Efa-1/LifA mediates intestinal colonization of calves by enterohaemorrhagic Escherichia coli O26 : H– in a manner independent of glycosyltransferase and cysteine protease motifs or effects on type III secretion

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Enterohaemorrhagic Escherichia coli (EHEC) comprise a group of animal and zoonotic pathogens of worldwide importance. Our previous research established that intestinal colonization of calves by EHEC serotypes O5 : H– and O111 : H– requires EHEC factor for adherence (Efa-1), also known as lymphostatin (LifA). Towards an understanding of the mode of action of Efa-1/LifA, chromosomal in-frame deletions of predicted glycosyltransferase (DXD) and cysteine protease (CHD) motifs were created in a Δstx1 derivative of EHEC O26 : H–. The magnitude and duration of faecal excretion of EHEC O26 : H– were significantly reduced by null mutation of efa-1/lifA, but were not impaired by ΔDXD or ΔCHD mutations, in contrast to observations made with truncated Efa-1/LifA mutants of Citrobacter rodentium in mice. Although C. rodentium Efa-1/LifA influences the induction of colonic hyperplasia in mice, EHEC O26 : H– Efa-1/LifA was not required for fluid accumulation or neutrophil recruitment in bovine ileal loops. In contrast to observations with EHEC O5 : H– or O111 : H– mutants, inactivation of efa-1/lifA in EHEC O26 : H– did not significantly affect adherence or secretion of type III secreted proteins that play pivotal roles in calf colonization. Lymphostatin activity could not be reliably demonstrated in lysates of EHEC O26 : H–; however, deletion of the glycosyltransferase and cysteine protease motifs in Efa-1/LifA from enteropathogenic E. coli O127 : H6 abolished lymphostatin activity. Our data uncouple the role of Efa-1/LifA in calf colonization from effects on type III secretion and reinforce the potential for pathotype- and serotype-specific phenotypes.

INTRODUCTION

Enterohaemorrhagic Escherichia coli (EHEC) comprise a subset of Shiga toxin-producing E. coli associated with acute gastroenteritis in humans (Gyles, 2007). Infections are frequently acquired by direct or indirect contact with ruminant faeces and may be complicated by haemorrhagic colitis and severe renal and neurological sequelae. Selected EHEC serotypes are also associated with diarrhoea in calves. The molecular mechanisms by which EHEC persist in the intestines of ruminants and produce pathology are incompletely understood. Targeted and genome-wide mutagenesis has revealed that the locus of enterocyte effacement (LEE)-encoded type III secretion system (T3SS) plays a key role in colonization of cattle by injection of bacterial proteins into enterocytes to form ‘attaching and effacing’ lesions (Dziva et al., 2004; van Diemen et al., 2005; Naylor et al., 2005). This requires a filamentous extension of the T3SS needle complex composed of EspA, which may also play a direct role in adherence by EHEC O26 (Ebel et al., 1998) and enteropathogenic E. coli (EPEC; Cleary et al., 2004). Interactions between the bacterial adhesin intimin and its injected receptor (Tir) are particularly significant in colonization of calves (Dean-Nystrom et al., 1998; Vlisidou et al., 2006). Studies with defined EHEC mutants in calves have established accessory roles in colonization for F9 fimbriae (Low et al., 2006), the secreted serine protease EspP (Dziva et al., 2007) and EHEC factor for adherence, Efa-1 (Stevens et al., 2002b).
Efa-1 was first identified by screening EHEC O111:H–Tn5phoA mutants for reduced adherence to CHO-K1 cells and is predicted to be a 365 kDa protein (Nicholls et al., 2000). EHEC O5:H– and O111:H– efa-1 mutants are attenuated in calves, but exhibit reduced expression and secretion of EspA and Tir (Stevens et al., 2002b). It was therefore not possible to conclude whether Efa-1 functions as an adhesin per se, owing to the potential for indirect effects on LEE function. Mutation of efa-1 in rabbit EPEC has not been reported to affect type III secretion, and Efa-1-specific antibodies labelled the bacterial surface and inhibited adherence to cultured cells, implying a direct role in adherence (Badea et al., 2003). However, in other studies, actin nucleation under adherent bacteria was taken as a measure of normal LEE function in efa-1 mutants (Nicholls et al., 2000; Klapproth et al., 2000), even though such activity can occur when type III secretion is reduced (Stevens et al., 2002b). The mode of action of Efa-1 is further complicated by the fact that it appears to be bifunctional. EHEC O111:H– Efa-1 is 97.4 % identical to EPEC O127:H6 LifA (lymphostatin), which confers upon EPEC an ability to inhibit the mitogen-activated proliferation of peripheral blood and intraepithelial lymphocytes and the synthesis of pro-inflammatory cytokines (Klapproth et al., 1995, 1996; Malstrom & James, 1998; Klapproth et al., 2000). Such activity has been reported for Efa-1/LifA in EHEC O103:H2 against bovine peripheral blood lymphocytes (Abu-Median et al., 2006).

