Escherichia coli O157:H7 forms attaching and effacing lesions at the terminal rectum of cattle and colonization requires the LEE4 operon

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Enterohaemorrhagic Escherichia coli O157:H7 is a human pathogen that causes no apparent disease in cattle, its primary reservoir host. Recent research has demonstrated that E. coli O157:H7 predominately colonizes the distal few centimetres of the bovine rectum, and in this study, the LEE4 operon encoding a type III secretion system translocon and associated proteins was shown to be essential for colonization. A deletion mutant of LEE4 failed to colonize cattle, in contrast to a co-inoculated strain containing a chromosomal complement of the operon, therefore fulfilling ‘molecular’ Koch's postulates for this virulence determinant. In addition, attaching and effacing (A/E) lesions were detectable in E. coli O157:H7 microcolonies from the terminal rectum of both naturally and experimentally colonized cattle when examined by transmission electron microscopy. This study proves that type III secretion is required for colonization of cattle by E. coli O157:H7, and that A/E lesion formation occurs at the bovine terminal rectum within E. coli O157:H7 microcolonies. The research confirms the value of using type III secreted proteins as vaccine candidates in cattle.

INTRODUCTION

Enterohaemorrhagic Escherichia coli (EHEC) has emerged in developed countries over the past 20 years as an important cause of human intestinal disease. In addition to bloody diarrhoea, intestinal infection can lead to potentially fatal systemic sequelae resulting from the activity of Shiga toxins. The majority of these infections in the USA, Canada, UK and Japan are caused by E. coli O157:H7 (Nataro & Kaper, 1987). It is widely acknowledged that controlling E. coli O157:H7 within the bovine population would be an effective method of reducing transmission to humans (Stevens et al., 2002).

In common with other EHEC and EPEC (enteropathogenic E. coli), E. coli O157:H7 contains a pathogenicity island, known as the locus of enterocyte effacement (LEE), that confers the attaching and effacing (A/E) phenotype (Frankel et al., 1998; McDaniel & Kaper, 1997). The LEE encodes a type III secretion system (TTSS) (Hueck, 1998), various translocators and effectors, the outer-membrane protein intimin (Jerse et al., 1990) and its receptor, termed Tir (translocated intimin receptor) (Kenny et al., 1997). The LEE is arranged into several polycistronic operons termed LEE1 to LEE5 (Elliott et al., 1998). The LEE4 operon encodes several proteins essential for the A/E phenotype. These include SepL (Kresse et al., 2000) and EscF (Wilson et al., 2001), both essential components of the LEE TTSS, and Esps (EPEC secreted proteins) A, B, D and F (Knutton et al., 1998; Taylor et al., 1998; Wachter et al., 1999; McNamara & Donnenberg, 1998). EspA forms filamentous extensions to the TTSS with a hollow central channel through which Tir and several other effector proteins are translocated into the host cell via a pore created by EspD and EspB. Amongst the translocated effector molecules are the LEE4-encoded EspF, the LEE5-encoded Tir, as well as EspG, EspH and Map (Elliott et al., 2001; Tu et al., 2003; Kenny & Jepson, 2000),

Abbreviations: A/E, attaching and effacing; EHEC, enterohaemorrhagic Escherichia coli; EPEC, enteropathogenic Escherichia coli; Esp, EPEC secreted protein; FAE, follicle associated epithelium; LEE, locus of enterocyte effacement; MPN, most probable number; TEM, transmission electron microscopy; Tir, translocated intimin receptor; TTSS, type III secretion system.
and the non-LEE-encoded Cif, EspI/NleA and TccP (Marches et al., 2003; Mundy et al., 2004; Gruenheid et al., 2004; Garmendia et al., 2004). Other than EspF, these are all likely to be expressed in a LEE4 knock-out strain, but not secreted or translocated into host cells. Deletion of LEE4 will preclude the formation of A/E lesions, as translocation of Tir into the host cells is prevented. Importantly, intimin expression on the bacterial surface will not be inhibited, thus preserving any alternative functions of this molecule that exist in addition to its interaction with Tir. The alternative functions may include involvement in direct adherence to the intestinal epithelium (Sinclair & O’Brien, 2002).

