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

Victoria Deacon,^{1,2} Francis Dziva,¹ Pauline M. van Diemen,¹ Gad Frankel² and Mark P. Stevens¹

Correspondence

Mark P. Stevens

mark-p.stevens@bbsrc.ac.uk

¹Enteric Bacterial Pathogens Laboratory, Institute for Animal Health, Compton, Berkshire RG20 7NN, UK

²Centre for Molecular Microbiology and Infection, Division of Cell and Molecular Biology, Imperial College, London SW7 2AZ, UK

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 Δ *stx1* 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 Δ DXD or Δ CHD 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.

Received 12 March 2010

Revised 30 April 2010

Accepted 12 May 2010

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).

Abbreviations: EHEC, enterohaemorrhagic *E. coli*; EPEC, enteropathogenic *E. coli*; LEE, locus of enterocyte effacement; PBL, peripheral blood lymphocyte; PMN, polymorphonuclear; T3SS, type III secretion system.

Efa-1 was first identified by screening EHEC O111:H–Tn5*phoA* 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–

Efa-1 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; Babbitt *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.*, 2005). These mutations were created by λ Red recombinase-mediated insertion of a kanamycin-resistance cassette in place of the motifs followed by flippase-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 (Babbitt *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 $\mu\text{g ml}^{-1}$), kanamycin (50 $\mu\text{g ml}^{-1}$), ampicillin (100 $\mu\text{g ml}^{-1}$) or chloramphenicol (25 $\mu\text{g ml}^{-1}$). The pHCT9-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 pCVD Δ stx1 was similarly used to create a deletion spanning *stx1AB* (Stevens *et al.*, 2002a). Plasmid pDK46 encoding the γ , β and *exo* genes of bacteriophage λ under the control of an arabinose-inducible promoter (Datsenko & Wanner, 2000) was obtained from the *E. coli* Genetic Stock Center, Yale University. Plasmids pCR4Blunt and pCR-TOPO-2.1 were obtained from Invitrogen. Oligonucleotides used to create and validate bacterial mutants are listed in Table 2.

Table 1. Bacterial strains used in this study

Strain	Description	Reference
HB101	Non-pathogenic <i>E. coli</i> K-12	Invitrogen
HB101 pIV-8-A	HB101 containing EPEC O127:H6 <i>lifA</i> in cosmid pHC79	Klapproth <i>et al.</i> (1995)
E2348/69	Prototypic EPEC O127:H6	Levine <i>et al.</i> (1978)
E2348/69 Δ <i>lifA</i>	E2348/69 <i>lifA</i> non-polar deletion mutant	Stevens <i>et al.</i> (2002b)
E45035N	EHEC O111:H– human clinical isolate	Nicholls <i>et al.</i> (2000)
EBK4	E45035N <i>efa-1::kan^R</i> insertion mutant	Nicholls <i>et al.</i> (2000)
S102-9	EHEC O5:H– calf dysentery isolate	Stevens <i>et al.</i> (2002b)
S102-9 Δ <i>efa-1</i>	S102-9 <i>efa-1/lifA</i> deletion mutant	Stevens <i>et al.</i> (2002b)
S17-12pir	<i>E. coli</i> K-12 host for <i>oriR6K mobRP4</i> replicons	Simon <i>et al.</i> (1983)
193 nal ^R	Nalidixic acid resistant derivative of EHEC O26:H– calf isolate 193	van Diemen <i>et al.</i> (2005)
193 nal ^R <i>escN::miniTn5Km2</i>	193 nal ^R with a transposon insertion in <i>escN</i>	van Diemen <i>et al.</i> (2005)
193 nal ^R Δ <i>stx1</i>	193 nal ^R with a partial deletion of <i>stx1AB</i>	This study
193 nal ^R Δ <i>stx1 efa-1/lifA::kan^R</i>	193 nal ^R Δ <i>stx1</i> with the <i>efa-1/lifA::kan^R</i> mutation from EBK4	This study
193 nal ^R Δ <i>stx1</i> Δ DXD::AAA	193 nal ^R Δ <i>stx1</i> with substitution of DXD for AAA	This study
193 nal ^R Δ <i>stx1</i> Δ CHD::AAA	193 nal ^R Δ <i>stx1</i> with substitution of CHD for AAA	This study
E2348/69 Δ DXD::AAA	E2348/69 with substitution of DXD for AAA	This study
E2348/69 Δ CHD::AAA	E2348/69 with substitution of CHD for AAA	This study

