Comparative analysis of EspF variants in inhibition of Escherichia coli phagocytosis by macrophages and inhibition of E. coli translocation through human- and bovine-derived M cells

Citation for published version:

Digital Object Identifier (DOI):
10.1128/IAI.00023-11

Link:
Link to publication record in Edinburgh Research Explorer

Document Version:
Publisher's PDF, also known as Version of record

Published In:
Infection and Immunity

Publisher Rights Statement:
Copyright © 2013 by the American Society for Microbiology.

General rights
Copyright for the publications made accessible via the Edinburgh Research Explorer is retained by the author(s) and / or other copyright owners and it is a condition of accessing these publications that users recognise and abide by the legal requirements associated with these rights.

Take down policy
The University of Edinburgh has made every reasonable effort to ensure that Edinburgh Research Explorer content complies with UK legislation. If you believe that the public display of this file breaches copyright please contact openaccess@ed.ac.uk providing details, and we will remove access to the work immediately and investigate your claim.

Download date: 02. Apr. 2018
Enterohemorrhagic *Escherichia coli* (EHEC) is an emerging zoonotic pathogen, particularly in industrialized countries (6). EHEC strains cause sporadic outbreaks of severe disease in humans, the most important being hemorrhagic colitis (HC) and hemolytic-uremic syndrome (HUS); the latter disease results in kidney damage and may lead to death (8, 28). Shiga toxins (Stx) produced by EHEC strains are the main factors responsible for these serious outcomes in humans. In contrast, *Enterohemorrhagic E. coli* (EPEC) strains cause sporadic outbreaks of severe disease in humans, although these infections are not usually associated with the colonization and persistence of the bacterium in the gastrointestinal tract (16, 19, 20, 34, 52, 64). The primary phenotype associated with the T3SS is intimate attachment between the bacterial outer membrane protein intimin and the T3SS translocated intimin receptor (Tir) (42). In both EHEC and EPEC, the genes encoding this protein secretion system are expressed from the locus of enterocyte effacement (LEE) pathogenicity island (33, 36). Although several effector proteins are also expressed from the LEE, a number of additional proteins are also expressed from the LEE, a number of additional...
secreted effector proteins have been identified that are expressed primarily from integrated phage elements scattered throughout the O157 chromosome (79). EHEC and EPEC strains have different combinations of effector proteins, potentially reflecting host adaptation and differences in pathogenesis.

EspF is a LEE-encoded effector protein that requires the CesF chaperone to be translocated by the T3SS into host cells (20). EspF has multiple proline-rich domains that act by binding to SH3 domains or enabled/VASP homology 1 (EVH1) domains of host cell signaling proteins (15, 55). For example, EPEC EspFO127 binds to sorting nexin 9 (SNX9) via its SH3 domains of host cell signaling proteins (15, 55). EspF is involved in disruption of tight junctions and increases monolayer permeability in part through the redistribution of occludins (54, 80). EspF sequences differ between EPEC and EHEC strains, and the EHEC O157 variant has a more modest impact on transepithelial electrical resistance (TER) (80). EspF in combination with other effectors inhibits the water transporter SGLT-1, highlighting the importance of effector interplay (16, 43). EPEC EspFO127 is targeted to mitochondria with the N-terminal region of EspF functioning as an import signal. EPEC EspFO127 causes an increase in mitochondrial membrane permeabilization in addition to the release of cytochrome c from mitochondria into the cytoplasm and subsequent caspase-9 and caspase-3 cleavage, leading to cell death (15, 58, 65, 66). More recent work has demonstrated that EspF can lead to loss of caspase-3 cleavage, leading to cell death (15, 58, 65, 66). More recent work has demonstrated that EspF can lead to loss of caspase-3 cleavage, leading to cell death (15, 58, 65, 66).

Functional variation in E. coli EspF

<table>
<thead>
<tr>
<th>Strain</th>
<th>Serotype</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli ZAP1139</td>
<td>O26:H11</td>
<td>Cattle isolate, Stx negative; Chris Low, Scottish Agricultural College, Penicuik, Scotland (21)</td>
</tr>
<tr>
<td>E. coli TUV93-0</td>
<td>O157:H7</td>
<td>Stx phage-negative derivative of the sequenced human strain EDL933; John Leong, Massachusetts Medical School, Boston, MA (10)</td>
</tr>
<tr>
<td>E. coli TUV93-0ΔespF</td>
<td>O157:H7</td>
<td>This study Mark Jepson (38)</td>
</tr>
<tr>
<td>S. enterica serovar (SL1344) Typhimurium</td>
<td>O157:H7</td>
<td>Mark Jepson (38)</td>
</tr>
<tr>
<td>E. coli EPEC E2348/69 (ΔespF)</td>
<td>O127:H6</td>
<td>Derivative of human isolate supplied by Brendan Kenny, University of Newcastle, Newcastle, United Kingdom (74)</td>
</tr>
<tr>
<td>E. coli AACE 185</td>
<td>O148</td>
<td>Laboratory stock</td>
</tr>
<tr>
<td>E. coli DH5α</td>
<td>Rough</td>
<td>Laboratory stock</td>
</tr>
</tbody>
</table>

**Materials and Methods**

**Bacterial strains and media.** EPEC, EHEC, and other *E. coli* strains used in the present study are detailed in Table 1. Bacteria were cultured in Luria-Bertani (LB) broth, minimal essential medium (MEM)-HEPES, or Dulbecco modified Eagle medium (DMEM) with antibiotics included when required at the following concentrations: chloramphenicol, 20 μg/ml; kanamycin, 50 μg/ml; and ampicillin, 100 μg/ml.