Recently, it has been demonstrated that the terminal rectum is the primary colonization site for EHEC O157 : H7 in cattle (Naylor et al., 2003; Rice et al., 2003). This has been confirmed in slaughter cattle (Low et al., 2005), and successful colonization can be induced by a rectal swab inoculated with EHEC O157 : H7 (Sheng et al., 2004), although the factors driving the rectal tropism remain unknown. A number of experimental studies on neonatal animals or weaned-fasted animals have demonstrated the formation of A/E lesions in the gastrointestinal tract of animals (Dean-Nystrom et al., 1997, 1999, 2000; Wakes et al., 2001), but such lesions have not been shown at the principal colonization site of the terminal rectum in experimentally or naturally colonized cattle. The importance of intimin has been shown for the colonization of cattle by several groups (Cookson & Woodward, 2003; Cornick et al., 2002; Dean-Nystrom et al., 1998), and an extensive series of signature-tagged mutagenesis studies on both EHEC O157 : H7 and EHEC O26 : H11 (Dziva et al., 2004; van Diemen et al., 2005) have identified over 100 genes required for colonization of the bovine intestine, including LEE genes, regulatory genes and surface structures. In these studies, a different model from the current study was used, in which very young calves (1–2 weeks of age) were colonized without the same apparent terminal rectal tropism. In addition, it is not known whether non-O157 EHEC have a tropism for this site. Two of the E. coli O157 mutants in LEE genes, escN and map, were tested in calves alongside the parent strain, and escN, but not map, was shown to compromise colonization ability. Neither of these mutations was complemented, and recent research has highlighted potentially profound changes in the genome that can occur as a result of the genetic manipulation. Therefore, despite the fact that EHEC type III secreted proteins are being tested as a vaccine to prevent colonization of cattle by EHEC O157 : H7 (Potter et al., 2004), there has not been a definitive study testing a chromosomally complemented deletion of the TTSS or demonstrating A/E lesion formation in normal cattle. The aim of this research was: (1) to characterize the colonization potential in calves and on in vitro organ culture of a LEE4-deleted E. coli O157 : H7 strain compared against the same strain with the LEE4 operon replaced within the LEE; (2) to determine if A/E lesions are formed by E. coli O157 : H7 at the predominant colonization site of both experimentally and naturally colonized cattle.

**METHODS**

**Strains, primers and plasmids.** A summary of the strains, primers and plasmids used is provided in Table 1.

**Generation of allelic exchange vectors.** Primer pairs KO LHS 5'/3’ and KO RHS 5'/3’ were used to amplify flanking regions of

| Table 1. Strains, oligonucleotide primers and plasmids used in this study |
|---------------------------|-----------------|-----------------|
| **Strain** | **Description** |
| ZAP198 | Nalidixic acid resistant, Shiga toxin-negative E. coli O157 : H7 (Naylor et al., 2003) |
| ZAP984 | ZAP198: LEE4 replaced with kanamycin resistance gene |
| ZAP985 | ZAP984: kanamycin resistance cassette replaced with cloned LEE4 |