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

EHEC O26:H– strain 193 nal^R was selected for this study as it efficiently colonizes the intestines of calves (van Diemen *et al.*, 2005, 2007). A deletion spanning the *stx1AB* genes was first created using the positive-selection suicide replicon pCVD Δ *stx1*, as described previously (Stevens *et al.*, 2002a). The resulting strain (193 nal^R Δ *stx1*) was verified by Southern hybridization and PCR with primers Stx1-3 and Stx1-6 (Table 2), and was not cytotoxic to VERO cells (data not shown). To create an *efa-1/lifA* null mutant in this strain, the *efa-1/lifA::kan^R* 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 nal^R Δ *stx1* harbouring pKD46 grown at 30 °C and induced to express λ Red recombinase by addition of 10 mM L-arabinose (Datsenko & 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 nal^R 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'-GCGGCCGCG-3' 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 spliced ~1.5 kb product was first recovered into pCR4Blunt by topoisomerase I-mediated cloning, then subcloned as a *SacI* fragment into pDM4. The resulting plasmid was introduced into 193 nal^R Δ *stx1* by conjugation from strain S17-12pir and merodiploids were selected on the basis of nalidixic acid and

Table 2. Oligonucleotides used in this study

Primer	Sequence (5'–3')
Stx1-3	ATATATGAGCTCTCTAACACATCTATTATCAG
Stx1-6	ATATATGAGCTCCTTGACCAGATATGTTAAGG
88T14	GAGA CTGCCAGAGAAAG
88T9	GGTATTGTTGCATGTTTCAG
DXD1	ATATATGAGCTCATGCCATATGGGTTGC
DXD2	TGCAGGCATCATCGCGCCGCTGTATAAATACCACCATG
DXD3	ATTTATACAGCGCGCCGCGATGATGCCTGCATACTCT
DXD4	ATATATGAGCTCGACATCATTAAACGCCG
CHD1	ATATATGAGCTCGGCCAGAGTGTTAATCC
CHD2	AATCTGCATGCGCGCCGCTTCTTGGGTTACCAATAG
CHD3	AACCCAAAGAAGCGGCCGCGCATGCAGATTTTCCTTTCA
CHD4	ATATATGAGCTCCAAGTACACCGGCGAG
M13 Forward	GTAAAACGACGGCCAG
M13 Reverse	CAGGAAACAGCTATGAC

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/lifA*. 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 allele or the reverse of 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-*NotI* 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^R Δ *stx1* as above. Allelic exchange was confirmed 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 Δ DXD::AAA and Δ CHD::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^R Δ *stx1* parent strain, *efa-1/lifA*::kan^R null mutant or Δ DXD::AAA and Δ CHD::AAA in-frame substitution mutants were separately amplified to stationary phase in Brain-Heart Infusion broth and ~10¹⁰ c.f.u. given in 20 ml of antacid [5% (w/v) MgO, 5% (w/v) Mg trisilicate, 5% (w/v) NaHCO₃ in double-distilled water] just before the morning feed. The magnitude and duration of faecal excretion of the bacteria were followed twice daily for 14 days by plating of triplicate serial dilutions of fresh faeces collected by rectal palpation to sorbitol MacConkey agar (Oxoid) supplemented with 2.5 µg potassium tellurite ml⁻¹ and 25 µg nalidixic acid ml⁻¹ (T-SMAC-nal; limit of detection by direct plating 2 log₁₀ c.f.u. g⁻¹). Samples that were negative by direct plating were enriched in LB medium overnight before plating to T-SMAC-nal, giving a theoretical lower limit of detection of 0.6 log₁₀ c.f.u. g⁻¹. Selected colonies recovered from calves were confirmed to have the expected genotype by PCR analysis of the mutated regions. Data presented are the least-square mean (LSM) daily faecal count (expressed as log₁₀ c.f.u. g⁻¹) from four calves for wild-type and three calves for each mutant strain.