**Cloning of espF alleles.** espF alleles were amplified from the different strains using the primers defined in Table 2 and the products cloned into pTS1 (Table 3). BamHI and HindIII sites were used to clone the alleles from EHEC O157:H7 and O26:H11 strains. For EPEC O127 espF, since this contains a natural HindIII site, an alternative cloning strategy was used: pMB102 (Table 3) was digested for the alleles giving the amino acid sequences are shown in Fig. 2. Gateway cloning of the espF alleles from the different *E. coli* strains was also carried out for the LUMIER assays. espF alleles were amplified by using a proofreading Taq polymerase with the primers defined in Table 2 and cloned into the Gateway vector pDONR-207 (Invitrogen) to form entry clones. The obtained clones were checked for the correct insert by DNA plasmid extraction and restriction digestion using BamHI. Once in the Gateway system, clones were easily transferred to the required destination vectors, including pTREX-DEST30 (protein A fusions) and pRenilla (luciferase fusions).

To analyze type III secretion profiles and EspF secretion from the EPEC
Cloning of espF was added to the macrophages (initially seeded at 10^5 cells/well the day before). EspF3R CTGATATCGATATAAAGAGGCATAAATTATGC 
EspF2R CTAAGCTTGATATAAAGAGGCATAAATTATGC

Induced with 1 mM IPTG (isopropyl-

microscopy, Confocal data were acquired using a 1.024 by 1.024 pixel image size, a Zeiss Plan Apochromat 1.4 NA ×63 oil immersion lens, and a multitrack (sequential scan) experimental setup on a Zeiss LSM 510. Image data, acquired at Nyquist sampling rates, were deconvolved using Huygens software (Scientific Volume Imaging, Netherlands), the resulting three-dimensional models were analyzed, and orthogonal views were created using NIH ImageJ software; the final figures were assembled in Adobe Photoshop.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence 5′-3′</th>
<th>Use</th>
</tr>
</thead>
<tbody>
<tr>
<td>EspF1F</td>
<td>CAGGATCCCTCAATGCAAATCTAAATTCG</td>
<td>Cloning of espF into pTS1</td>
</tr>
<tr>
<td>EspF1R</td>
<td>CTAAAGCTTGATATAAAGAGGCATAAATTATGC</td>
<td>Cloning of espF into pTS1</td>
</tr>
<tr>
<td>EspF2F</td>
<td>CAGGATCCACACACAAATTTGTATGCTCGT</td>
<td>Cloning of espF into pTS1</td>
</tr>
<tr>
<td>EspF2R</td>
<td>CTAAGCTTGATATAAAGAGGCATAAATTATGC</td>
<td>Gateway cloning of espF into pTS1</td>
</tr>
<tr>
<td>EspF3F</td>
<td>CAGGATCCACACACAAATTTGTATGCTCGT</td>
<td>Gateway cloning of espF into pTS1</td>
</tr>
<tr>
<td>EspF3R</td>
<td>CTAAAGCTTGATATAAAGAGGCATAAATTATGC</td>
<td>Gateway cloning of espF into pTS1</td>
</tr>
<tr>
<td>EspF4F</td>
<td>ATGCCCTAATGGAATTAGTACGC</td>
<td>Gateway cloning of espF into pTS1</td>
</tr>
<tr>
<td>EspF4R</td>
<td>CTACACAAACCGCATAG</td>
<td>Gateway cloning of espF into pTS1</td>
</tr>
<tr>
<td>EspF5F</td>
<td>ATGCCCTAATGGAATTAGTACGC</td>
<td>Gateway cloning of espF into pTS1</td>
</tr>
<tr>
<td>EspF5R</td>
<td>CTACACAAACCGCATAG</td>
<td>Gateway cloning of espF into pTS1</td>
</tr>
</tbody>
</table>

* Restriction sites are underlined.

<table>
<thead>
<tr>
<th>Table 2. Primers used in the study</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primer</td>
</tr>
<tr>
<td>--------</td>
</tr>
<tr>
<td>EspF1F</td>
</tr>
<tr>
<td>EspF1R</td>
</tr>
<tr>
<td>EspF2F</td>
</tr>
<tr>
<td>EspF2R</td>
</tr>
<tr>
<td>EspF3F</td>
</tr>
<tr>
<td>EspF3R</td>
</tr>
<tr>
<td>EspF4F</td>
</tr>
<tr>
<td>EspF4R</td>
</tr>
</tbody>
</table>

O127:H6 espF and complemented strains, previous procedures were used (50, 61, 71). EspD was detected by using a monoclonal antibody kindly supplied by T. Chakraborty (Giessen, Germany). EspF was detected by using polyclonal antibodies supplied from C. Sasakawa (Tokyo, Japan) (58), and G. Hecht (University of Illinois at Chicago) (80).

**Phagocytosis assays.** The murine macrophage cell line (RAW 264.7) was cultured in Dulbecco modified Eagle medium (DMEM; Sigma) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Sigma), 1 U/mL of penicillin, 1 mM l-glutamine (final concentrations). Cells were grown at 37°C in 5% CO2 and moisture. The bacteria, transformed with pUC-gfp (Table 3), were inoculated from LB broth overnight cultures into DMEM to an optical density at 600 nm (OD600) of 0.3. When analyzed, the espF alleles were induced with 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside). At an OD600 of 0.7, the bacterial cultures were diluted 1:5 into prewarmed DMEM, and 300 μL was added to the macrophages (initially seeded at 10^6 cells/well the day before). The cells were then incubated at 37°C in 5% CO2 in a moist box for the desired incubation time. The cells were washed three times with sterile phosphate-buffered saline (PBS) and fixed using 4% paraformaldehyde (PFA) for 1 h. After several washes, the slides were then washed three times with PBS, incubated with Alexa Fluor 594-conjugated goat anti-rabbit IgG, and coverslips were applied. All bacteria, internal and external to the macrophages, were stained with the anti-O157 LPS antibody. This antibody was a polyclonal antibody supplied from C. Sasakawa (Tokyo, Japan) (58), and G. Hecht (University of Illinois at Chicago) (80).

