

An investigation of the expression and adhesin function of H7 flagella in the interaction of *Escherichia coli* O157 : H7 with bovine intestinal epithelium

Arvind Mahajan,^{1*} Carol G. Currie,² Shona Mackie,² Jai Tree,¹ Sean McAteer,¹ Iain McKendrick,³ Tom N. McNeilly,² Andrew Roe,⁴ Roberto M. La Ragione,⁵ Martin J. Woodward,⁵ David L. Gally¹ and David G. E. Smith^{2,6*}

¹Division of Infection and Immunity, The Roslin Institute, Royal (Dick) School of Veterinary Studies, University of Edinburgh, Pentlands Science Park, Roslin, Midlothian, EH25 9EZ, UK.

²Microbial-Cellular Interactions Group, Moredun Research Institute, Pentlands Science Park, Midlothian, Pentlands Science Park, Midlothian, EH26 0PZ, UK.

³Biomathematics and Statistics Scotland (BioSS), James Clerk-Maxwell Building, The King's Buildings, Edinburgh EH9 3JZ, UK.

⁴Division of Infection and Immunity, Institute of Biomedical and Life Sciences, University of Glasgow, Glasgow G12 8QQ, UK.

⁵Department of Food and Environmental Safety, Veterinary Laboratories Agency (VLA), Weybridge, New Haw, Addlestone KT15 3NB, UK.

⁶Institute for Comparative Medicine, Faculty of Veterinary Medicine, University of Glasgow, Glasgow G61 1QH, UK.

Summary

Enterohaemorrhagic *Escherichia coli* O157 : H7 is a bacterial pathogen that can cause haemorrhagic colitis and haemolytic uremic syndrome. In the primary reservoir host, cattle, the terminal rectum is the principal site of *E. coli* O157 colonization. In this study, bovine terminal rectal primary epithelial cells were used to examine the role of H7 flagella in epithelial adherence. Binding of a *fliC_{H7}* mutant O157 strain to rectal epithelium was significantly reduced as was binding of the flagellated wild-type strain following incubation with H7-specific antibodies. Complementation of *fliC_{H7}* mutant O157 strain with *fliC_{H7}* restored the adherence to

wild-type levels; however, complementation with *fliC_{H6}* did not restore it. High-resolution ultra-structural and immunofluorescence studies demonstrated the presence of abundant flagella forming physical contact points with the rectal epithelium. Binding to terminal rectal epithelium was specific to H7 by comparison with other flagellin types tested. In-cell Western assays confirmed temporal expression of flagella during O157 interaction with epithelium, early expression was suppressed during the later stages of microcolony and attaching and effacing lesion formation. H7 flagella are expressed *in vivo* by individual bacteria in contact with rectal mucosa. Our data demonstrate that the H7 flagellum acts as an adhesin to bovine intestinal epithelium and its involvement in this crucial initiating step for colonization indicates that H7 flagella could be an important target in intervention strategies.

Introduction

Enterohaemorrhagic *Escherichia coli* (EHEC) can cause serious infections ranging in symptoms from diarrhoea and haemorrhagic colitis to life-threatening complications including haemolytic uraemic syndrome and thrombocytopenic purpura (Karmali *et al.*, 1983a; Riley *et al.*, 1983; Paton and Paton, 1998). EHEC serotype O157 : H7 was first recognized as an emerging human pathogen in two outbreaks of haemorrhagic colitis in 1982 (Bitzan *et al.*, 1993; Nauschuetz, 1998) and since then it has been associated with many food-borne outbreaks worldwide. Infection can be fatal and morbidity long-term (Karmali *et al.*, 1983b). *E. coli* O157 : H7 strains are estimated to cause around 73 480 cases and 60 deaths per annum in the USA (Mead *et al.*, 1999). In addition to the human cost, the economic impact of preventing food chain transmission, especially in USA where screening occurs routinely and contaminated material condemned, along with the costs in controlling outbreaks are vast.