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<tr>
<th><strong>Primers</strong></th>
<th><strong>Sequence</strong></th>
<th><strong>Construct</strong></th>
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<td>pAJR162</td>
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<tr>
<td>Repair 3’</td>
<td>cgctgcccccctgctggctagtcttacca</td>
<td>pAJR162</td>
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<th><strong>Plasmids</strong></th>
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<tr>
<td>pIB307</td>
<td>pMAK705-based vector for allelic exchange; temperature-sensitive replicon (Blomfield et al., 1991)</td>
</tr>
<tr>
<td>pUC4K</td>
<td>Source of kanamycin resistance cassette (Pharmacia)</td>
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<tr>
<td>pH3</td>
<td>pIB307 with 5’ and 3’ LEE4 flanking regions inserted</td>
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<tr>
<td>pH10</td>
<td>pHY10 with kanamycin resistance cassette inserted between flanking regions</td>
</tr>
<tr>
<td>pAJR162</td>
<td>pIB307 with cloned LEE4 inserted</td>
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the LEE4 operon of E. coli O157:H7 (stx−) ZAP198 (Naylor et al., 2003) under standard conditions. These were cloned into the temperature-sensitive vector pBl307 (Blomfield et al., 1991), creating pHY3. A sacB-kan cassette was cloned between the flanking regions at the BamHI restriction site (Roe et al., 2003) to create plasmid pHY10. To allow subsequent ‘repair’ of any deletion, the LEE4 operon was amplified using primer pair Repair 5’/3’ by long-range PCR (Roche) and cloned into pBl307 to create construct pAR162.

Creation of the LEE4 deletion and complemented strains. The allelic exchange method of Hamilton et al. (1989) was used to delete and then repair the entire LEE4 operon from E. coli O157:H7 (ZAP198), generating strains ZAP984 and ZAP985, respectively. To create ZAP984 (ΔLEE4), plasmid pHY10 was electroporated into ZAP198 and transformants selected on Luria–Bertani (LB, Melford Laboratories, UK)/chloramphenicol (C) (30 μg ml−1) agar plates incubated at 30 °C. Transformants were passaged repeatedly in LB/kanamycin (K) (12-5 μg ml−1) broth at 42 °C to obtain co-integrates. Further passage at 30 °C in LB/K followed by replica plating on LB/K and LB/C plates allowed identification of clones in which a secondary recombination event resulted in the LEE4 operon being replaced by the sacB-kan cassette. To generate the complemented strain ZAP985, plasmid pAR162 was electroporated into ZAP984 and the above procedure repeated with the following differences: primary integrates at 42 °C were selected by C instead of K, the second set of cultures at 30 °C was performed without antimicrobial selection, and the correct clones were identified by sensitivity to both K and C on replica plates.

Validation of genetically engineered strains. Both the LEE4 deletion mutant and its complemented derivative were compared against the parent strain for their ability to grow in two liquid media. Overnight stationary phase cultures in LB broth were inoculated at a dilution of 1 in 100 into both LB broth and minimal essential medium (MEM)/HEPES (Sigma-Aldrich). Growth curves at 37 °C (aerated) for all strains were shown to be similar by measurement of OD600 (1 cm cuvette path length in a Cecil 2021 spectrophotometer). Expression of LEE-encoded proteins was assessed by the labelling of bacteria by indirect immunofluorescence. Samples of mid-exponential-phase MEM/HEPES cultures were fixed in paraformaldehyde (4 %, w/v) and processed for immunofluorescence microscopy using specific EspA and intimin antibodies (Roe et al., 2004). Larger volumes (50 ml) of cultures grown under the same conditions were used to obtain secreted protein preparations for SDS-PAGE followed by colloidal blue staining and Western blotting with anti-EspD antibody (Roe et al., 2003).