**LUMIER assays.** Proteins were transiently expressed in HEK-293 cells as hybrid proteins with the Staphylococcus aureus protein A tag or Renilla reniformis luciferase fused to their amino termini. Portions (20 ng) of each expression construct were transfected into HEK-293 cells using 0.5 μL of Lipofectamine 2000 (Invitrogen) in 96-well plates. After 48 h, the medium was removed, and the samples were lysed on ice in 10 μL of ice-cold lysis buffer (20 mM Tris pH 7.5, 250 mM NaCl, 1% Triton X-100, 10 mM EDTA, 10 mM dithiothreitol [DTT], protease inhibitor cocktail and phosphatase inhibitor cocktail [both from Roche], and benzamidine [Novagen]). Sheep anti-rabbit IgG-coated magnetic beads were also added (Dynabeads M280 [Invitrogen]; 2 μg/mL, final concentration), followed by incubation on ice for 15 min. Then, 100 μL of washing buffer (PBS, 1 mM DTT) was added per well, and 10% of the diluted lysate was removed to determine the luciferase activity present in each sample before washing. The rest of the sample was washed six times in washing buffer. Luciferase activity was measured in the lysate as well as in washed beads. Negative controls (nc) were wells transfected with the plasmid expressing the luciferase fusion protein and a vector expressing a dimer of protein A. For each sample, four values were measured: the luciferase activity present in each sample before washing, the luciferase activity present on the beads after washing (“bound”), and the same two values for the negative controls (i.e., “input nc” and “bound nc”). Normalized signal-to-noise ratios were calculated as published previously (4, 9): normalized signal-to-noise ratio = (bound - input nc) / input nc.

**Microscopy.** Confocal data were acquired using a 1.024 by 1.024 pixel image size, a Zeiss Plan Apochromat 1.4 NA ×63 oil immersion lens, and a multitrack (sequential scan) experimental setup on a Zeiss LSM 510. Image data, acquired at Nyquist sampling rates, were deconvolved using Huygens software (Scientific Volume Imaging, Netherlands), the resulting three-dimensional models were analyzed, and orthogonal views were created using NIH ImageJ software; the final figures were assembled in Adobe Photoshop.
The trypsinized cells were fixed and permeabilized in 500 μl PBS, and the specific monoclonal antibodies were detected using fluorescein isothiocyanate (FITC)- or TRITC (tetramethyl rhodamine isothiocyanate)-labeled second antibody. After washing in PBS, the stained cells were mounted in fluorescence mounting medium Fluoromount. The cell nuclei were stained with TO-PRO iodide (Molecular Probes). The cell nuclei were washed in PBS with 0.5% bovine serum albumin and 0.1% sodium azide.

The percentages of cells expressing vimentin in the bovine terminal rectal primary epithelial cell cultures were determined using flow cytometry. Briefly, the trypsinized cells were fixed in 500 μl of MEM-HEPES without antibiotic for 1 h before the bacterial culture reached an OD560 of 0.4, and the wells were centrifuged at 90 rpm for 1 min. The infected cells were incubated at 37°C with 5% CO₂ and moisture for 1.5 h. Bacteria were prepared as described for the phagocytosis assays, and 100 μl of bacterial suspension was added to each upper chamber. Bacterial counts from the lower chamber for 6 days (44). The TER was measured every day before adding the Raji-B cells until it become consistent at 200 to 300 Ω/cm²; however, it was not changed by coculture with Raji-B cells. Bacteria were prepared as described for the phagocytosis assays, and 100 μl of bacterial suspension was added to each upper chamber. Bacterial counts from the lower chamber were determined at 60 min relative to the levels inoculated.

Characterization of bovine primary rectal cultured cells. The epithelial origin of the cells was confirmed by immunostaining for cytokeratin intermediate fila-
ments. The cells at 5 days of culture were fixed with 2% PFA, permeabilized with cold acetone for 5 min, washed with PBS, and stained with a pan-cytokeratin and or anti-vimentin antibodies (Sigma, 1:300) for 3 h at 25°C. The cells were washed with PBS, and the specific monoclonal antibodies were detected using fluorescein isothiocyanate (FITC)- or TRITC (tetramethyl rhodamine isothiocyanate)-la-
beled secondary anti-rabbit or anti-mouse monoclonal antibody (Sigma) at 1:80. The cell nuclei were stained with TO-PRO iodide (Molecular Probes). The stained cells were mounted in fluorescent mounting medium Fluoromount (Dako) and examined by using a Leica DMLB epifluorescence microscope. To ascertain whether lymphoid cells were present in primary cell cultures from the crypts isolated from lymphoid rich mucosal tissue, immunostaining was carried out with a panel of seven monoclonal antibodies (Table 4) specific for different immune cell types of cattle.

The percentages of cells expressing vimentin in the bovine terminal rectal primary epithelial cell cultures were determined using flow cytometry. Briefly, the trypsinized cells were fixed and permeabilized in 500 μl of cell permeabilization solution (BD Pharmingen) for 10 min at room temperature. The cells were washed in PBS with 0.5% bovine serum albumin and 0.1% sodium azide.