Although data vary with geographical location and the detection methods used, presence of efa-1/lifA has been correlated with severe clinical presentation of atypical EPEC and EHEC serogroup O26 isolates (Bielaszewska et al., 2007; Afset et al., 2006). Remnants of the gene are also found in pathogenic Chlamydia species, where they are associated with cytotoxicity (Belland et al., 2001). A truncated copy of efa-1/lifA is present in the sequenced genomes of EHEC O157:H7 and forms part of an O-island associated with virulence (Morabito et al., 2003; Karmali et al., 2003). Mutation of the truncated E. coli O157:H7 efa-1 gene impaired adherence to cultured cells but not intestinal colonization of calves (Stevens et al., 2004). EHEC O157:H7 strains also contain a homologue of Efa-1/LifA on their large plasmid (ToxB). ToxB promotes adherence of EHEC O157:H7 to cultured cells and, as with efa-1/lifA in EHEC O5:H– and O111:H–, mutation of the gene impairs the expression and secretion of LEE4-encoded proteins at a post-transcriptional level (Tatsuno et al., 2001). However, ToxB was not required for intestinal colonization of calves (Stevens et al., 2004) and neither it, nor the truncated Efa-1, appears to confer lymphostatin-like activity (Abu-Median et al., 2006).

The relative importance of intrinsic adhesin activity, lymphostatin activity or indirect effects on the LEE-encoded T3SS in the ability of Efa-1/LifA to promote intestinal colonization of calves by EHEC is unknown. A need exists to uncouple these activities and to identify functional domains. The N-termini of EHEC O111:H– and EPEC O127:H6 LifA share approximately 40 % similarity over 470 amino acids to the N-terminal catalytic domain of large clostridial toxins (LCTs). The N-terminal sequence of 546 amino acids of C. difficile toxin B collapses the actin cytoskeleton by glucosylating Rho-family GTPases in a manner dependent on a DXD motif (Busch et al., 1998; Hofmann et al., 1997). Such a motif is conserved in Efa-1/LifA (amino acid residues 557–559), although effects of Efa-1/LifA on the actin cytoskeleton and glucosylation of Rho GTPases have so far not been detected (Abu-Median et al., 2006; Babbin et al., 2009). Analysis of the Efa-1/LifA primary sequence has also revealed the presence of a cysteine protease motif (C1480 H1581 D1596) that is conserved in the YopT family of secreted proteins implicated in bacterial virulence (Shao et al., 2002).

Mutation of glycosyltransferase and cysteine protease motifs of Citrobacter rodentium efa-1/lifA has been reported to impair intestinal colonization and crypt cell hyperplasia in mice (Klapproth et al., 2003). These mutations were created by βRed recombinase-mediated insertion of a kanamycin-resistance cassette in place of the motifs followed by flipase-mediated excision of the cassette to leave an in-frame scar (Klapproth et al., 2005). Close inspection reveals that the second codon of the scar is an in-frame stop codon; therefore published phenotypes relate to proteins truncated at the point of deletion, not to the motifs per se. Glycosyltransferase and cysteine protease motifs of C. rodentium Efa-1/LifA have since been deleted by another method and implicated in disruption of intestinal barrier integrity in mice via effects on Rho GTPase function (Babbin et al., 2009). However, the impact of such mutations on lymphostatin activity, adhesion, intestinal colonization and inflammation was not reported.

Here we created in-frame chromosomal deletions to evaluate the role of Efa-1/LifA glycosyltransferase and cysteine protease motifs in the ability of EHEC O26:H– to colonize bovine intestines, induce enteritis, adhere to cells and inhibit lymphocyte proliferation.

**METHODS**

**Bacterial strains, plasmids and oligonucleotides.** Strains used in the present study are listed in Table 1; they were amplified in Luria–Bertani (LB) medium supplemented as appropriate with nalidixic acid (25 μg mL−1), kanamycin (50 μg mL−1), ampicillin (100 μg mL−1) or chloramphenicol (25 μg mL−1). The pH79-based cosmid containing the EPEC O127:H6 strain E2348/29 lifA gene (pIV-8-A) has been described (Klapproth et al., 1995, 2000). Plasmid pDM4 is a positive-selection suicide replicon (oriR6K mobRP4 cat sacBR; Milton et al., 1996) and was used to create chromosomal deletions by double homologous recombination. Plasmid pCVD836txl was similarly used to create a deletion spanning stxlAB (Stevens et al., 2002a). Plasmid pDK46 encoding the stx1AB was similarly used to create a deletion spanning stxlAB (Stevens et al., 2002a). Plasmid pDK46 encoding the stx1AB was similarly used to create a deletion spanning stxlAB (Stevens et al., 2002a).
Table 1. Bacterial strains used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Description</th>
<th>Reference</th>
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<tbody>
<tr>
<td>HB101</td>
<td>Non-pathogenic E. coli K-12</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>E2348/69</td>
<td>Prototypic E. coli O127:H6</td>
<td>Levine et al. (1978)</td>
</tr>
<tr>
<td>E2348/69 H10fA</td>
<td>E2348/69 lifA non-polar deletion mutant</td>
<td>Stevens et al. (2002b)</td>
</tr>
<tr>
<td>EBK4</td>
<td>E45035N efa-1::kanR insertion mutant</td>
<td>Nicholls et al. (2000)</td>
</tr>
<tr>
<td>S102-9</td>
<td>EHEC O5:H- calf dysentery isolate</td>
<td>Stevens et al. (2002b)</td>
</tr>
<tr>
<td>S102-9 Δefa-1</td>
<td>S102-9 efa-1/lifA deletion mutant</td>
<td>Stevens et al. (2002b)</td>
</tr>
<tr>
<td>S17-1/pZip</td>
<td>E. coli K-12 host for oriR6K mobRP4 replicons</td>
<td>Simon et al. (1983)</td>
</tr>
<tr>
<td>193 nalR</td>
<td>Nalidixic acid resistant derivative of EHEC O26:H- calf isolate 193</td>
<td>van Diemen et al. (2005)</td>
</tr>
<tr>
<td>193 nalR::escN::miniTn5Km2</td>
<td>193 nalR with a transposon insertion in escN</td>
<td>van Diemen et al. (2005)</td>
</tr>
<tr>
<td>193 nalRΔstxl</td>
<td>193 nalR with a partial deletion of stxlAB</td>
<td>This study</td>
</tr>
<tr>
<td>193 nalRΔstxl efa-1/lifA::kanR</td>
<td>193 nalR Δstxl with the efa-1/lifA::kanR mutation from EBK4</td>
<td>This study</td>
</tr>
<tr>
<td>193 nalRΔstxl ΔDXD::AAA</td>
<td>193 nalR Δstxl with substitution of DXD for AAA</td>
<td>This study</td>
</tr>
<tr>
<td>193 nalRΔstxl ΔCHD::AAA</td>
<td>193 nalR Δstxl with substitution of CHD for AAA</td>
<td>This study</td>
</tr>
<tr>
<td>E2348/69ADXD::AAA</td>
<td>E2348/69 with substitution of DXD for AAA</td>
<td>This study</td>
</tr>
<tr>
<td>E2348/69ACHD::AAA</td>
<td>E2348/69 with substitution of CHD for AAA</td>
<td>This study</td>
</tr>
</tbody>
</table>