In vitro adherence of ZAP198 wild-type, the LEE4 deletion and complemented strains to bovine intestinal epithelium. ZAP198, 984 and 985 were prepared for inoculation of mucosal explants. Fresh colonies on LB agar plates were used to inoculate 5 ml MEM/HEPES that was incubated with shaking overnight (37 °C). Fresh pre-warmed MEM/HEPES (4 ml) was inoculated with 1 ml of overnight culture and incubated for 3 h (37 °C). All strains were standardized to OD600 0.5 immediately prior to application to tissue explants. Tissue was obtained from two conventionally reared, weaned Holstein/Friesian male calves aged 10 and 12 weeks. Following euthanasia with intravenous pentobarbitone, full-thickness pieces of normal terminal ileum and ileal Peyer’s patch were excised and placed into ice-cold MEM/HEPES with minimal delay. After transport to the laboratory, 4 × 4 mm pieces of intestinal mucosa were cut from the underlying muscle layers and placed on individual sterile foil pads. Pre-warmed culture medium [90 % RPMI (Gibco-BRL) and 10 %, v/v, fetal calf serum (Sigma–Aldrich)] was added until the fluid surface was in contact with the tissue edge. A 50 μl portion of standardized bacterial culture for each strain in the mid-exponential growth phase was added to the explant surface of different blocks (n = 4 for each strain), and the samples were initially flushed with 100 % O2, sealed, and then incubated in 5 %, v/v, CO2 for 5 h. Culture medium was changed every 2 h. At the end of the culture period, the samples were washed briefly in fresh culture medium and fixed in 4 %, w/v, paraformaldehyde in PBS (Oxoid). Fixed tissue was embedded in paraffin blocks and routine histological sections prepared. Bacteria on the sections were labelled by indirect immunofluorescence using rabbit anti-O157 antisera (1 in 100) (Mast Diagnostics) and FITC-conjugated goat anti-rabbit secondary antibody (1 in 1000) (Sigma–Aldrich). Tissue was counterstained with propidium iodide (1 μg ml−1) (Sigma–Aldrich) and viewed on a Leica DMLB fluorescence microscope using a 40 × objective. The proportion of fields containing bacteria adhering to intact epithelium was determined blind by assessing 10 sections from each explant. One explant per strain per tissue type per calf was assessed in this way.

Animals and experimental challenge. Experimental calf challenges were performed at the Moredun Research Institute (MRI) in containment level 3 large-animal housing facilities under Home Office licence number 60/3179. Ethical approval was obtained from the MRI Animal Experiments Committee. Calves were reared conventionally on the farm of origin until at least 2 weeks post-weaning and transported to MRI, where they were acclimatised for 1 week prior to challenge. Faecal samples from each calf prior to challenge were confirmed negative for E. coli O157:H7 by immunomagnetic separation (IMS), performed as per manufacturer’s instructions (Dynal). At the time of challenge, the mean age of the calves was 19 weeks (range 13–25 weeks). The challenge bacterial strains were grown separately in LB broth (18 h at 37 °C, with aeration) and diluted in sterile PBS to achieve an inoculum of 106 c.f.u. of each strain per animal in a total volume of 20 ml. The inoculum was administered to the calves via a stomach tube and washed down with 500 ml sterile PBS (Naylor et al., 2003).

Tissue for microscopy was obtained from separate studies. Two tissue blocks were from calves (aged 3 and 5 months) challenged with the wild-type E. coli O157:H7 strain (ZAP198) administered orally as described above. Post-mortem tissues were performed at 14 and 21 days, respectively, post challenge. The third tissue block was obtained from a naturally colonized 12 month old steer, as described previously (Naylor et al., 2003). Briefly, a known positive herd was identified by field epidemiology conducted by the Scottish Agricultural College. Faecal samples were screened on CT-SMAC plates, and an individual shedding >104 c.f.u. g−1 non-sorbitol-fermenting E. coli O157 was selected for post-mortem the following day.