The cells were incubated with primary anti-vimentin and the respective species-
specific isotype control antibodies, diluted in flow cytometry (FC) medium (DMEM, 1% FBS, 0.1% sodium azide) at 4°C for 30 min, centrifuged (2,500 rpm for 3 min), and washed twice in FC medium. The cells were then labeled with FITC-labeled secondary antibodies at 4°C for 30 min, centrifuged again, and washed twice in FC medium. The cell pellets were suspended in 200 μl of FC medium, and vimentin-expressing cells were quantified by using Calibur flow cytometer (Becton Dickinson).

Uptake of inert microparticles and Salmonella enterica serovar Typhimurium has been used as in vitro functional assay to ascertain M cells in cultures (14, 44). Similar technique was adapted to identify M-cell subsets in bovine rectal epithelial cell culture. Briefly, FITC-conjugated latex beads with a 0.2-μm pore size (Polysciences, Inc., Germany) were diluted (1:1,000) in DMEM containing 2% FBS. Aliquots (100 μl) of diluted beads were pipetted evenly onto 6-day-old cultures, followed by incubation at 37°C for 45 min; the cells were then washed three times in PBS and further incubated with Salmonella Typhimurium (pUC18GFP-labeled SL1344 strain) (multiplicity of infection [MOI] of 1:100) for 10 min. The cells were washed three times in PBS, fixed, and permeabilized with 2% (vol/vol) PFA-0.25% (vol/vol) Triton X-100 at room temperature for 20 min. Staining of F-actin was carried out with Phalloidin-647 (diluted 1:40 in PBS; Molecular Probes) for 45 min at room temperature. For colocalization studies with vimentin, the cells were incubated overnight at 4°C with mouse anti-vimen-
tin monoclonal antibody (1:100; Sigma). This primary antibody was detected with Alexa Fluor 594-tagged rabbit anti-mouse or goat anti-mouse polyclonal anti-
bodies according to the manufacturer’s instructions (Invitrogen). The cell nuclei were stained with either TO-PRO iodide or DAPI (4',6-diamidino-2-phenyl-
dole; Merck) nuclear stains. The mounted slides were examined by confocal microscopy. To examine the position of the beads, 0.4-μm optical sections were acquired and processed using the Imaris Surpuss module (Bitplane) computer software program.

Coculture of Caco-2 cells with Raji-B cells and measurement of bacterial translocation. Caco-2 cells were grown for 14 days in DMEM supplemented with 10% FBS, 15 mM L-glutamine, and 1% penicillin-streptomycin (Sigma) trans-
well polycarbonate inserts (3-μm pore size). This time allows the differentiation and development of microvilli. These cells were then cocultured with 0.5 × 10⁵ Raji-B cells in the basal compartment for 6 days (44). The TER was measured every day before adding the Raji-B cells until it became consistent at 200 to 300 Ω/cm²; however, it was not changed by coculture with Raji-B cells. Bacteria were prepared as described for the phagocytosis assays, and 100 μl of bacterial sus-
pension was added to each upper chamber. Bacterial counts from the lower chamber were determined at 60 min relative to the levels inoculated.

Kanamycin protection assay to detect intracellular E. coli. Bacteria were prepared as described for the phagocytosis assays. The bovine primary epithelial cells were washed twice with MEM-HEPES without antibiotic for 1 h before the bacterial culture reached an OD560 of 0.4, and the wells were centrifuged at 90 rpm for 1 min. The infected cells were incubated at 37°C with 5% CO₂ and moisture for 1.5 h. The bacterial suspension was removed, and the wells were washed four times with PBS to remove the nonattached bacteria. A total of 500 μl of MEM-HEPES containing 750 μg of kanamycin/ml was added to each well of the infected cells. The infected cells were incubated for 3 h and then washed three times with PBS. Then, 300 μl of 0.1% Triton X-100 (in PBS) was added to each well to lyse the cells. The bacteria were collected after scraping of the cells, serially diluted in PBS, and triplicate plated onto LB plates with appropriate antibiotics. The plates were incubated at 37°C for overnight, and the colonies were counted the next day.

### TABLE 4. Monoclonal antibodies used in immunocytochemical screening of bovine rectal primary epithelial cell cultures

<table>
<thead>
<tr>
<th>Monoclonal antibody</th>
<th>Specificity</th>
<th>Cellular expression</th>
<th>Source</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>CC21</td>
<td>CD21</td>
<td>Follicular dendritic cells, mature B cells</td>
<td>IAH</td>
<td>57</td>
</tr>
<tr>
<td>CC20</td>
<td>Bovine CD1b</td>
<td>Dendritic cells</td>
<td>IAH</td>
<td>31</td>
</tr>
<tr>
<td>CC15</td>
<td>Bovine WC1</td>
<td>γδ T cell</td>
<td>IAH</td>
<td>32</td>
</tr>
<tr>
<td>ILA-12</td>
<td>CD4</td>
<td>T helper cells</td>
<td>ILRAD</td>
<td>78</td>
</tr>
<tr>
<td>ILA-51</td>
<td>CD8</td>
<td>Cytotoxic T cells</td>
<td>ILRAD</td>
<td>78</td>
</tr>
<tr>
<td>ILA-156</td>
<td>CD40</td>
<td>B cells, antigen-presenting cells</td>
<td>ILRAD</td>
<td>63</td>
</tr>
<tr>
<td>ILA-111</td>
<td>CD25</td>
<td>Activated T and B cells, macrophages</td>
<td>ILRAD</td>
<td></td>
</tr>
<tr>
<td>ILA-43</td>
<td>CD2</td>
<td>ß T cells, natural killer cells</td>
<td>ILRAD</td>
<td>78</td>
</tr>
<tr>
<td>Anti-hPH</td>
<td>β-subunit of hPH</td>
<td>Fibroblasts</td>
<td>Acris GmbH</td>
<td>3</td>
</tr>
</tbody>
</table>
Statistical design and analyses. All statistical analyses were carried out in R (v2.10.1; R Foundation for Statistical Computing). All binding, uptake, and translocation assays were repeated on at least separate occasions. For all analyses, a similar methodology was adopted. Overall differences between strains were first assessed, and if there were statistically significant differences, then post hoc Tukey pairwise comparisons were carried out.