Procedures for construction of bovine ligated ileal loops under terminal general anaesthesia are described in detail elsewhere (Stevens *et al.*, 2002a). Each strain was tested in triplicate loops in a semi-randomized order in each of four separate calves. Inocula comprised ~5 × 10⁹ c.f.u.

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 ($\pm$ 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 oxinate 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 $\pm$ standard error of the LSM across four calves is presented.

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 × 10⁵ 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% CO₂ 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(NO₃)₂, 0.1% (w/v) glucose and 1% (w/v) D-(+)-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 Hemacolor 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 ($\pm$ 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 supernatant 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 T3SS null mutant of 193 nal^R with a transposon insertion in *escN* (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 glutamine l⁻¹, 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 × 10⁵ per well) were tested in the presence of either ConA (1 µg ml⁻¹) or ConA combined with various final concentrations

of bacterial protein (0.625, 1.25 or 2.5 $\mu\text{g ml}^{-1}$). Each treatment was tested in triplicate and cells were incubated for 4 days at 37 °C, 5 % CO_2 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 $[(\text{c.p.m.}_{\text{ConA}} + \text{lysate}) / \text{c.p.m.}_{\text{ConA}}] \times 100$. The LSM ($\pm$ 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 $\log_{10}$ transformation of the number of excreted bacteria, with daily values taken as repeated measurements. Where the strain was identified as a significant variable, pair-wise 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 nal^R) 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). *Stx* 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 nal^R Δstx1 following oral inoculation of calves did not reveal obvious attenuation relative to datasets accrued for the parent strain (van Diemen *et al.*, 2005, 2007). A null *efa-1/lifA::kan^R* mutation from EHEC O111 : H– strain EBK4 (Nicholls *et al.*, 2000) was transferred to strain 193 nal^R Δstx1 by λ Red recombinase-mediated integration of a PCR amplicon. Following oral inoculation of age-matched calves, we detected a significant reduction in the number of the *efa-1/lifA::kan^R* mutant excreted in the faeces relative to the parent strain from day 7 post-inoculation (Fig. 1; *P*-values ≤ 0.05). Numbers of the mutant per g faeces fell below the limit of detection by direct plating (2 $\log_{10}$ c.f.u. g^{-1}) from 10 days post-inoculation, whereas the parent continued to be excreted at $\sim 4 \log_{10}$ c.f.u. g^{-1} (Fig. 1). The magnitude of the attenuating effect was comparable to that described for EHEC O5 : H– $\Delta\text{efa-1/lifA}$ and EHEC O111 : H– *efa-1/lifA::kan^R* mutants in calves (Stevens *et al.*, 2002b).

Overlapping PCR was used to create amplicons in which the glycosyltransferase motif (residues 557–559) and cysteine protease motif (residues 1480–1596) were each replaced by three alanine residues and a unique *NotI* restriction site. The mutated alleles were transferred to the chromosome of 193 nal^R Δstx1 by homologous recomb-

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 nal^R Δstx1 (diamonds), and from three calves inoculated with 193 nal^R Δstx1 *efa-1/lifA::kan^R* (squares), 193 nal^R Δstx1 $\Delta\text{DXD::AAA}$ (triangles) or 193 nal^R Δstx1 $\Delta\text{CHD::AAA}$ (crosses) are shown. Bacterial recoveries from faeces were measured twice each day and the least-square means (LSM $\pm$ standard error of the LSM) of the combined daily $\log_{10}$ -transformed data are presented. Samples below the limit of detection by direct plating (2 $\log_{10}$ c.f.u. g^{-1}) were enriched 1 : 1 in LB then plated, giving a theoretical limit of detection of 0.6 $\log_{10}$ c.f.u. g^{-1} . Asterisks denote significant differences from the parent strain at *P* ≤ 0.05 ; † denotes *P* ≤ 0.1 .