Cattle are the principal reservoir of *E. coli* O157 : H7 and most disease outbreaks have been associated with the consumption of meat or dairy products as well

Received 17 April, 2008; revised 15 September, 2008; accepted 17 September, 2008. *For correspondence. E-mail david.g.e.smith@moredun.ac.uk or a.mahajan@ed.ac.uk; Tel. (+44) 131 650 8827; Fax (+44) 131 445 5770.

as water, fruit and vegetables contaminated with cattle faeces or manure (Borczyk *et al.*, 1987; Orskov *et al.*, 1987; Locking *et al.*, 2001; O'Brien *et al.*, 2001; Hussein, 2007). Recent surveys of cattle presented for slaughter in the USA and UK have revealed faecal prevalence rates of *E. coli* O157 : H7 ranging from approximately 5% to approximately 30% (Elder *et al.*, 2000; Paiba *et al.*, 2002), indicating that there is high risk for contamination of the food chain and environment. Recent studies have shown that *E. coli* O157 : H7 colonization of the bovine gastrointestinal tract is primarily associated with the last few centimetres of the terminal rectal mucosa (Naylor *et al.*, 2003; Rice *et al.*, 2003; Low *et al.*, 2005; Cobbold *et al.*, 2007). Expression of specific bacterial adhesin(s) or cell receptor(s) may account for this tropism. A knowledge of such factors could help in designing effective control strategies and a reduction in the carriage of EHEC in ruminants is likely to lead to a decline in the incidence of human EHEC infections (Jordan *et al.*, 1999; Matthews *et al.*, 2006).

Escherichia coli O157 : H7 possesses an array of adhesins that could play a role in intestinal colonization. Proteins associated with the LEE-encoded type III secretion system (T3SS) including EspA, intimin and other proteins are required for intimate adherence and formation of characteristic attaching and effacing (A/E) lesion (McDaniel and Kaper, 1997; Frankel *et al.*, 1998). Numerous other factors including outer membrane proteins (Iha, OmpA) (Tarr *et al.*, 2000; Torres and Kaper, 2003), fimbrial proteins (curli, LPF1, LPF2, F9, ECP) (Torres *et al.*, 2002; Uhlich *et al.*, 2002; Dziva *et al.*, 2004; Jordan *et al.*, 2004; Kim and Kim, 2004; Low *et al.*, 2006; Rendon *et al.*, 2007), proteases (StcE, EspP) (Dziva *et al.*, 2007; Grys *et al.*, 2005) and Efa1 (Nicholls *et al.*, 2000; Stevens *et al.*, 2002) have been identified as contributing to *E. coli* O157 : H7 adherence in various experimental systems.

Although a substantial amount of data has been generated in recent years regarding the interaction of *E. coli* O157 : H7 with host cells, so far type III secretory proteins are the only O157 : H7 virulence determinants demonstrated to play a role in intestinal colonization of cattle *in vivo* (Dean-Nystrom *et al.*, 1998; Cornick *et al.*, 2002; Dziva *et al.*, 2004; Naylor *et al.*, 2005; Sheng *et al.*, 2006; Vlisidou *et al.*, 2006; Bretschneider *et al.*, 2007). The role of many T3SS-dependent factors is likely to be limited to intimate adherence and effacement (Donnenberg *et al.*, 1993; McKee *et al.*, 1995; Tzipori *et al.*, 1995). Thus, factors conferring the initial interaction of EHEC with intestinal epithelium remain to be clearly defined.