Three types of statistical models were used. Differences between strains in the percents of intracellular bacteria at either set time intervals, as determined by fluorescence microscopy or as a measure of phagocytosis, were examined by general linear models with binomial errors to account for the percentage nature of the data. To ensure that any differences between experiments were accounted for in the time interval analysis, which of the three experiments the data came from was also entered as a covariate. When we considered differences in the number of bacteria bound per microscopic slides between the three strains, general linear models with Poisson errors were used to account for the integer nature of the data.

For all other statistical analyses, analysis of covariance of the log_{10}-transformed number of bacteria posttranslocation was carried out to assess the differences between strains. To adjust for differences in pretranslocation levels and experiments, the number of bacteria pretranslocation and which of the three experiments the translocation carried out were entered as covariates. Log transformation was undertaken to normalize the residuals. Statistical significance was taken when P was <0.05.

RESULTS

Strain-specific susceptibility to phagocytosis by cultured macrophages. Previous research has demonstrated that EspF is able to inhibit bacterial uptake into macrophages, although the majority of the previous research examining this inhibition has focused on the EspF_{O127} variant from EPEC O127 E2348/69. Initially, EHEC O157, EHEC O26, and EPEC O127 were compared for their capacity to inhibit their nonopsonized phagocytosis into cultured macrophages. EPEC O127 bound in significantly higher numbers to the macrophages (Fig. 1A, P < 0.001), and the majority remained external (Fig. 1B and C). EHEC O26 exhibited an adherence level similar to that of EHEC O157 but significantly less than that of EPEC O127 (Fig. 1A, P < 0.001); however, it resisted uptake by macrophages in a way comparable to that of the EPEC strain (Fig. 1B and C). In contrast, EHEC O157 adhered at lower levels, and a significantly higher proportion was phagocytosed (Fig. 1B and C). For example, at 90 min after addition 63% ± 3.3% of EHEC O157 bacteria were internalized compared to only 33% ± 3.5% of EPEC O127 or 31% ± 6.3% of EHEC O26 (Fig. 1B). By 6 h after addition to the cultures, the macrophages lost their structural integrity in the presence of EPEC O127. In contrast, macrophages exposed to EHEC were still healthy at this time point, with relatively very few bacteria evident (data not shown).

Strain-specific susceptibility to phagocytosis is associated with espF allele expression. EHEC and EPEC serotypes express different variants of EspF (Fig. 2). EHEC O157 contains four polyproline repeat regions (PRRs) compared to three in EPEC O127 and EHEC O26. In addition, the three sequences differ within the polyproline repeat regions and in the amino terminus of the protein shown to be important for organelle targeting (1, 17). To determine the relative contributions of the different espF alleles in inhibiting phagocytosis, these were amplified from E. coli O157, O127, and O26 and cloned into pMB102 (Table 2) under the control of a pTAC promoter that is inducible with IPTG. The sequences were confirmed to be identical to those published in the National Center for Biotechnology Information (NCBI) database for these serotypes.
resulting in the predicted amino acid sequences shown in Fig. 2. The clones were transformed into EPEC E2349/69/H9004 \(espF\) (Table 1), and the secretion profiles were examined. All strains secreted comparable levels of the translocon protein EspD; EspF secretion was detected from all three complemented strains by Western blotting (Fig. 3B) but with potentially lower levels detectable for the O26 variant. The complemented strains were then compared in a macrophage phagocytosis assay at 90 min postinfection (Fig. 3A). All three alleles were able to complement the \(espF\) knockout (Fig. 3, \(P < 0.001\)), although the \(espF\) gene from the \(E. coli\) serotype O157 (\(espF\)O157) allele was significantly less effective at inhibiting uptake compared to the \(espF\)O127 and \(espF\)O26 alleles (Fig. 3A, \(P < 0.001\)). There were no significant differences in the adherence levels for the complemented strains (data not shown). These results indicate that the capacity of the different strains to inhibit phagocytosis correlates with the activity of the respective \(espF\) allele.

**Strain-specific variation in M-cell translocation is associated with \(espF\) variation.** Coculturing of human intestinal epithelial Caco-2 cells and Raji-B cells in vitro leads to the differentiation of a subset of epithelial cells into antigen-sampling cells (M cells) (44, 47). This in vitro “M-cell” culture system can then be used to analyze bacterial translocation and the capacity of bacteria to inhibit this trafficking (53). This trafficking is presumed to occur by transcytosis, but this and other research does not rule out the possibility of paracellular transport. Evidence for M-cell translocation in the assay is provided by comparison of translocation through a standard epithelial Caco-2 monolayer which occurs at significantly lower levels (Fig. 4A to C). To determine the contribution of \(espF\) to the translocation of EHEC O157 through this M-cell culture system, a defined deletion of \(espF\) was constructed in \(E. coli\) O157:H7 TUV93-0 (Table 1). Translocation of the EHEC O157/espF strain was significantly higher than that of the wild-type strain across both the Caco-2/Raji-B coculture and the Caco-2 monolayer. The levels of translocation were restored to those of the wild type by complementation with \(espF\)O157 in trans (Fig. 4A).