Bacterial enumeration in faeces. Faeces were sampled and separated into surface and core components, as described by Naylor et al. (2003). Ten-gram quantities of faeces were suspended in 90 ml sterile PBS and serially diluted in 10-fold steps in PBS. These serial dilutions were cultured as 100 μl aliquots spread in triplicate onto both sorbitol MacConkey agar plates containing 15 μg ml−1 nalidixic acid (N-SMAC) (Oxoid) and SMAC plates containing 15 μg nalidixic acid ml−1 and 12.5 μg kanamycin ml−1 (NK-SMAC). All inoculated media were incubated overnight at 37 °C. Non-sorbitol-fermenting colonies were counted, and a colony from each sample tested for O157 LPS using a latex agglutination test kit (Oxoid). The most probable number (MPN) of recovered bacteria was determined as described previously (Naylor et al., 2003). The LEE4 deletion mutant was enumerated by the MPN obtained from NK-SMAC plates, whereas the complemented strain was enumerated by subtracting the NK-SMAC MPN from the N-SMAC MPN. Enrichment cultures of samples negative by direct culture were established from 1 ml of the neat faecal suspension added to 9 ml of LB containing 15 μg nalidixic acid ml−1. Following 37 °C incubation for 24 h, 100 μl of enrichment culture was spread onto N-SMAC and NK-SMAC plates and incubated overnight at 37 °C.
**Electron microscopy.** Blocks chosen for processing had \(>10^4\) c.f.u. cm\(^{-2}\), correlating with similar levels per gram in faeces. No *E. coli* O157:H7 microcolonies were detected on tissues from which the levels of *E. coli* O157 were below \(1 \times 10^8\) cm\(^{-2}\). Blocks were formalin fixed and paraffin-embedded (Wales *et al*., 2001). Sections (5 \(\mu\)m) were prepared for immunofluorescence analysis using anti-O157 antisera (Mast Diagnostics). From this, the positions of *E. coli* O157 microcolonies within the tissue blocks were determined. In total, three such regions from two animals were then deparaffinized with xylene, rehydrated in graded dilutions of ethanol, and post-fixed in osmium tetroxide. After dehydration in graded dilutions of acetone, the samples were infiltrated and embedded in Araldite. Seven ultrathin sections (80 nm) were cut from each of the three regions identified above and mounted on copper grids (Agar Scientific). Random regions negative for *E. coli* O157 microcolonies were similarly processed, and no A/E lesions were detectable by transmission electron microscopy (TEM). Specimens were viewed and photographed on a Philips CM12 transmission electron microscope. Tissue blocks from experimentally challenged calves and a naturally colonized steer described previously (Naylor *et al*., 2003) were processed by this method.

**RESULTS**

**Examination of LEE4 phenotype in wild-type, deletion and complemented strains**

The presence or absence of *LEE4*-encoded proteins in the wild-type and derived strains (ZAP984 and ZAP985) was confirmed by two methods. The first involved direct labelling of EspA filaments on whole bacterial cells using immunofluorescence. Both the parent and the complemented strains produced EspA filaments under culture conditions (MEM/HEPES) known to stimulate expression of *LEE*-encoded factors (Roe *et al*., 2003). No filaments were observed in the *LEE4*-deleted strain ZAP984 (Fig. 1A–C). In comparison, intimin was detected on the surface of all three strains by indirect immunofluorescence, indicating that the genetic manipulation of *LEE4* did not prevent the production of the adjacent *LEE5* proteins (data not shown).

Secreted proteins from these strains cultured in MEM/HEPES were harvested and examined by SDS-PAGE. Colloidal blue staining of secreted proteins demonstrated a marked reduction in levels produced by the deletion mutant (Fig. 1D). Western blotting for EspD confirmed the absence of this *LEE4*-encoded protein from the deletion mutant and its presence in the supernatant fluids from the wild-type and complemented strains (Fig. 1E). Several other proteins were absent from the secretion profile of the deletion strain but present in the parent and complemented strain preparations.

**Bacterial adherence to bovine tissue explants**

The wild-type and both derived strains were placed on tissue explants of ileal Peyer’s patch, containing a high proportion of follicle associated epithelium (FAE), and non-Peyer’s patch terminal ileum containing no FAE. The level of adherent bacteria on intact epithelium was determined (Fig. 2). For both tissue types, the level of adherence was significantly decreased for the *LEE4*-deletion and restored to wild-type levels for the complemented strain.