A role in adherence to epithelial cells has also been described in certain bacteria for flagella, primarily a motility organelle. For example, *Pseudomonas aeruginosa* (Arora *et al.*, 1998), *Clostridium difficile* (Tasteyre *et al.*, 2001), *Burkholderia pseudomallei* (Inglis *et al.*, 2003), *Aeromonas* spp. (Kirov *et al.*, 2004) and *Listeria monocyto-*

genes (Dons *et al.*, 2004). Recently, evidence has been presented to support a role for *E. coli* flagella in epithelial colonization not merely via motility/chemotaxis but directly as an adhesin. Specifically, flagella of enteropathogenic *E. coli* of several serotypes [including O127 : H6 (E2348/69), O119 : H6, O128 : H2 and O127 : H40], were directly implicated in promotion of adherence and formation of microcolonies on HeLa or Hep-2 cells (Giron *et al.*, 2002). Of note, these H-types are present on various *E. coli* pathotypes including EHEC (<http://www.lugo.usc.es/ecoli/>). Earlier flagella of EHEC O157 : H7 has also been shown to play a role in persistent colonization of chicks (Best *et al.*, 2005). More recently, H7 flagellin has been demonstrated to bind to bovine mucus potentially via muc2 (Erdem *et al.*, 2007a). Together, these findings indicate that the general contribution of flagella as adherence organelles cannot be overlooked.

In this investigation, we focused specifically on *E. coli* O157 : H7, the major EHEC serotype in many countries, to determine the extent to which H7 flagella play a role in direct adherence to epithelium at the terminal rectum of cattle, the site for which this bacterium shows a clear tropism. We demonstrate that H7 flagella promote adherence to terminal rectum epithelial cells not only through motility/chemotaxis but as a primary adherence organelle. Deletion of *fliC_{H7}* from *E. coli* O157 significantly reduced bacterial adherence to rectal epithelium as did incubation of the parental strain with H7-specific antibodies. To address the specificity of H7 binding, we complemented the *E. coli* O157 *fliC* mutant with both *fliC_{H6}* and *fliC_{H7}*, with only H7 restoring adherence to wild-type levels. As H7 flagella were downregulated following contact with epithelium as microcolonies formed and conversely T3SS upregulated, this work shows that these flagella contribute to initiating the colonization process of the bovine host.

Results

E. coli O157 lacking flagella exhibited diminished adherence to bovine rectal epithelium

Following demonstration of terminal rectal mucosa as the principal site of *E. coli* O157 : H7 colonization in cattle by us and others (Naylor *et al.*, 2003; Rice *et al.*, 2003; Low *et al.*, 2005; Cobbold *et al.*, 2007), we developed a primary epithelial cell culture *in vitro* system to investigate factors that contribute to the colonization of terminal rectal epithelium of cattle. To examine the possible role of H7 as an adhesin, the adherence of an *E. coli* O157 : H7 flagellate strain (NCTC 12900, termed MCI24), and its *fliC* mutant (MCI25) (Best *et al.*, 2005) to bovine rectal primary epithelial cells was compared. The average growth rate of wild type and mutant strains were indistinguishable, and

phenotypic characterization of these strains for the expression of flagella was confirmed via motility assay, microscopy and immunoblotting (Best *et al.*, 2005).

At 3 h the parental strain MCI24 showed localized adherence, with abundant expression of flagella and substantial microcolony formation (Fig. 1A and C) while the aflagellate MCI25 strain adhered sparsely and expressed no flagella (Fig. 1B and D). Quantification of colony forming units revealed that wild-type MCI24 adhered at statistically significant higher mean levels than the *fliC* mutant MCI25 at both 1 and 3 h post infection ($P < 0.001$) (Fig. 1E). It must be noted that each primary cell culture is unique and reflects the source animal tissue. We measured considerable variation in adherence levels of *E. coli* O157 : H7 between primary cell culture batches; however, with multiple repeats and appropriate statistical analysis valid comparisons were made.

To determine whether this lower initial adherence of the *fliC* mutant was due to loss of motility, binding assays were carried out in which bacterial cells were centrifuged onto bovine rectal primary epithelial cells. Following slow-speed centrifugation coupled with a short incubation of 15 min, adherent bacteria were enumerated. Compared with adherence without centrifugation, the mild centrifugation significantly enhanced mean binding of both the flagellate parent strain MCI24 and the *fliC* mutant MCI25 strain ($P < 0.001$), but the mean adherence for MCI24 remained significantly higher than that for the *fliC* mutant (MCI25; $P < 0.001$) (Fig. 1F), indicative that H7 flagellin actively contributes to adherence.