The EHEC O157, EHEC O26, and EPEC O127 strains were then compared in the same coculture translocation assay. EHEC O157 demonstrated significantly higher levels of translocation compared to EPEC O127 and EHEC O26 (Fig. 4B, \(P < 0.001\)). To examine whether the \(espF\) alleles have an effect on the level of translocation inhibition, EPEC E2349/69/H9004 \(espF\) transformed with the three amplified and cloned \(espF\) alleles was analyzed in the same assay. All three complemented the \(espF\) mutation in the EPEC/espF strain to a significant level (Fig. 4C, \(P < 0.001\)). As with the phagocytosis assay, the \(espF\)O157 allele was the least effective of the three, being significantly less effective than the \(espF\)O127 allele at inhibiting translocation. This was the case for translocation across both the Caco-2/Raji-B coculture and the Caco-2 monolayers, although translocation through the coculture system always occurred at significantly higher levels (\(P < 0.001\)). Given the

---

**Fig. 2.** Alignment of EspF amino acid sequences from EHEC O157:H7 (EDL933); EPEC O127:H6 (E2348/69), and EHEC O26:H11 (ZAP1139). The proline-rich repeats (PRRs) are boxed with EHEC O157 containing an additional fourth repeat. Amino acid differences from the EHEC O157 sequence are indicated in boldface. A binding site for SNX9 is highlighted in gray within the PRRs (1). The putative N-WASP binding region is within the middle of the PRRs (1), although the most significant sequence diversity between these variants is at the ends of each PRR. The leucine at position 16 (arrow) has been shown to be essential for EspF translocation into mitochondria, and this is changed to the similar aliphatic amino acid isoleucine in EHEC O26 (38). Analysis of NCBI \(E. coli\) O157:H7 sequences showed no significant variation in the predicted EspF amino acid sequence. The predicted EspF from an \(E. coli\) O26:H2 strain was identical to that shown for \(E. coli\) O26:H11 (data not shown).
correlation with strain origin, these differences again indicate variation in EspF activity between the different espF alleles.

The significance of espF alleles on the interaction of E. coli with primary cultures of bovine rectal epithelial cells. Previous work has established that EHEC O157:H7 colonizes the terminal rectum of cattle, the main reservoir host (60). The tissue at this site contains a high number of lymphoid follicles and M-like cells that are present in the FAE (49). Primary cells were cultured from a mixed population of crypts isolated from this bovine terminal rectum (see Materials and Methods). The cells in the monolayers expressed cytokeratins (Fig. 5A) indicative of epithelial cells. Screening with a panel of antibodies (Table 4) provided no indication of contaminating cells, such as fibroblasts (data not shown). It was apparent that a small proportion of cells expressed vimentin, an intermediate filament protein indicative of M cells (Fig. 5A and B). A subset of vimentin-expressing cells endocytosed latex fluorescent micro particles (Fig. 5C and C1). S. enterica serovar Typhimurium preferentially targets M cells in the gut (14) and therefore was used to test whether the bacteria and the latex particles were internalized by vimentin-expressing cells in culture. Indeed, during early stages of infection, Salmonella Typhimurium interacted primarily with vimentin-expressing cells in culture (Fig. 5D) that also had internalized the latex beads (Fig. 5E), a further indication that these cells can be considered M cells based on previous M-cell characterization studies (44). Taken together, these data indicate that primary cells cultured from crypts isolated from bovine rectal FAE do contain a subset of cells with characteristics of M cells and are capable of taking up particles, including bacteria.

To determine the significance espF has on the interaction of E. coli O157 with these primary cells, translocation across these primary cell cultures was then assessed for the wild-type strains, the EHEC O157 ΔespF deletion, and EPEC ΔespF strain complemented with the three different alleles. In agreement with the coculture translocation assay, espF significantly contributed to inhibition of translocation through these cultured cells (Fig. 6A); however, in clear contrast to the “human” coculture system, EHEC O157 was significantly better in the bovine assay at inhibiting translocation compared to EPEC O127 and EHEC O26 (P < 0.001) (Fig. 6B). In agreement with this, the espFO157 allele showed the highest activity in restrict-
FIG. 4. Translocation of EPEC and EHEC strains across a Caco-2 and lympho-epithelial M-cell coculture system (black columns [A, C, and E]) and Caco-2 cells only (white columns [B, D, and F]). (A and B) espF is required to inhibit EHEC O157 TUV93-0 translocation through M cells. The translocation of EHEC O157 TUV93-0 was compared to an isogenic espF deletion mutant and complemented with espF0157 (pAT1, Table 2). (C and D) Comparative translocation of EPEC O127:H6 strain E2348/69, EHEC O157:H7 ZAP198, and EHEC O26:H11 across the coculture system. (E and F) Comparative translocation of an EPEC O127ΔespF mutant complemented with the three defined espF alleles. *, **, and $$$, statistical significances of $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively.
FIG. 5. Characterization of bovine primary rectal epithelial cells. (A) Heterogeneous population of epithelial cells in a primary cell culture from the bovine terminal rectum. A 5-day-old culture was prepared and immunolabeled to detect vimentin (green), pan-cytokeratins (red), and nuclei (blue) as described in Materials and Methods. A subset of the cells expressed the intermediate filament protein, vimentin (green), which is indicative of M cells. (B) Vimentin-expressing cells were quantified by using flow cytometry from four independent primary cultures (standard deviation, ±0.3). (C) Microparticle colocalization with vimentin-expressing cells. A subset of vimentin expressing (red) cells interacted with fluorescent microparticles (green). Panel C1 shows the inset image in panel C digitally magnified by a factor of 4. The primary rectal epithelial cells were incubated with latex particles (green, 0.2 μm in size) at 37°C and 5% CO₂ for 45 min, fixed, permeabilized, and labeled with anti-vimentin (red) and TO-PRO nuclear stain (blue). (D) Orthogonal section demonstrating the combined uptake of latex particles (green) and microparticles (yellow arrow, green) by vimentin-positive cells (red) in a bovine rectal primary culture. The cells were incubated with latex particles (green) for 45 min, washed (3× PBS), and further infected with mid-log-phase S. Typhimurium strain. All three espF alleles reduced uptake significantly (Fig. 7C, P < 0.001) with the espF_{O157} variant showing significantly higher activity than the espF_{O127} (P < 0.05) and espF_{O26} (P < 0.001) alleles (Fig. 6E).