Mutagenesis of EHEC O26:H- and EPEC O127:H6 efa-1/lifA.

EHEC O26:H- strain 193 nalR was selected for this study as it efficiently colonizes the intestines of calves (van Diemen et al., 2005, 2007). A deletion spanning the stxlAB genes was first created using the positive-selection suicide replicon pCVDaXt xl, as described previously (Stevens et al., 2002a). The resulting strain (193 nalR Δstxl) was verified by Southern hybridization and PCR with primers Stxl-3 and Stxl-6 (Table 2), and was not cytoxic to VERO cells (data not shown). To create an efa-1/lifA null mutant in this strain, the efa-1/lifA::kanR mutation from EHEC O111:H- strain EBK4 was amplified by PCR using primers 88T9 and 88T14 flanking the insertion site (Nicholls et al., 2000). The amplicon was then electroporated into 193 nalR Δstxl harbouring pKD46 grown at 30 °C and induced to express λRed recombinase by addition of 10 mM l-arabinose (Datysnko & Wanner, 2000). A recombinant was selected on medium containing kanamycin and the helper plasmid cured by culture at 37 °C in the absence of ampicillin. The mutation was verified by PCR and Southern hybridization (data not shown). Use of probes specific to the 5’ end of efa-1/lifA confirmed that strain 193 nalR has a single full-length copy of the gene, with no evidence of the truncated variant present in some EHEC strains (data not shown).

To create in-frame deletions of the glycosyltransferase and cysteine protease motifs an overlapping PCR strategy was used (Horton et al., 1989). The DXD motif at residues 557–559 was removed by separately amplifying ~750 bp regions flanking the motif with primers DXD1 and DXD2 or DXD3 and DXD4 (Table 2), using Vent proof-reading polymerase (New England Biolabs). The sequence encoding DXD within overlapping primers DXD2 and DXD3 was replaced by 5'-GCCGCCGCGC3’ to simultaneously create a triple alanine substitution and a unique NotI restriction endonuclease site (underlined). To splice the amplicons, the ~750 bp products were combined in equimolar proportions and a further round of PCR performed with primers DXD1 and DXD4. The splice ~1.5 kb product was first recovered into pCR4Blunt by topoisomerase I-mediated cloning, then subcloned as a SacI fragment into pMD4. The resulting plasmid was introduced into 193 nalR Δstxl by conjugation from strain S17-1/pZip and merodiploids were selected on the basis of nalidixic acid and ampicillin.