ination using positive-selection suicide replicons. Mutant strains were verified as described in Methods and separately administered to age-matched calves as above. No statistically significant differences were detected in the magnitude of faecal excretion of the $\Delta\text{DXD::AAA}$ or $\Delta\text{CHD::AAA}$ mutants 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 ^{111}In -labelled neutrophils (Fig. 2b) was significantly elevated in loops inoculated with 193 nal^R Δstx1 compared to control

loops filled with sterile medium (P -values ≤ 0.05). Efa-1/LifA null or motif mutants of 193 nal^R Δ *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 Δ *efa-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 Δ DXD::AAA and Δ CHD::AAA substitutions significantly affected adherence of strain 193 nal^R Δ *stx1* to HeLa cells (Fig. 3a). A strain 193 nal^R mutant lacking the T3SS inner membrane ATPase EscN exhibited a significant reduction in adherence (Fig. 3a; $P \leq 0.05$), 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– Δ *efa-1/lifA* and EHEC O111:H– *efa-1/lifA*::kan^R mutants tested in parallel (Fig. 3c) and previously (Stevens *et al.*, 2002b). The data raise the possibility 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 nal^R Δ *stx1* and its *efa-1/lifA*::kan^R 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 nal^R Δ *stx1* ($P \leq 0.05$).

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 Δ DXD::AAA and Δ CHD::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-

Fig. 2. EHEC O26:H– induces intestinal secretory and inflammatory responses independently of Efa-1/LifA in bovine ileal loops. (a) Fluid accumulation. The ratio of the volume of fluid accumulated to loop length (V/L) for each strain was derived from triplicate determinations in each calf. Values shown are the least-square mean ($\pm$ standard error of the LSM) for each strain from four calves. BHI, Brain Heart Infusion medium. (b) Neutrophil infiltration. Total ¹¹¹In-associated γ -radioactivity in the contents and mucosa of test loops is expressed as a ratio of the total activity in loops containing sterile BHI medium (PMN influx). The least-square mean PMN influx ($\pm$ standard error of the LSM) for each strain from four calves is shown. Asterisks denote significant differences from the parent strain at $P \leq 0.05$.

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 ($\pm$ standard error of the LSM) of three independent experiments is shown. Significant differences are marked with an asterisk ($P \leq 0.05$). (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-.

tion that were significantly higher than those obtained with lysates of the parent strain (Fig. 5; $P \leq 0.05$ at each protein concentration), indicating that Efa-1/LifA-mediated inhibition had been relieved. Levels of proliferation for cells treated with lysates of the $\Delta DXD::AAA$ and $\Delta CHD::AAA$ 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 effacing 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

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 $\Delta efa-1/lifA$ mutant (squares) or HB101 (triangles). (b) Lysates of the *efa-1/lifA::kan^R* mutant of EHEC O26:H- strain 193 nal^R 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 $\pm$ standard error of the LSM. Asterisks denote significant difference at $P \leq 0.05$.

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; Babbitt *et al.*, 2009). Although such motifs have since been implicated in the ability of Efa-1/LifA to modulate intestinal barrier integrity in mice (Babbitt *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/lifA::kan^R* mutation that impairs intestinal colonization

Fig. 5. Glycosyltransferase or cysteine protease motifs of EPEC O127:H6 Efa-1/LifA are required for lymphostatin activity. Inhibition of ConA-stimulated proliferation of bovine PBLs by lysates of EPEC O127:H6 strain E2348/69 (diamonds) and isogenic $\Delta efa-1/lifA$ (squares), $\Delta DXD::AAA$ (triangles) and $\Delta CHD::AAA$ (crosses) mutants was tested at final protein concentrations of 0.625, 1.25 and 2.5 $\mu\text{g ml}^{-1}$. Data are presented as percentage of the proliferative response of bovine PBLs to ConA alone and represent the least-square mean of six biological replicates $\pm$ standard error of the LSM. Asterisks denote significant differences from the parent strain at $P \leq 0.05$.