The fact that the relative activities of the espF clones are reversed in the bovine assays indicates that the differences measured between the variants are not due to expression or secretion levels. This is supported by the correlations with the relative activity levels of the parental strains in the different assays. Taken together, it is likely that the relative activities are due to different functional capacities of the EspF variants and, in turn, these are dependent on the host cell type in which the variants are acting.

Molecular basis to the comparative activity of EHEC and EPEC espF alleles. Previous work has established that EspF has a number of interacting partners in eukaryotic cells including interactions with both sorting nexin 9 (SNX9) and N-WASP. Since SNX9 is important for endocytosis dynamics and N-WASP is central to actin polymerization, we investigated by LUMIER binding assay whether differences in the interactions of the EspF variants with these proteins could account for the differences in functional levels measured in the present study. To quantify the binding activity, the three EspF variants, as well as their binding partners SNX9 (human) and N-WASP (human), were cloned into plasmids that express either protein A-tagged complexes were removed by using antibody-coated beads. Total fluorescence and captured fluorescence were measured and normalized, and z-scores for each interaction were determined as outlined in Materials and Methods and as previously published (4). The results confirmed the interaction of all of the EspF variants with both SNX9 and N-WASP (Fig. 8). The EspF_{O157} had the lowest interaction score of the three variants with luciferase-linked SNX9 and also had the lowest interaction score of the three with respect to binding to N-WASP.

**DISCUSSION**

EPEC is known to inhibit phagocytosis via inhibition of PI3K activity, and this has been shown to be due to the activity of the type III-secreted effector EspF (11, 70). Although more recent research has established that a number of secreted proteins can also function to inhibit uptake into cells, including EspB (34), EspJ (52), and EspH (18). The inhibition of EPEC translocation through M cells has been shown to be dependent...
on espF, presumably in a manner analogous to its activity on macrophages. Comparison of EspF protein sequences from EPEC O127, EHEC O26, and EHEC O157 shows a number of differences in both the localization domain and in the number and sequence of proline-rich repeats (PRRs). The aim of the present study was to investigate whether these differences affected the capacity of the EspF variants to (i) inhibit bacterial phagocytosis into macrophages, (ii) inhibit M-cell translocation in a human-derived Caco-2/Raji-B coculture system, and (iii) inhibit uptake into bovine primary epithelium, containing

FIG. 6. Interaction of EHEC and EPEC strains with cultured epithelial cells from the bovine terminal rectum. (A) espF limits EHEC O157 TUV93-0 uptake into rectal primary cells. The transcytosis levels (%) of EHEC O157 TUV93-0 were compared to an isogenic espF deletion mutant and complemented with espFO157 (pAT1, Table 2). (B) Comparative transcytosis levels (%) of wild-type strains EPEC O127:H6 E2348/69, EHEC O157:H7 TUV93-0, and EHEC O26:H11 on interaction with bovine rectal primary cells. (C) Comparative transcytosis levels (%) of an EPEC O127ΔespF mutant complemented with the three defined espF alleles. *, and ***, statistical significances of $P < 0.05$ and $P < 0.001$, respectively.
M-like cells, cultured from the terminal rectum of cattle, the predominant colonization site of EHEC O157:H7 in cattle.

Strain comparisons demonstrated that both EPEC O127 E2348/69 and E. coli O26:H11 had a much greater capacity to block nonopsonized phagocytosis into cultured murine macrophages compared to EHEC O157:H7. Both the EHEC O157 and the EHEC O26 strains adhered to the macrophages at lower levels than the EPEC O127 strain (Fig. 1). To determine whether this strain difference could be accounted for by variation in the EspF effector protein, the genes from the three

![Graph A](image1)

**FIG. 7. Interaction of EHEC and EPEC strain with cultured epithelial cells from the bovine terminal rectum.** (A) espF limits EHEC O157 TUV93-0 uptake into rectal primary cells. The intracellular levels of EHEC O157 TUV93-0 were compared to an isogenic espF deletion mutant and complemented with espF<sub>O157</sub> (pAT1, Table 2). (B) Comparative intracellular levels of wild-type strains EPEC O127:H6 E2348/69, EHEC O157:H7 TUV93-0, and EHEC O26:H11 on interaction with bovine rectal primary cells. (C) Comparative intracellular levels of an EPEC O127ΔespF mutant complemented with the three defined espF alleles. * and ***, statistical significances of P < 0.05 and P < 0.001, respectively.
have been postulated to be an important cell for the initial uptake or colonization by different enteric bacterial pathogens such as Salmonella, Shigella, and E. coli (37, 45). For EHEC O157:H7, it is a cell type that the bacteria will encounter when colonizing the terminal rectum (49, 60) and, consequently, inhibiting translocation may promote colonization, for example, by providing an initial attachment site and/or limiting bacterial presentation to the host’s immune system. The origin of M cells is unclear since studies either support their derivation from enterocytes by extrinsic stimuli (luminal antigens and/or lymphoid-follicle derived signals) or conclude that they originate from lineage-specific precursor cells (25, 72, 73, 75).