Table 2. Oligonucleotides used in this study

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’–3’)</th>
</tr>
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<tbody>
<tr>
<td>Stxl-3</td>
<td>ATATATGAGCTCTCTAACAACATCTATTATCAG</td>
</tr>
<tr>
<td>Stxl-6</td>
<td>ATATATGAGCTCTTTGACCAAGATATGTTAAC</td>
</tr>
<tr>
<td>88T14</td>
<td>GAGACGTCGCCAGAAGAAG</td>
</tr>
<tr>
<td>88T9</td>
<td>GGTATTGTGTCATGTTTCAG</td>
</tr>
<tr>
<td>DXD1</td>
<td>ATATATGAGCTCTAGCATATATTGTTGC</td>
</tr>
<tr>
<td>DXD2</td>
<td>TCCGAGCATCATGGCCGGCGCGCGGTATATAAATACCAATTAG</td>
</tr>
<tr>
<td>DXD3</td>
<td>ATTTATACAGCGCGCGCGATGTCGCTGACTACTCT</td>
</tr>
<tr>
<td>DXD4</td>
<td>ATATATGAGCTGCACTATATTACAGCGCG</td>
</tr>
<tr>
<td>CHD1</td>
<td>ATATATGAGCTCCGCGCAGAGTTTAAATCC</td>
</tr>
<tr>
<td>CHD2</td>
<td>AATCTGAGCAGGCGGGCGCGCCGTTTGTTGTTTCAATAG</td>
</tr>
<tr>
<td>CHD3</td>
<td>AACCCCGAAGAGCAGCGCGCGAGTGCAGATCTCTGCTTCTTCA</td>
</tr>
<tr>
<td>CHD4</td>
<td>ATATATGAGCTCAGACTTACACTTAAAGCCGCC</td>
</tr>
<tr>
<td>M13 Forward</td>
<td>GTAAAACGACGCGCCAG</td>
</tr>
<tr>
<td>M13 Reverse</td>
<td>CAGGAAAAACGCTATGAC</td>
</tr>
</tbody>
</table>
chloramphenicol resistance. To confirm insertion of the suicide replicon at the expected site, genomic DNA of merodiploids was subjected to Southern hybridization using a probe specific to both the native and pDM4-encoded region of efa-1/I\(\alpha\)A. A merodiploid strain was then amplified in LB broth in the absence of plasmid selection and plated to LB agar containing 6% (w/v) sucrose but no sodium chloride at 30 °C. Sucrose-resistant chloramphenicol-sensitive bacteria are inferred to have excised the plasmid, leading to either replacement of the native allelic or the reverse integration. Colonies were screened for the former by digestion of amplicons of the DXD::AAA region with NotI. No rearrangements were detected –5 kb either side of the site of substitution by Sau3AI digestion of long-range PCR amplicons (data not shown). A ~1.5 kb amplicon spanning the substitution was amplified from the genome with primers DXD1 and DXD4, cloned in pCR-TOPO-2.1, sequenced with M13 forward and reverse primers and confirmed to be identical to the predicted sequence except for the 9 bp substitution (data not shown).

A 369 bp region encoding the cysteine protease motif (C1480 H1581 D1596) was similarly replaced by a triple alanine-ef\(\alpha\)-1 substitution by first-round amplification of flanking sequences with primers CHD1 and CHD2 or CHD3 and CHD4 (Table 2), followed by mixing of the two products and amplification of the spliced ~1.3 kb product with primers CDH1 and CHD4. The resulting amplicon was cloned in pDM4 and transferred to the chromosome of 193 nal\(\text{R}\)::\(\alpha\)t\(\text{X}\)I substitution by NotI digestion and sequencing of a ~900 bp region spanning the deletion site as above. The same constructs and procedures were used to create chromosomal DXD::AAA and DXD::AAA substitutions in EPEC O127:H6 strain E2348/69, which is predicted to have an identical sequence to the EHEC orthologues in these regions. Faithful exchange of the chromosomal allele for the variant containing substituted residues was again confirmed by sequencing. Growth kinetics of the mutant strains were sensitively measured using a Bioscreen C real-time spectrophotometer (Thermo) and were unaltered relative to the parent (data not shown).

**Calf infection studies.** Animal experiments were conducted according to the requirements of the UK Animals (Scientific Procedures) Act 1986 (licence 30/2463) with the approval of the local Ethical Review Committee. A total of 17 Friesian bull calves were used, aged ~14 days for oral challenge or ~28 days for construction of ligated ileal loops. Calves were confirmed to be culture-negative for EHEC as described previously (Stevens et al., 2004) and were fed twice daily with reconstituted dried milk and unmedicated weaner nuts, with access to water ad libitum. For oral inoculation, the 193 nal\(\text{R}\)::\(\alpha\)t\(\text{X}\)I parent strain, efa-1/I\(\alpha\)A::kan\(\text{R}\) null mutant or DXD::AAA and DXD::AAA in-frame substitution mutants were separately amplified to stationary phase in Brain-Heart Infusion broth and ~10\(^{10}\) c.f.u. given in 20 ml of 0.1% (w/v) glucose and 1% (w/v) D-\((\pm)\)-mannose. Cells were infected with 50 μl fresh culture grown in the same medium for 3.5 h and adjusted to the same optical density. Cells were washed five times with fresh medium at 3 and 6 h post-inoculation. After a total of 8 h monolayers were washed seven times with PBS, fixed and stained with Hemacolour rapid staining solutions (Merck). For each independent assay a total of 100 cells were examined at a magnification of ×400 across at least 10 randomly selected fields, and the mean number of microcolonies (comprising ten or more bacteria) per cell was determined. The LSM number of microcolonies per cell (± standard error of the LSM) from three independent experiments was calculated.

**Cell adherence assays.** Adherence of mutant and parent strains to epithelial cells was quantified by visual observation, essentially as described previously (Stevens et al., 2004). HeLa cells (ATCC CCL2) were seeded at 2 × 10\(^5\) on 22 mm glass coverslips in Dulbecco’s Modified Eagle Medium supplemented with 10% (v/v) fetal calf serum (FCS) at 37 °C in a 5% CO\(_2\) atmosphere. Prior to infection the cell culture medium was replaced with serum-free Minimal Essential Medium supplemented with 25 mM HEPES, 0.25 μM Fe(NO\(_3\))\(_2\), 0.1% (w/v) glucose and 1% (w/v) D-\((\pm)\)-mannose. Cells were infected with 50 μl fresh culture grown in the same medium for 3.5 h and adjusted to the same optical density. Cells were washed five times with fresh medium at 3 and 6 h post-inoculation. For each independent assay a total of 100 cells were examined at a magnification of ×400 across at least 10 randomly selected fields, and the mean number of microcolonies (comprising ten or more bacteria) per cell was determined. The LSM number of microcolonies per cell (± standard error of the LSM) from three independent experiments was calculated.