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 nal^R 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* (Klapproth *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 Δ DXD or Δ CHD 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 nal^R strain by intramuscular immunization with the N-terminal and central thirds of Efa-1 expressed in *E. coli* (van Diemen *et al.*, 2007). Antigen-specific humoral responses were detected and it is conceivable that they may mediate protection against natural exposure but not the high experimental challenge doses used (van Diemen *et al.*, 2007). Given the important role of Efa-1/LifA in calf colonization, the high degree of conservation of Efa-1/LifA in non-O157 strains and the emergence of such serogroups in cattle and humans (Bettelheim 2007), further studies using multivalent vaccines or other vaccination formulae or regimens may be warranted.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge the support of the Biotechnology & Biological Research Council, UK (doctoral training account to V.D.).

REFERENCES

- Abu-Median, A. B., van Diemen, P. M., Dziva, F., Vlisidou, I., Wallis, T. S. & Stevens, M. P. (2006). Functional analysis of lymphostatin homologues in enterohaemorrhagic *Escherichia coli*. *FEMS Microbiol Lett* **258**, 43–49.
- Afset, J. E., Bruant, G., Brousseau, R., Harel, J., Anderssen, E., Bevanger, L. & Bergh, K. (2006). Identification of virulence genes linked with diarrhea due to atypical enteropathogenic *Escherichia coli* by DNA microarray analysis and PCR. *J Clin Microbiol* **44**, 3703–3711.
- Arbeloa, A., Bulgin, R. R., MacKenzie, G., Shaw, R. K., Pallen, M. J., Crepin, V. F., Berger, C. N. & Frankel, G. (2008). Subversion of actin dynamics by EspM effectors of attaching and effacing bacterial pathogens. *Cell Microbiol* **10**, 1429–1441.
- Arbeloa, A., Garnett, J., Lillington, J., Bulgin, R. R., Berger, C. N., Lea, S. M., Matthews, S. & Frankel, G. (2010). EspM2 is a RhoA guanine nucleotide exchange factor. *Cell Microbiol* **12**, 654–664.
- Babbin, B. A., Sasaki, M., Gerner-Schmidt, K. W., Nusrat, A. & Klapproth, J. M. (2009). The bacterial virulence factor lymphostatin compromises intestinal epithelial barrier function by modulating rho GTPases. *Am J Pathol* **174**, 1347–1357.
- Badea, L., Doughty, S., Nicholls, L., Sloan, J., Robins-Browne, R. M. & Hartland, E. L. (2003). Contribution of Efa1/LifA to the adherence of enteropathogenic *Escherichia coli* to epithelial cells. *Microb Pathog* **34**, 205–215.
- Belland, R. J., Scidmore, M. A., Crane, D. D., Hogan, D. M., Whitmire, W., McClarty, G. & Caldwell, H. D. (2001). *Chlamydia trachomatis* cytotoxicity associated with complete and partial cytotoxin genes. *Proc Natl Acad Sci U S A* **98**, 13984–13989.