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**Fig. 1.** Phenotypic confirmation of *LEE4* deletion and complementation. Immunofluorescence staining of EspA filaments: (A) wild-type *E. coli* O157:H7 ZAP198; (B) ∆*LEE4* (ZAP984); (C) ∆*LEE4* complemented with *LEE4* (ZAP985). (D) Colloidal blue staining of supernatant proteins: lanes as above.
Experimental colonization of weaned calves

The LEE4 deletion and complemented strains were simultaneously inoculated orally into six calves, and faecal samples obtained daily until day 14 post challenge. Bacterial counts for both the faecal-surface and core samples were calculated for the two strains. The level of shedding and therefore colonization within an animal using the surface counts (Fig. 3A, B), and the surface : core ratio provide information on terminal rectal-restricted colonization (Fig. 3C). Shedding by the LEE4-deleted strain was impaired severely relative to the complemented strain. This strain was not detected from any of the calves beyond day 2 post challenge, indicating a total failure to establish mucosal colonization. In contrast, the complemented strain was consistently shedding beyond day 7 post challenge in four of the six calves. This difference in colonization ability was significant, as determined by Fisher’s exact test (one-sided) with a P value of 0.03. In separate calf experiments, the parent strain has been shown to establish persistent infections (S. W. Naylor and others, unpublished results), and shedding of the LEE4 mutant was clearly reduced when compared to this strain: for example, the same oral challenge dose of the wild-type strain results in faecal shedding (>10^3 c.f.u. ml^-1) beyond 5 days in 17/22 animals (77 %), compared to 0/6 for the LEE4 deletion in this study. However, as the experiments were not carried out under identical conditions, the extent of attenuation caused by deletion of LEE4 cannot be stated conclusively. It is clear however that trans-complementation of the LEE4 mutant

Fig. 2. Adherence of wild-type (ZAP198), ΔLEE4 (ZAP984) and LEE4-complemented (ZAP985) E. coli O157:H7 strains to bovine explant tissues. COMP, complemented; KO, knockout; L4, LEE4; PP, Peyer’s patch; TI, non-Peyer’s patch terminal ileum; WT, wild-type. Assays were carried out as described in Methods. The percentage of fields containing adherent bacteria on intact epithelium is shown by the black bar area. Numbers above the bars show the ratio of positive fields to the total counted. Error bars show the standard error of the between-section mean for each strain (n=10).

Fig. 3. Experimental colonization of weaned calves. The MPN (c.f.u. g^-1) of each strain within surface faecal samples from six calves orally inoculated with (A) the ΔLEE4 E. coli O157:H7 (ZAP984) strain and (B) its LEE4 chromosomal complement (ZAP985). Individual calves were designated A to F: ○, A; ●, B; □, C; ■, D; △, E; ▲, F. A y axis value of 0 represents samples negative by broth enrichment and giving no c.f.u. by direct plating. (C) ZAP985 data with the surface : core ratio plotted against the surface count. This only includes samples in which at least one of the separated components contained 10^2 c.f.u. g^-1 or greater. Samples with negative core components are assigned a default ratio of 10^3. Three different post-challenge time ranges are indicated.
enhances colonization markedly, confirming a key role for LEE4 in intestinal colonization.

The temporal pattern of terminal rectal-restricted colonization exhibited by the complemented strain was qualitatively similar to that observed for the parent strain in our calf colonization model (unpublished data). As indicated in Fig. 3C, the surface:core ratio was low at early time points, but in almost all samples beyond day 6 post challenge, the ratio was greater than 10, indicating that the majority of E. coli O157:H7 within these samples originated from the terminal rectal mucosa (Naylor et al., 2003).