An M-cell coculture system was first defined in 1997 (44) and makes use of the capacity of Raji-B cells to signal differentiation of human colon-derived Caco-2 cells. Initial experiments demonstrated that espF from EHEC O157 is required to limit EHEC O157 translocation through M cells but that the EHEC O157 espF allele was again reduced in its capacity to limit translocation of E. coli compared to the O127 and O26 alleles. This finding mirrored the relative capacity of the specific strains to inhibit their translocation, again demonstrating the significance of the espF allele for the strain phenotype.

The strains and espF alleles were then compared on primary cells cultured from crypts isolated from the bovine terminal rectum. These cultures were further characterized in the present study. A subset of the primary cells expressed the intermediate filament protein, vimentin, that is found in rabbit intestinal M cells (13). M cells in the terminal rectum of cattle have been demonstrated to express vimentin as the predominant intermediate filament protein (49), and the same staining was apparent in the cultured primary cells. A subset of vimentin-expressing cells was also able to take up Salmonella Typhimurium (Fig. 5D) alone and S. Typhimurium and beads together (Fig. 5E).

Therefore, the bovine rectal primary cultures contain a subset of cells that express vimentin and have characteristics of M cells. There was a significant role for espF in inhibiting bacterial uptake into cells, and translocation through these cells and the activity of the strains correlated with that of the espF alleles. An important result was that, in contrast to the previous experiments on mouse macrophages and the human-derived M-cell coculture system, EHEC O157 was the most effective strain at inhibiting uptake and translocation and this correlated with the relative activity of the espF alleles (Fig. 6). Taken together, these results indicate that EHEC O157 and the espF allele are more effective at inhibiting uptake into bovine M cells than the O26 and O127 strains and alleles, a reversal of the situation observed for the interactions with murine macrophages and the human coculture system.

There are multiple phenotypes associated with EspF in addition to inhibition of phagocytosis, including inhibition of water transport and disruption of tight junctions mitochondria and the nucleolus (17, 29, 58, 80). Uptake functions are likely to be related to EspF interactions with N-WASP and SNX9 (1). N-WASP stimulates actin filament assembly by direct activation of the Arp2/3 complex, and SNX9 is essential for clathrin-coated pits (CCPs) at the late stages of vesicle formation. SNX9 binds to β2 appendages of adaptor protein complex 2 (AP-2) and interacts with clathrin and dynamin-2, two other important molecules in the endocytic process (48, 77). SNX9 has two lipid interaction domains; a phospholipid-bind-
ing region termed the phox (PX) domain, followed by a putative Bin/amphiphysin/Rvs (BAR) lipid-binding domain. The BAR domain is a banana-shaped helical dimer that senses membrane curvature and can reconfigure lipid vesicles or sheets into membrane tubules (1). SNX9 also possesses an N-terminal Src homology-3 (SH3) protein interaction region that was recently shown to bind WASP (2) and to functionally activate dynamin at CCPs. Therefore, SNX9 is an important factor in remodeling the membrane and cytoskeleton during endocytosis (1, 77). The SH3 domain of SNX9 binds to the PPRs of EspF (1, 17). Despite both direct and indirect (via SNX9) activation of N-WASP, EspF is not considered to have a role in A/E lesion formation (54, 55). To determine whether the different EspF variants have different affinities for SNX9 and N-WASP, the interactions were assayed inside transfected HEK293 cells using a LUMIER assay (4; described in Materials and Methods). This in-cell assay confirmed that all three EspF variants bound to both N-WASP and SNX9, although EspF\textsubscript{O157} showed a lower interaction score with both SNX9 and N-WASP compared to the other two variants. Whether this is due to a reduced affinity of EspF\textsubscript{O157} for these two proteins remains to be determined. A specific region in the PRRs of EspF (shaded gray in Fig. 2) interacts directly with SNX9 through its SH3 domain-binding motif. There are only minor differences in this region between the serotypes examined here, and we cannot determine whether these or their presentation, due to sequence changes in flanking regions, account for the differences observed.

The published Bos taurus sequence for SNX9 (NCBI) contains a number of changes over the human variant used in the LUMIER assays; however, these differences are mainly present in the amino terminus of the predicted bovine SNX9 and lie outside the SH3 domain. There are also very few sequence differences between the predicted Bos taurus N-WASP protein sequence (NCBI) and the human variant. Taken together, our results indicate that there are likely to be other protein interactions involving EspF that could also contribute to the host specificity demonstrated here. The greatest region of diversity between the variants lies in the motifs preceding the second and third PRRs (amino acids 110 to 118 and amino acids 157 to 165 in Fig. 2), with no established function for these regions. Future work will seek to identify other interacting partners for EspF and address how important M-cell interactions are for colonization of cattle at the terminal rectum or whether other factors may explain the tropism of the EHEC O157:H7 for this gastrointestinal site.

ACKNOWLEDGMENTS
A.T. thanks the Egyptian government for Ph.D. research support. D.L.G. and A.M. acknowledge the core support of the BBSC at the Roslin Institute. K.S. was funded by a research grant from the BBSC (15/D19613).

REFERENCES