- Bettelheim, K. A. (2007). The non-O157 shiga-toxigenic (verocytotoxigenic) *Escherichia coli*; under-rated pathogens. *Crit Rev Microbiol* **33**, 67–87.
- Bielaszewska, M., Sonntag, A. K., Schmidt, M. A. & Karch, H. (2007). Presence of virulence and fitness gene modules of enterohemorrhagic *Escherichia coli* in atypical enteropathogenic *Escherichia coli* O26. *Microbes Infect* **9**, 891–897.
- Busch, C., Hofmann, F., Selzer, J., Munro, S., Jeckel, D. & Aktories, K. (1998). A common motif of eukaryotic glycosyltransferases is essential for the enzyme activity of large clostridial cytotoxins. *J Biol Chem* **273**, 19566–19572.
- Cleary, J., Lai, L. C., Shaw, R. K., Straatman-Iwanowska, A., Donnenberg, M. S., Frankel, G. & Knutton, S. (2004). Enteropathogenic *Escherichia coli* (EPEC) adhesion to intestinal epithelial cells: role of bundle-forming pili (BFP), EspA filaments and intimin. *Microbiology* **150**, 527–538.
- Datsenko, K. A. & Wanner, B. L. (2000). One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proc Natl Acad Sci U S A* **97**, 6640–6645.
- Dean-Nystrom, E. A., Bosworth, B. T., Moon, H. W. & O'Brien, A. D. (1998). *Escherichia coli* O157:H7 requires intimin for enteropathogenicity in calves. *Infect Immun* **66**, 4560–4563.
- Dziva, F., van Diemen, P. M., Stevens, M. P., Smith, A. J. & Wallis, T. S. (2004). Identification of *Escherichia coli* O157:H7 genes influencing colonization of the bovine gastrointestinal tract using signature-tagged mutagenesis. *Microbiology* **150**, 3631–3645.
- Dziva, F., Mahajan, A., Cameron, P., Currie, C., McKendrick, I. J., Wallis, T. S., Smith, D. G. & Stevens, M. P. (2007). EspP, a Type V-secreted serine protease of enterohaemorrhagic *Escherichia coli* O157:H7, influences intestinal colonization of calves and adherence to bovine primary intestinal epithelial cells. *FEMS Microbiol Lett* **271**, 258–264.
- Ebel, F., Podzadel, T., Rohde, M., Kresse, A. U., Krämer, S., Deibel, C., Guzmán, C. A. & Chakraborty, T. (1998). Initial binding of Shiga toxin-producing *Escherichia coli* to host cells and subsequent induction of actin rearrangements depend on filamentous EspA-containing surface appendages. *Mol Microbiol* **30**, 147–161.
- Gyles, C. L. (2007). Shiga toxin-producing *Escherichia coli*: an overview. *J Anim Sci* **85**, E45–E62.
- Hofmann, F., Busch, C., Prepens, U., Just, I. & Aktories, K. (1997). Localization of the glucosyltransferase activity of *Clostridium difficile* toxin B to the N-terminal part of the holotoxin. *J Biol Chem* **272**, 11074–11078.
- Horton, R. M., Hunt, H. D., Ho, S. N., Pullen, J. K. & Pease, L. R. (1989). Engineering hybrid genes without the use of restriction enzymes: gene splicing by overlap extension. *Gene* **77**, 61–68.