To further confirm the role of flagella in adherence of *E. coli* O157 : H7 to bovine gut, *ex vivo* binding studies were carried out using tissue explants from the terminal rectum mucosa. On explants the parent flagellate strain MCI24 formed large and compact microcolonies (Fig. S1A) in comparison with the *fliC* mutant strain (MCI25) that exhibited sparse adherence and only occasional microcolonies (Fig. S1B). For strain MCI24 (flagellate), flagella expression was apparent for some of the bacteria present as single cells but not within microcolonies.

Flagella antiserum inhibited E. coli O157 : H7 binding to bovine rectal primary epithelial cell

To confirm the role of H7 in adherence, binding inhibition assays were carried out in the presence of anti-H7 antibody. MCI24 and its *fliC* mutant MCI25 were treated with anti-H7 polyclonal antibody (1:10 dilution; Mast Diagnostics) for 30 min prior to infection of epithelial cells, and after 1 h incubation with cells the adherent bacteria were enumerated. Rabbit H7-antiserum statistically significantly inhibited the mean binding of flagellate parental strain MCI24 ($P < 0.001$) but had no effect on the *fliC* mutant MCI25 ($P = 0.89$) (Fig. 2A). In the presence of

bovine sera containing anti-H7 antibodies the mean binding of both strains was statistically significantly reduced, but the reduction in mean binding for the flagellate strain (MCI24) (Fig. 2B) was statistically significantly greater than that for the aflagellate mutant (MCI25) ($P = 0.02$). Pre-immune sera from cattle were used as a control.

Isolated flagella possessed adhesive properties and inhibited bacterial binding

On the basis of the above data, it was postulated that the flagella of *E. coli* O157 may possess adhesive properties *per se*. To examine this, purified flagella from EHEC serotypes O157 : H7, O26 : H11 and O113 : H21 (Fig. 3I) were incubated with bovine rectal primary epithelial cells for 3 h and bound flagella were detected by immunofluorescence (IF) using type-specific anti-H antibodies after thorough washing to remove loosely associated flagella. Of the three flagella types examined, only the H7 flagella bound to the bovine rectal epithelial cells at detectable levels (Fig. 3IIA). Neither H11 nor H21 flagella could be observed in association with rectal epithelium (Fig. 3IIB and C), demonstrating that of these three flagella types, only H7 flagella directly adheres to rectal epithelium.

To further confirm that H7 acts as an adhesin, and that the inhibition exhibited by anti-H7 reagents is not due to occlusion of other adhesins, the capacity of purified H7 flagella to inhibit *E. coli* O157 : H7 binding to rectal primary epithelial cells was determined. After pre-treatment of cells for 30 min with purified flagella, adherence of *E. coli* O157 : H7 was decreased. Although there was much variability associated with the different flagella doses in different experimental replicates and no apparent dose-dependent pattern, there was a statistically significant decrease in mean adherence observed at a concentration of $0.25 \mu\text{g ml}^{-1}$ when compared with mock flagella (prepared from MCI25)-treated control cells ($P = 0.007$) (Fig. 3IID).