**Analysis of type III secreted proteins.** Strains were amplified in serum-free Eagle’s Modified Essential Medium buffered with 25 mM HEPES to late-exponential phase and supernatants passed through 0.45 μm low-protein-binding filters (Millipore). Proteins were then recovered by addition of 30 μl StrataClean resin (Agilent Technologies), which was collected by centrifugation, washed in PBS then heated in Laemmli buffer. Protein samples derived from equivalent volumes of culture supernatants were analysed by SDS-PAGE and Western blotting performed with a monoclonal antibody specific to EHEC O157 EspA as described by Stevens et al. (2002b). A TSS null mutant of 193 nal\(\text{R}\) with a transposon insertion in esc\(\text{V}\) (van Diemen et al., 2005) was included as a negative control.

**Lymphocyte proliferation assay.** The effect of the bacterial lysates on the mitogenic activity of concanavalin A (ConA; Sigma) toward bovine peripheral blood lymphocytes (PBLs) was quantified essentially as described by Abu-Median et al. (2006). PBLs were separately isolated from heparinized venous blood from six healthy Friesian calves using Histopaque 1083 (Sigma) and resuspended in RPMI 1640 medium buffered with 2 g sodium bicarbonate l\(^{-1}\) and supplemented with 20 mM HEPES, 10% (v/v) heat-inactivated FCS, 0.3 g l\(^{-1}\) glucose, nystatin, penicillin and streptomycin. Bacterial lysates were prepared from strains cultured to stationary phase in 50 ml Tryptone Soya Broth for 16 h at 37 °C. Cells were collected by centrifugation, washed and resuspended in 5 ml PBS, then disrupted by sonication (for a total of 2 min with 2 s intervals using a Sonicator XL ultrasonic processor). Lysates were centrifuged at 1000 g for 10 min at 4 °C to remove cell debris and the total protein concentration was determined by colorimetric assay using the bicinchoninic acid method (Pierce Biotechnology). Lysates were stored in single-use aliquots at −70 °C.

Isolated PBLs (2 × 10\(^5\) per well) were tested in the presence of either ConA (1 μg ml\(^{-1}\)) or ConA combined with various final concentrations of stationary-phase Brain Heart Infusion cultures; sterile medium was included as a negative control. Fluid accumulation was analysed after incubation of the loops for 12 h *in situ* and is recorded as the LSM (± standard error of the LSM) volume (V, ml)/loop length (L, cm) ratio. Neutrophil recruitment was measured by isolation of polymorphonuclear (PMN) cells from jugular blood, labelling with 111In oxide followed by reinjection into the donor calf as described previously (Stevens et al., 2002a). PMN influx denotes the ratio of total γ-radioactivity associated with the contents and mucosa of test loops relative to negative control loops inoculated with sterile medium. PMN influx was derived from triplicate determinations per calf, and the LSM PMN influx ± standard error of the LSM across four calves is presented.
of bacterial protein (0.625, 1.25 or 2.5 μg ml⁻¹). Each treatment was
tested in triplicate and cells were incubated for 4 days at 37 °C, 5% CO₂
in a humidified atmosphere. Sixteen hours before cells were harvested,
0.4 μCi (14.8 kBq) [methyl-³H]thymidine (Amersham Pharmacia
Biotech) was added. Cells were harvested using a Mach III harvester
96 (Tomtec) and [³H]thymidine uptake was determined with a Perkin-
Elmer β-scintillation counter. Mean counts per minute (c.p.m.) were
derived for each lysate and dose, then the effect of bacterial lysates on
ConA proliferation was calculated as a percentage of the response to
ConA alone [(c.p.m. ConA + lysate/c.p.m. ConA) × 100]. The LSM (± standard
error of the LSM) of the percentage ConA response is shown.

Statistical analysis. All measured traits were analysed for the effect
of strain by two-way analysis of variance (Statistical Analysis System
1995, SAS Institute). The magnitude and duration of faecal excretion
of strains were analysed after a log₁₀ transformation of the number of
excreted bacteria, with daily values taken as repeated measurements.
Where the strain was identified as a significant variable, pairwise
comparisons of least-square means were performed. P-values ≤0.05
were considered significant.

RESULTS

Efa-1/LifA, but not internal glycosyltransferase or
cysteine protease motifs, is required for intestinal
colonization of calves by EHEC O26 : H–

We created a panel of efa-1/lifA mutants in an EHEC
O26 : H– strain proven to efficiently colonize the intestines of
calves (van Diemen et al., 2005, 2007). This strain (193 nalR)
was first rendered incapable of producing Shiga toxin, as Stx1
inhibits proliferation of bovine lymphocytes (Menge et al.,
1999) and may mask lymphostatin activity in EHEC (Stevens
et al., 2002b; Abu-Median et al., 2006). Stx1 has been reported
to influence intestinal colonization of mice by EHEC
O157 : H7 (Robinson et al., 2006); however, the course of
faecal excretion of 193 nalR Δstx1 following oral inoculation
of calves did not reveal obvious attenuation relative to the parent
strain for the duration of the trial (Fig. 1). Thus, the glycosyltransferase and cysteine
protease motifs are not required for Efa-1/LifA-mediated
intestinal colonization, as previously inferred from studies
with truncated Efa-1/LifA mutants of C. rodentium in mice
(Klapproth et al., 2005).