- Karmali, M. A., Mascarenhas, M., Shen, S., Ziebell, K., Johnson, S., Reid-Smith, R., Isaac-Renton, J., Clark, C., Rahn, K. & Kapur, J. B. (2003). Association of genomic O island 122 of *Escherichia coli* EDL933 with verocytotoxin-producing *Escherichia coli* seropathotypes that are linked to epidemic and/or serious disease. *J Clin Microbiol* **41**, 4930–4940.
- Klapproth, J. M., Donnenberg, M. S., Abraham, J. M., Mobley, H. L. & James, S. P. (1995). Products of enteropathogenic *Escherichia coli* inhibit lymphocyte activation and lymphokine production. *Infect Immun* **63**, 2248–2254.
- Klapproth, J. M., Donnenberg, M. S., Abraham, J. M. & James, S. P. (1996). Products of enteropathogenic *E. coli* inhibit lymphokine production by gastrointestinal lymphocytes. *Am J Physiol* **271**, G841–G848.
- Klapproth, J. M., Scaletsky, I. C., McNamara, B. P., Lai, L. C., Malstrom, C., James, S. P. & Donnenberg, M. S. (2000). A large toxin from pathogenic *Escherichia coli* strains that inhibits lymphocyte activation. *Infect Immun* **68**, 2148–2155.
- Klapproth, J. M., Sasaki, M., Sherman, M., Babbitt, B., Donnenberg, M. S., Fernandes, P. J., Scaletsky, I. C., Kalman, D., Nusrat, A. & Williams, I. R. (2005). *Citrobacter rodentium* *lifA/efa1* is essential for colonic colonization and crypt cell hyperplasia in vivo. *Infect Immun* **73**, 1441–1451.
- Levine, M. M., Berquist, E. J., Nalin, D. R., Waterman, D. H., Hornick, R. B., Young, C. R. & Stoman, S. (1978). *Escherichia coli* that cause diarrhoea but do not produce heat-labile or heat-stable enterotoxins and are non-invasive. *Lancet* **i**, 1119–1122.
- Low, A. S., Dziva, F., Torres, A. G., Martinez, J. L., Rosser, T., Naylor, S., Spears, K., Holden, N., Mahajan, A. & other authors (2006). Cloning, expression, and characterization of fimbrial operon F9 from enterohemorrhagic *Escherichia coli* O157:H7. *Infect Immun* **74**, 2233–2244.
- Malstrom, C. & James, S. (1998). Inhibition of murine splenic and mucosal lymphocyte function by enteric bacterial products. *Infect Immun* **66**, 3120–3127.
- Menge, C., Wieler, L. H., Schlapp, T. & Baljer, G. (1999). Shiga toxin 1 from *Escherichia coli* blocks activation and proliferation of bovine lymphocyte subpopulations in vitro. *Infect Immun* **67**, 2209–2217.
- Menge, C., Stamm, I., van Diemen, P. M., Sopp, P., Baljer, G., Wallis, T. S. & Stevens, M. P. (2004). Phenotypic and functional characterization of intraepithelial lymphocytes in a bovine ligated intestinal loop model of enterohaemorrhagic *Escherichia coli* infection. *J Med Microbiol* **53**, 573–579.
- Milton, D. L., O'Toole, R., Horstedt, P. & Wolf-Watz, H. (1996). Flagellin A is essential for the virulence of *Vibrio anguillarum*. *J Bacteriol* **178**, 1310–1319.
- Morabito, S., Tozzoli, R., Oswald, E. & Caprioli, A. (2003). A mosaic pathogenicity island made up of the locus of enterocyte effacement and a pathogenicity island of *Escherichia coli* O157:H7 is frequently present in attaching and effacing *E. coli*. *Infect Immun* **71**, 3343–3348.
- Naylor, S. W., Roe, A. J., Nart, P., Spears, K., Smith, D. G., Low, J. C. & Gally, D. L. (2005). *Escherichia coli* O157:H7 forms attaching and effacing lesions at the terminal rectum of cattle and colonization requires the LEE4 operon. *Microbiology* **151**, 2773–2781.
- Nicholls, L., Grant, T. H. & Robins-Browne, R. M. (2000). Identification of a novel genetic locus that is required for in vitro adhesion of a clinical isolate of enterohaemorrhagic *Escherichia coli* to epithelial cells. *Mol Microbiol* **35**, 275–288.
