severe diarrhea and slightly better intestinal colonization in the early stages of disease. In addition, intimate bacterial adherence to the gut mucosa was indistinguishable in morphology, location, and number between the wild type and the lpf mutants of REPEC 83/39 (26). For EHEC O157:H7, the most important animal reservoir is cattle, although animals do not get sick from this organism. Jordan et al. (19) studied lpfA1 and lpfA2 mutants of O157:H7 in sheep, conventional pigs, and gnotobiotic piglets. In gnotobiotic piglets, a model of early colonization, the lpfA1 mutant caused fewer attaching/effacing lesions in the spiral colon than the wild-type parent. Using conventional pigs and sheep to study long-term colonization over 60 days, lpf mutants were recovered at lower levels than those for the parent strain (19). Thus, LPF appears to contribute to the long-term persistence, rather than virulence, of O157:H7 in species such as sheep (19), which have been implicated as an important reservoir of O157:H7 in the farm environment (27). Thus, although we failed to detect a role for LPF in EPEC or C. rodentium pathogenesis, experience with other A/E pathogenic E. coli strains indicates that LPF contributes to intestinal colonization and disease in some hosts but to a substantially lesser degree than does the intimin adhesin, which is essential for formation of the A/E lesion. Therefore, it appears that LPF is not an essential virulence factor but may be an accessory virulence determinant in some A/E pathogens.

lpf genes also have been identified in non-A/E pathogenic E. coli, although the genes have undergone considerable divergence (16). Mutation of the lpf genes in EHEC O113:H21, which is a more significant cause of hemolytic-uremic syndrome in Australia than O157:H7, resulted in a significant reduction in adherence to CHO-K1 cells (10). An lpf mutant of a meningitis-associated extraintestinal pathogenic E. coli strain was significantly reduced in adherence to a kidney cell line (HEK 293) (16). LPF may therefore play a more important role in disease caused by E. coli strains that lack intimin and the rest of the LEE than in disease caused by E. coli strains that possess intimin and the LEE, such as EPEC and C. rodentium.

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