Efa-1/LifA is not required for induction of enteritis
by EHEC O26 : H– in bovine ileal loops

Efa-1/LifA has been reported to influence crypt cell
proliferation and inflammation elicited by C. rodentium in
mice (Klapproth et al., 2005). Such phenotypes may reflect
reduced bacterial load owing to the role of Efa-1/LifA in
intestinal colonization rather than direct effects. We have
developed a bovine ligated ileal loop model to evaluate the
role of bacterial and host factors in the induction of
intestinal inflammatory and secretory responses by EHEC
(Stevens et al., 2002a; Vlisidou et al., 2004). Consistent with
these studies, fluid secretion (Fig. 2a) and recruitment of
[¹¹In]-labelled neutrophils (Fig. 2b) was significantly elevated
in loops inoculated with 193 nalR Δstx1 compared to control

Fig. 1. Efa-1/LifA influences the course of faecal excretion of
EHEC O26 : H– in a manner independent of the glycosyltransferase
and cysteine protease motifs. Mean daily faecal shedding data
from four age-matched Friesian bull calves inoculated with 193
nalR Δstx1 (diamonds), and from three calves inoculated with 193
nalR Δstx1 efa-1/lifA::kanR (squares), 193 nalR Δstx1 ΔDXD::AAA (triangles) or 193 nalR Δstx1 ΔCHD::AAA
(crosses) are shown. Bacterial recoveries from faeces were measured
twice each day and the least-square means (LSM ± standard
error of the LSM) of the combined daily log₁₀-
transformed data are presented. Samples below the limit of
detection by direct plating (2 log₁₀ c.f.u. g⁻¹) were enriched 1 : 1 in
LB then plated, giving a theoretical limit of detection of 0.6 log₁₀
 c.f.u. g⁻¹. Asterisks denote significant differences from the parent
strain at P≤0.05; † denotes P≤0.1.
loops filled with sterile medium (P-values ≤0.05). Efa-1/ LifA null or motif mutants of 193 nalR ∆stx1 produced secretory and inflammatory responses in this model that were not statistically different from the parent strain (Fig. 2).

**Mutation of efa-1/lifA in EHEC O26:H– does not affect adherence or type III secretion**

Adherence of the EHEC O26: H– Efa-1/LifA null and motif mutants to HeLa cells was compared to the parent strain by quantitative microscopy. EHEC strains S102-9 (O5: H–) and E45035N (O111: H–), and their respective efa-1/lifA null mutants S102-9 Δefa-1 and EBK4 (Table 1), were included as controls. Consistent with previous observations (Stevens et al., 2002b), null efa-1/lifA mutants of EHEC serotypes O5: H– and O111: H– exhibited a statistically significant reduction in adherence to HeLa cells (Fig. 3a; P-values ≤0.05). Unexpectedly, neither mutation of efa-1/lifA nor the ΔDXD::AAA and ΔCHD::AAA substitutions significantly affected adherence of strain 193 nalR ∆stx1 to HeLa cells (Fig. 3a). A strain 193 nalR mutant lacking the T3SS inner membrane ATPase EscN exhibited a significant reduction in adherence (Fig. 3a; P≤0.05), as expected (van Diemen et al., 2005). Fluorescent actin staining indicated that all wild-type and efa-1/lifA mutant strains were able to nucleate actin at the site of bacterial adherence (data not shown). Null and motif mutations of EHEC O26: H– efa-1/lifA did not affect the secretion of the LEE4-encoded protein EspA (Fig. 3b). This is in contrast to the reduced secretion of EspA detected in EHEC O5: H– Δefa-1/lifA and EHEC O111: H– efa-1/lifA::kanR mutants tested in parallel (Fig. 3c) and previously (Stevens et al., 2002b). These results suggest that the adherence defects previously described for the EHEC O5: H– and O111: H– efa-1/lifA null mutants may be a consequence of indirect effects on type III secretion. Further, the data suggest that attenuation of the EHEC O26: H– efa-1/lifA null mutant is unlikely to be a consequence of effects on the T3SS, which is known to be vital for colonization of calves by this strain (van Diemen et al., 2005).

**Lymphostatin activity cannot be detected in EHEC O26: H–**

Consistent with previous data (Stevens et al., 2002b; Abu-Median et al., 2006), mitogen-activated proliferation of bovine PBLS was significantly inhibited by lysates of EPEC O127: H6 strain E2348/69 or E. coli K-12 strain HB101 harbouring the E2348/69 efa-1/lifA gene on a cosmid relative to isogenic strains lacking efa-1/lifA (Fig. 4a; P-values ≤0.05 at each protein concentration). These data are consistent with effects of Efa-1/LifA on human PBLS (Klapproth et al., 2000), and confirm that the assay is able to specifically detect Efa-1/LifA activity. Although lysates of EHEC O26: H– strain 193 nalR ∆stx1 and its efa-1/lifA::kanR null mutant produced some concentration-dependent inhibition of ConA-stimulated lymphocyte proliferation, no statistically significant difference between the strains could be detected (Fig. 4b). Lysates of E2348/69 were significantly more inhibitory than those of 193 nalR ∆stx1 (P≤0.05).