- Ogura, Y., Ooka, T., Iguchi, A., Toh, H., Asadulghani, M., Oshima, K., Kodama, T., Abe, H., Nakayama, K. & other authors (2009). Comparative genomics reveal the mechanism of the parallel evolution of O157 and non-O157 enterohemorrhagic *Escherichia coli*. *Proc Natl Acad Sci U S A* **106**, 17939–17944.
- Robinson, C. M., Sinclair, J. F., Smith, M. J. & O'Brien, A. D. (2006). Shiga toxin of enterohemorrhagic *Escherichia coli* type O157:H7 promotes intestinal colonization. *Proc Natl Acad Sci U S A* **103**, 9667–9672.
- Shao, F., Merritt, P. M., Bao, Z., Innes, R. W. & Dixon, J. E. (2002). A *Yersinia* effector and a *Pseudomonas* avirulence protein define a family of cysteine proteases functioning in bacterial pathogenesis. *Cell* **109**, 575–588.
- Simon, R., Preifer, U. & Puhler, A. (1983). A broad host range mobilisation system for *in vivo* genetic engineering: transposon mutagenesis in Gram-negative bacteria. *Biotechnology (N Y)* **1**, 784–791.
- Stevens, M. P., Marchès, O., Campbell, J., Huter, V., Frankel, G., Phillips, A. D., Oswald, E. & Wallis, T. S. (2002a). Intimin, Tir, and Shiga toxin 1 do not influence enteropathogenic responses to Shiga toxin-producing *Escherichia coli* in bovine ligated intestinal loops. *Infect Immun* **70**, 945–952.
- Stevens, M. P., van Diemen, P. M., Frankel, G., Phillips, A. D. & Wallis, T. S. (2002b). *Efa1* influences colonization of the bovine intestine by Shiga toxin-producing *Escherichia coli* serotypes O5 and O111. *Infect Immun* **70**, 5158–5166.
- Stevens, M. P., Roe, A. J., Vlisidou, I., van Diemen, P. M., La Ragione, R. M., Best, A., Woodward, M. J., Gally, D. L. & Wallis, T. S. (2004). Mutation of *toxB* and a truncated version of the *efa-1* gene in *Escherichia coli* O157:H7 influences the expression and secretion of locus of enterocyte effacement-encoded proteins but not intestinal colonization in calves or sheep. *Infect Immun* **72**, 5402–5411.
- Tatsuno, I., Horie, M., Abe, H., Miki, T., Makino, K., Shinagawa, H., Taguchi, H., Kamiya, S., Hayashi, T. & Sasakawa, C. (2001). *toxB* gene on pO157 of enterohemorrhagic *Escherichia coli* O157:H7 is required for full epithelial cell adherence phenotype. *Infect Immun* **69**, 6660–6669.
- Tozzoli, R., Caprioli, A. & Morabito, S. (2005). Detection of *toxB*, a plasmid virulence gene of *Escherichia coli* O157, in enterohemorrhagic and enteropathogenic *E. coli*. *J Clin Microbiol* **43**, 4052–4056.
- van Diemen, P. M., Dziva, F., Stevens, M. P. & Wallis, T. S. (2005). Identification of enterohemorrhagic *Escherichia coli* O26:H– genes required for intestinal colonization in calves. *Infect Immun* **73**, 1735–1743.
- van Diemen, P. M., Dziva, F., Abu-Median, A., Wallis, T. S., van den Bosch, H., Dougan, G., Chanter, N., Frankel, G. & Stevens, M. P. (2007). Subunit vaccines based on intimin and Efa-1 polypeptides induce humoral immunity in cattle but do not protect against intestinal colonisation by enterohaemorrhagic *Escherichia coli* O157:H7 or O26:H–. *Vet Immunol Immunopathol* **116**, 47–58.
- Vlisidou, I., Lyte, M., van Diemen, P. M., Hawes, P., Monaghan, P., Wallis, T. S. & Stevens, M. P. (2004). The neuroendocrine stress hormone norepinephrine augments *Escherichia coli* O157:H7-induced enteritis and adherence in a bovine ligated ileal loop model of infection. *Infect Immun* **72**, 5446–5451.
- Vlisidou, I., Dziva, F., La Ragione, R. M., Best, A., Garmendia, J., Hawes, P., Monaghan, P., Cawthraw, S. A., Frankel, G. & other authors (2006). Role of intimin-Tir interactions and the Tir-cytoskeleton coupling protein in the colonization of calves and lambs by *Escherichia coli* O157:H7. *Infect Immun* **74**, 758–764.

Edited by: P. H. Everest