Electron microscopy

Previous research has demonstrated the terminal rectum as the principal site of colonization for E. coli O157:H7 in cattle (Naylor et al., 2003). LEE-mediated A/E lesions have not been demonstrated at this important site in naturally or experimentally colonized cattle. Therefore, TEM was carried out on terminal rectal mucosa obtained from two animals experimentally colonized with the wild-type strain and one naturally colonized steer. Initially, immunofluorescence was used to locate regions containing E. coli O157 microcolonies that were reprocessed for electron microscopy. A/E lesions were detected in all positive regions examined (n = 21) from the three animals (Fig. 4). No attached bacteria were detected in regions negative by immunofluorescence (data not shown). E. coli O157:H7 microcolonies at the terminal rectum comprise bacteria that intimately attached to the epithelial surface (Fig. 4). Cells with attached bacteria display the typical features of an effaced brush border and clear pedestal formation below the attached bacteria.

DISCUSSION

In this study, the role of the E. coli O157:H7 LEE4 operon in the colonization of cattle is examined by the use of allelic exchange for both deletion and complementation. The data clearly confirm the role of LEE4 in mediating the colonization of calves in a model that reproduces the restricted colonization of the terminal rectum described previously in both experimentally challenged and naturally colonized cattle (Naylor et al., 2003). A strain lacking LEE4, which encodes factors essential for the translocation of Tir and other effector proteins, failed to colonize conventionally reared, weaned calves. When the LEE4 operon was exchanged back into the deletion strain, this restored the capacity of the strain to secrete effector proteins, produce EspA filaments, adhere to bovine epithelium in vitro and colonize cattle at the terminal rectum. As the distribution of a LEE among A/E pathogens has been clearly demonstrated (McDaniel et al., 1995), the present study completes ‘molecular’ Koch’s postulates (Falkow, 2004) for this determinant in the E. coli O157:H7 colonization of the main natural host, cattle.

A common deficiency in many previous animal studies is the lack of any form of molecular complementation. To assess the functional requirement of a micro-organism for genes or groups of genes, the region of interest can be deleted. This manipulation can introduce unknown but significant mutations. To ensure that this has not occurred, it has become established practice to complement the mutation with the region of interest. This is most commonly achieved by transformation with a plasmid including the cloned region. However, this approach has a number of limitations, including plasmid stability and copy number. Therefore, single-copy chromosomal complementation is more desirable, as it can replace the region of interest into its original site. In this study, the whole of LEE4 was deleted and the complement engineered to precisely replace the operon. To our knowledge, this is the first time that this approach has been used for the assessment of a bacterial colonization determinant in vitro and in vivo. Moreover, comparison of mutant and complement shedding profiles within the same animals removed between-animal variation in the experiment, and therefore limited the number of study animals required. One possible limitation with the
approach taken in this study is that in order to complement back the wild-type LEE4 region into the strain containing the deletion and compare direct lineage strains in the experimental colonization study, the deletion strain contained a sacB-kan cassette. This also marked the strain for comparison with the complement. While there is no evidence in the literature that high sucrose levels would be present in the bovine gastrointestinal tract, it is possible that the sacB gene could have a negative impact on the strain. Against this, viable bacteria containing the cassette were still detected in the faeces at early time points following oral inoculation, yet no colonization at the terminal rectum occurred (Fig. 3A).

TEM of E. coli O157 microcolonies confirmed the presence of A/E lesions for the first time in a naturally colonized animal, and for the first time at the terminal rectum of experimentally colonized animals. A high level of colonization is required to locate microcolonies by this method. The affinity of E. coli O157 : H7 for the terminal rectal mucosa results in sufficiently high levels to locate A/E lesions. In addition, lesions were detected within individuals exhibiting sustained colonization, rather than the transient widespread distribution likely to occur soon after a high-dose oral challenge (e.g. Dean-Nystrom et al., 1999). A/E lesions were detected in all regions containing E. coli O157 microcolonies and not in regions where E. coli O157 was absent. Taken together with the LEE4 data, these images confirm the importance of type III secretion and A/E lesion formation in the colonization of cattle by E. coli O157 : H7. Our findings support the investigation of type III secreted proteins as vaccine candidates to limit colonization of cattle (Potter et al., 2004).