**Glycosyltransferase or cysteine protease motifs of EPEC O127: H6 Efa-1/LifA are required for lymphostatin activity**

To evaluate the role of predicted catalytic motifs of Efa-1/ LifA in lymphostatin activity, the ΔDXD::AAA and ΔCHD::AAA substitutions were transferred to the chromosome of EPEC O127: H6 strain E2348/69 using the same strategy as above. The ability of lysates of these strains to inhibit the ConA-stimulated proliferation of bovine peripheral blood lymphocytes was then tested (Fig. 5). Use of lysates from the glycosyltransferase and cysteine protease motif mutants resulted in levels of lymphocyte prolifera-
tion that were significantly higher than those obtained with lysates of the parent strain (Fig. 5; $P<0.05$ at each protein concentration), indicating that Efa-1/LifA-mediated inhibition had been relieved. Levels of proliferation for cells treated with lysates of the ΔDXD::AAA and ΔCHD::AAA mutants were higher than observed for the null mutant, albeit not significantly so. The data indicate that the glycosyltransferase and cysteine protease motifs are required for lymphostatin activity of EPEC O127::H6.

**DISCUSSION**

Our data reinforce the important role of Efa-1/LifA in intestinal colonization by attaching and efacing pathogens (Stevens et al., 2002b; Klapproth et al., 2005) and advance knowledge of the mechanistic basis of the effect. A previous report assigning roles to the glycosyltransferase and cysteine protease motifs of C. rodentium Efa-1/LifA in colonization of the murine intestines and colonic hyperplasia was flawed owing to truncation of the proteins at the point of deletion (Klapproth et al., 2005; Babbin et al., 2009). Although such motifs have since been implicated in the ability of Efa-1/LifA to modulate intestinal barrier integrity in mice (Babbin et al., 2009), their role in intestinal colonization, inflammation and lymphostatin activity has not been described. Our analysis of EHEC O26::H− mutants with in-frame chromosomal deletions indicated that the glycosyltransferase and cysteine protease motifs are not essential for Efa-1/LifA-mediated colonization of the bovine intestines or cell adherence.

A significant finding of this study was that an identical efa-1::kanR mutation that impairs intestinal colonization pro tease motifs of C. rodentium Efa-1/LifA in colonization of the murine intestines and colonic hyperplasia was flawed owing to truncation of the proteins at the point of deletion (Klapproth et al., 2005; Babbin et al., 2009). Although such motifs have since been implicated in the ability of Efa-1/LifA to modulate intestinal barrier integrity in mice (Babbin et al., 2009), their role in intestinal colonization, inflammation and lymphostatin activity has not been described. Our analysis of EHEC O26::H− mutants with in-frame chromosomal deletions indicated that the glycosyltransferase and cysteine protease motifs are not essential for Efa-1/LifA-mediated colonization of the bovine intestines or cell adherence.

A significant finding of this study was that an identical efa-1::kanR mutation that impairs intestinal colonization

![Fig. 3. Mutation of Efa-1/LifA and internal glycosyltransferase and cysteine protease motifs does not affect adherence of EHEC O26::H− to HeLa cells or type III secretion. (a) Adherence of wild-type and mutant strains of EHEC serotypes O26::H−, O111::H− and O5::H− to HeLa cells. The number of microcolonies per cell was quantified in at least 10 fields comprising 100 cells and the least-square mean (± standard error of the LSM) of three independent experiments is shown. Significant differences are marked with an asterisk ($P<0.05$). (b) EspA secretion by EHEC O26::H− wild-type and isogenic efa-1/lifA null and motif mutants as detected by Western blotting. (c) EspA secretion by control efa-1/lifA null mutants of EHEC serotypes O5::H− and O111::H− compared to EHEC O26::H−.](http://mic.sgmjournals.org/)