To further confirm specificity of H7 as adhesin, we repeated the binding studies on bovine rectal primary epithelial cells with the wild-type *E. coli* O157 : H7 (MCI24), the *fliC* mutant (MCI25) and the *fliC* mutant (MCI25) complemented with *fliC*_{H6} (MCI25-H6) and *fliC*_{H7} (MCI25-H7). FliC H6 was chosen for the complementation studies due to its known differential binding properties (Giron *et al.*, 2002; Erdem *et al.*, 2007a). The wild-type MCI24 and the complemented *fliC*_{H7} (MCI25-H7) adhered at statistically significant higher mean levels than the *fliC* mutant MCI25 ($t_{5,8} = 2.47$; $P = 0.05$) ($t_{5,8} = 3.00$; $P = 0.025$) respectively. However, there was no significant difference in adherence of the non-flagellated (MCI25) and the complemented *fliC*_{H6} (MCI25-H6) strain ($t_{5,8} = 1.77$; $P = 0.13$) (Fig. 4C). The complemented MCI25-H6

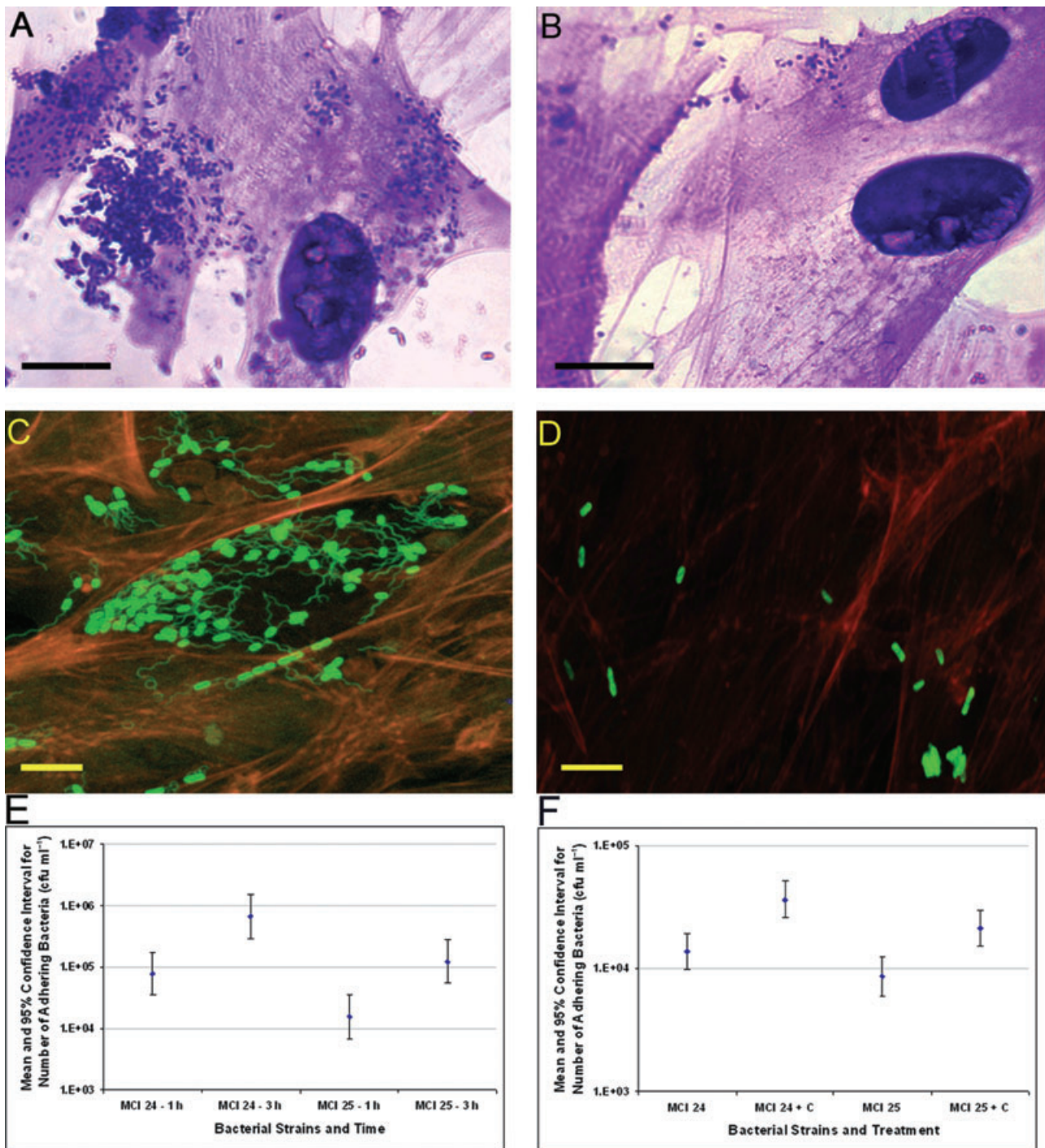


Fig. 1. Adherence of wild-type *E. coli* O157:H7 strain MCI24 and the isogenic *fliC* mutant MCI25 to bovine rectal primary epithelial cells. The cells were infected with bacterial cultures in mid-log phase growth at an approximate multiplicity of infection of 100 for 1 and 3 h at 37°C, 5% CO₂ and the adherence pattern was visualized by Diff-Quik (A and B) and immunofluorescent staining (C and D). MCI24 (FliC+) showed localized adherence (A) and most of the adherent bacteria expressed flagella – seen as wavy green filaments (C). MCI25 (FliC-) showed sparse adherence (B) and no expression of flagella (D). To enumerate adherent bacteria these were solubilized with PBS-0.1% Triton X-100, serially diluted and plated to enumerate colony-forming units (cfu). Mean MCI24 adherence was significantly higher than that for the aflagellate strain at 1 and 3 h post infection ($P < 0.001$) (E). Mild centrifugation (1000 r.p.m., 3 min) although significantly enhanced mean adherence of both the strains ($P < 0.001$), but mean adherence of MCI24 remained statistically significant higher than MCI25 ($P < 0.001$) (F). The results shown represent the average of three separate experiments. For IF, epithelial cells were stained with actin-specific phalloidin-TRITC (red) and nuclear stain TO-PRO (blue). The bacteria labelled with anti-O157 and anti-H7 antibodies were detected with secondary FITC (green) conjugated antibody. Scale bar, 2.5 μ m.