Extensive work by a number of groups has shown the importance of intimin in the colonization of animals by EHEC (Cornick et al., 2002; Dean-Nystrom et al., 1998, 2002; Judge et al., 2004; McKee et al., 1995), but it is unclear from previous work whether its role is limited to Tir interaction or to binding to host cellular receptors, such as nucleolin (Sinclair & O’Brien, 2002). The present study has demonstrated that in the absence of type III secretion, but with intimin expressed, the bacteria are unable to colonize cattle, confirming the pivotal role of the intimin–Tir interaction. By comparison with other studies, the absence of a type III secretion system appears to have a more profound effect on colonization than either intimin (Cornick et al., 2002) or Tir (Stevens et al., 2004) deletions alone. This implies a role for other type III translocated effector proteins in ruminant colonization. While Map has been shown not to be essential for colonization in cattle (Dziva et al., 2004), a number of other secreted proteins have been identified in attaching and effacing pathogens (Kenny et al., 1997; Knutton et al., 1998; Taylor et al., 1998; Wachter et al., 1999; McNamara & Donnenberg, 1998; Elliott et al., 2001; Tu et al., 2003; Kenny & Jepson, 2000; Marches et al., 2003; Mundy et al., 2004; Gruenheid et al., 2004; Garmendia et al., 2004) and some of these may be translocated by E. coli O157 : H7 into host cells. Most evidence from cell culture and mouse model systems points to translocated effector proteins modifying host cell functions to favour bacterial persistence in the host: for example, EspF inhibiting apoptosis and disrupting tight junctions (Crane et al., 2001; McNamara & Donnenberg, 1998). These effects may be essential to ensure that any adherent E. coli O157 : H7 are able to survive within a hostile niche, regardless of the mechanism of adherence.

All three strains were compared for attachment to ileal explant cultures, and these studies confirmed the importance of LEE4 for attachment over a 5 h incubation period. While the LEE4 deletion would need to be tested by direct application to the terminal rectum of cattle to confirm an essential role for type III secretion in the colonization of this site by E. coli O157 : H7, the combined colonization, explant and microscopy data presented here do confirm a key role for type III secretion at the bovine terminal rectum. Our ongoing research suggests an increased affinity of E. coli O157 : H7 for other FAE-rich sites along the gastrointestinal tract, and we cannot rule out that initial localization at these or other sites increases the likelihood of terminal rectal colonization. An analogous situation has been demonstrated recently for Citrobacter rodentium in mice (Wiles et al., 2004). Ileal FAE was used for the explant studies rather than terminal rectal FAE, as the latter cannot be reliably obtained (Mahajan et al., 2005). FAE contains M or M-like cells that sample antigens from the gastrointestinal lumen (Owen, 1999; Nicoletti, 2000). All bacteria in the gut may be sampled by these cells, but certain bacteria, such as Salmonella, Shigella, EPEC and EHEC may use them to invade or establish colonization (Siebers & Finlay, 1996). The type III secretion system is likely to be a key factor in the interaction of these bacteria with this cell type: for example, inhibiting function to allow colonization or usurping function to allow invasion.

The electron micrographs show classical brush border effacement and degeneration of the cells to which the bacteria are attached, and the host response to and role in this pathology are currently being investigated. Ongoing research into the epidemiology of E. coli O157 : H7 and shedding dynamics supports the concept of ‘supershedders’, a small proportion of E. coli O157 : H7-positive animals that contribute the majority of environmental load (Low et al., 2005). These animals are shedding in excess of $10^8$ c.f.u. (g faeces)$^{-1}$ and exhibit the terminal rectal colonization previously observed. Specific treatment of this animal subset or vaccination approaches to prevent supershedding, for example using preparations including EHEC type III secreted proteins, are likely to prevent cattle-to-cattle transmission and protect human health.

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