![Fig. 4. (a) Inhibition of ConA-stimulated proliferation of bovine PBLs by lysates of EPEC O127::H6 strain E2348/69 (diamonds) and E. coli K-12 strain HB101 containing EPEC O127::H6 lifA on cosmid pIV-8-A (crosses). Significantly greater proliferation (less inhibition) was observed with lysates of the isogenic E2348/69 Δefa-1/lifA mutant (squares) or HB101 (triangles). (b) Lysates of the efa-1/lifA::kanR mutant of EHEC O26::H− strain 193 nalR Δstx1 (squares) did not significantly affect the ConA-stimulated proliferation of bovine PBLs relative to lysates of the parent strain (diamonds). Data are presented as a percentage of the proliferative response of bovine PBLs to ConA alone and represent the least-square mean of at least six biological replicates ± standard error of the LSM. Asterisks denote significant difference at $P<0.05$.](http://mic.sgmjournals.org/)
of calves by EHEC O26:H– (Fig. 1) and EHEC O111:H– (Stevens et al., 2002b) produces a reduction in the secretion of EspA in EHEC O111:H– but not EHEC O26:H– (Fig. 3c). The efa-1/lifA gene is encoded immediately downstream of the LEE4 operon in some non-O157 EHEC strains (Ogura et al., 2009), and even where such linkage does not occur, efa-1/lifA is carried by LEE-positive strains at a high frequency. The reasons why efa-1/lifA mutations affect expression and secretion of type III secreted proteins in some EHEC strains but not others require further investigation. It is possible that functional redundancy may exist, whereby loss of Efa-1/LifA in some strains may be compensated for by other proteins that are similarly able to modulate type III secretion, such as ToxB (Tatsuno et al., 2001). Indeed, we observed that EHEC O26 strain 193 harbours toxB, whereas EHEC O111 strain E45035N and EHEC O5 strain S102-9 did not contain the gene as assessed by PCR with toxB-specific primers (Tozzoli et al., 2005; data not shown). Thus ToxB may compensate for the loss of efa-1/lifA and support normal type III secretion in strain 193. LEE regulation is highly complex, and the repertoire of other regulators is also known to vary between strains. It is noteworthy that effects of efa-1/lifA mutation on adherence were seen only when effects of type III secretion were detected, and further work is needed to establish if Efa-1/LifA plays a role as an adhesin per se in some strains. We have generated antibodies directed against the N-terminal and central thirds of Efa-1; however, we have so far been unable to detect production of the full-length protein in EHEC O26:H–, although weak reactivity to a ~365 kDa protein can be observed with lysates of HB101 pIV-8-A (data not shown). Thus, the absence of detectable phenotypes may be due to poor expression under the growth conditions tested and we cannot exclude the possibility that Efa-1/LifA acts directly as an adhesin in vivo. Irrespective of the basis of the effect, our data suggest that Efa-1/LifA influences colonization of the bovine intestines by EHEC O26:H– independently of effects on type III secretion, which is known to be vital for this process (Dziva et al., 2004; van Diemen et al., 2005; Naylor et al., 2005).

The absence of detectable lymphostatin activity in EHEC O26:H– strain 193 nalR also leads one to question how widespread such activity is among attaching and effacing E. coli and whether varying activity is a consequence of expression levels. We previously detected lymphostatin activity in an EHEC O103:H2 strain (Abu-Median et al., 2006), but deletion of efa-1/lifA in EHEC O5:H– strain S102-9 did not relieve inhibition of bovine PBLs (Stevens et al., 2002b). It was inferred that this was due to the presence of Stx1 in S102-9, which is a potent inhibitor of bovine lymphocyte function (Menge et al., 1999), and it is possible that the phenotype of the efa-1/lifA mutant of EHEC O26:H– studied herein may be masked by the presence of other molecules inhibitory to bovine lymphocytes.

Although lymphostatin activity has been detected against murine intestinal intraepithelial lymphocytes ex vivo (Klapperth et al., 1996; Malstrom & James, 1998), evidence of suppression of mucosal immunity by attaching and effacing pathogens in vivo is lacking. Indeed, intra-epithelial lymphocytes exposed to EHEC O103:H2 strains in situ for 12 h in a bovine ligated intestinal loop model did not exhibit differences in proliferative capacity or cytokine production that could be attributed to lymphostatin activity (Menge et al., 2004). In the present study, no evidence was found for effects of Efa-1/LifA on intestinal inflammatory and secretory responses in a modified version of this assay (Fig. 2). As with studies in orally inoculated mice, it will prove difficult to separate effects on the bovine mucosal immune system from reduced bacterial load as a consequence of the role of Efa-1/LifA in intestinal colonization.

Although we were unable to reliably detect lymphostatin activity in the EHEC O26:H– strain studied, concentration-dependent inhibition of ConA-stimulated proliferation of bovine PBLs could be detected with lysates of EPEC O127:H6 strain E2348/69. We therefore evaluated the role of the glycosyltransferase and cysteine protease motifs in this background by adopting the same mutagenesis strategy. Motif substitutions resulted in proliferative responses that were similar to those of the efa-1/lifA null mutant and significantly higher than for PBLs exposed to a lysate of the parent strain (Fig. 5). It may therefore be inferred that the glycosyltransferase and cysteine protease motifs play a role in lymphostatin activity. Assuming that the same would be true in EHEC O26:H–, it may be inferred that lymphostatin activity of this strain (if it exists...
in vivo) is unlikely to explain the role of Efa-1/LifA in calf colonization, since no significant attenuation was seen for ΔDXD or ΔCHD substitution mutants.

It has been reported that Efa-1/LifA influences intestinal barrier integrity by modulating the function of Rho-family GTPases (Babbin et al., 2009). This has been associated with the ability of C. rodentium to translocate from the intestines to the spleen, liver and mesenteric lymph nodes (Babbin et al., 2009); however, it is unclear if the role of Efa-1/LifA in systemic dissemination can be separated from its role in intestinal colonization of mice (Klapproth et al., 2005). We observed no increase in the amount of activated RhoA in cells infected with EPEC strain E2348/69, EHEC O26 strain 193 or null efa-1/lifA mutants thereof using an ELISA specific for activated RhoA (data not shown). The assay was validated by detection of activated RhoA upon infection of cells with EPEC E2348/69 expressing the type III secreted effector EspM2, which is known to be a guanine nucleotide exchange factor for RhoA (data not shown; Arbeloa et al., 2008, 2010).

Although we have established an important role for Efa-1/LifA in colonization of the reservoir host by EHEC O26: H− and other non-O157 EHEC (Stevens et al., 2002b), we were previously unable to protect calves against challenge with the 193 nalR strain by intramuscular challenge with the 193 nalR strain by intramuscular

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