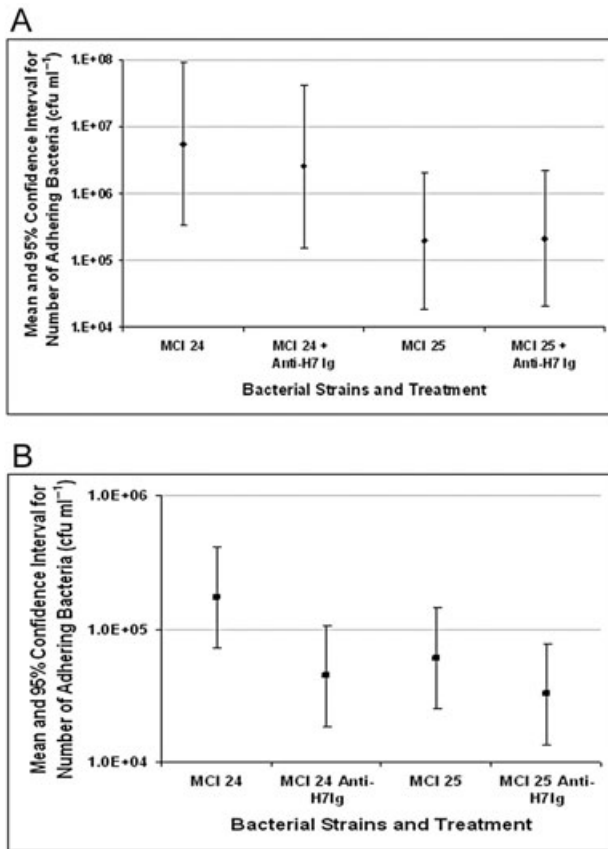


Fig. 2. Binding of *E. coli* O157:H7 to bovine rectal epithelial cells is inhibited by type-specific flagellin antiserum. Wild-type flagellate strain MCI24 and isogenic *fliC* mutant MCI25 in the mid-log phase of growth were pre-treated with anti-H7 polyvalent antisera (1:10) from rabbit (A) and cattle (B) at RT for 30 min before infecting the cells at an approximate multiplicity of infection of 100 at 37°C, 5% CO₂ for 1 h. Adherent bacteria were solubilized with PBS-0.1% (v/v) Triton X-100, serially diluted and enumerated as colony forming units (cfu). In the presence of anti-H7 rabbit antisera the mean binding of the flagellate strain (MCI24) was significantly decreased ($P < 0.001$) with no significant effect on the aflagellate mutant (MCI25) ($P = 0.89$). In the presence of anti-H7 bovine antisera the mean binding of both strains was statistically significantly reduced, but the reduction in mean binding for the flagellate strain (MCI24) was statistically significantly greater than that for the aflagellate mutant (MCI25) ($P = 0.02$). Pre-immune sera from the same cattle were used as a control. Figure represents the apparent statistical significance of the effect of antibody because of a positive correlation between the estimates of the two means, which is not apparent in the confidence intervals.

and MCI25-H7 strains expressed specific flagella types and exhibited comparable motility phenotypes (Fig. 4A and B).

To ascertain if the difference in the adhesive properties of H6, H7, H11 and H21 flagellin filaments is due to structural difference between the FliC subunits, a comparison of the amino acid sequence of the FliC proteins was done (Fig. S3). The amino acid residues at the N- and C-terminal regions of D0–D1 domain were highly conserved across different FliC proteins. However, a high

degree of variability at the central D2–D3 domain region may have accounted for the differential binding properties of H7 FliC protein.

E. coli O157:H7 expressed flagella during attachment to rectal epithelium

Upon adherence to bovine rectal primary epithelial cells *E. coli* O157:H7 strain (MCI66) abundantly expressed flagella (Fig. 5). In these immunofluorescent micrographs flagella can be seen as filamentous structures on the cell surface extending from bacterial poles (Fig. 5A) and forming an extensive network between adjacent bacteria (Fig. 5C). Flagella appeared to mediate direct interaction with the epithelial cells, forming contact points directly at the apical surface of epithelial cells as observed in colocalization (dual labelling) studies (Fig. 5B and D). Some of the bacterial cells were observed apparently using these flagellar appendages to anchor to epithelial cells, therefore presumably playing a role during early bacterial–epithelial interaction(s) (Fig. 5B, D1 and D2).

Interaction of flagella with infected bovine rectal epithelial cells was further examined by scanning electron microscopy (SEM). The long filament resembling flagella can be seen intercalated within the microvilli (Fig. 6A and B). The identity of these structures was confirmed to be H7 flagella by immunogold labelling and high-resolution field SEM using anti-H7 antibodies and anti-rabbit IgG conjugated 10 nm gold particles (Fig. 6C1 and D1). At the contact points with the epithelial cell the flagellar surface was not apparently accessible for immuno-gold labelling, and were evident only as unlabelled intercepts on the otherwise uniformly labelled flagella (Fig. 6C and D). Expression of flagella *in situ* was then investigated to determine whether flagella were expressed during interaction of *E. coli* O157:H7 with the bovine gut. Terminal rectal tissue from a calf colonized with *E. coli* O157:H7 following experimental infection was processed for electron microscopy. Individual bacteria expressing flagella-like appendages at the poles were visible adhering to the rectal epithelium (Fig. 6E and F).

Together these fluorescent and electron microscopic observations provide support to the postulate that H7 flagella act as an adherence organelle for bovine rectal epithelial cells.

Temporal expression of flagella during *E. coli* O157:H7 adherence

Initial observations at 3 h post infection showed flagella expression by individual or small clusters of *E. coli* O157:H7 but not larger compact groups or microcolonies on adherence to bovine rectal primary epithelial cells (Fig. 71B); therefore, we reasoned that expression of

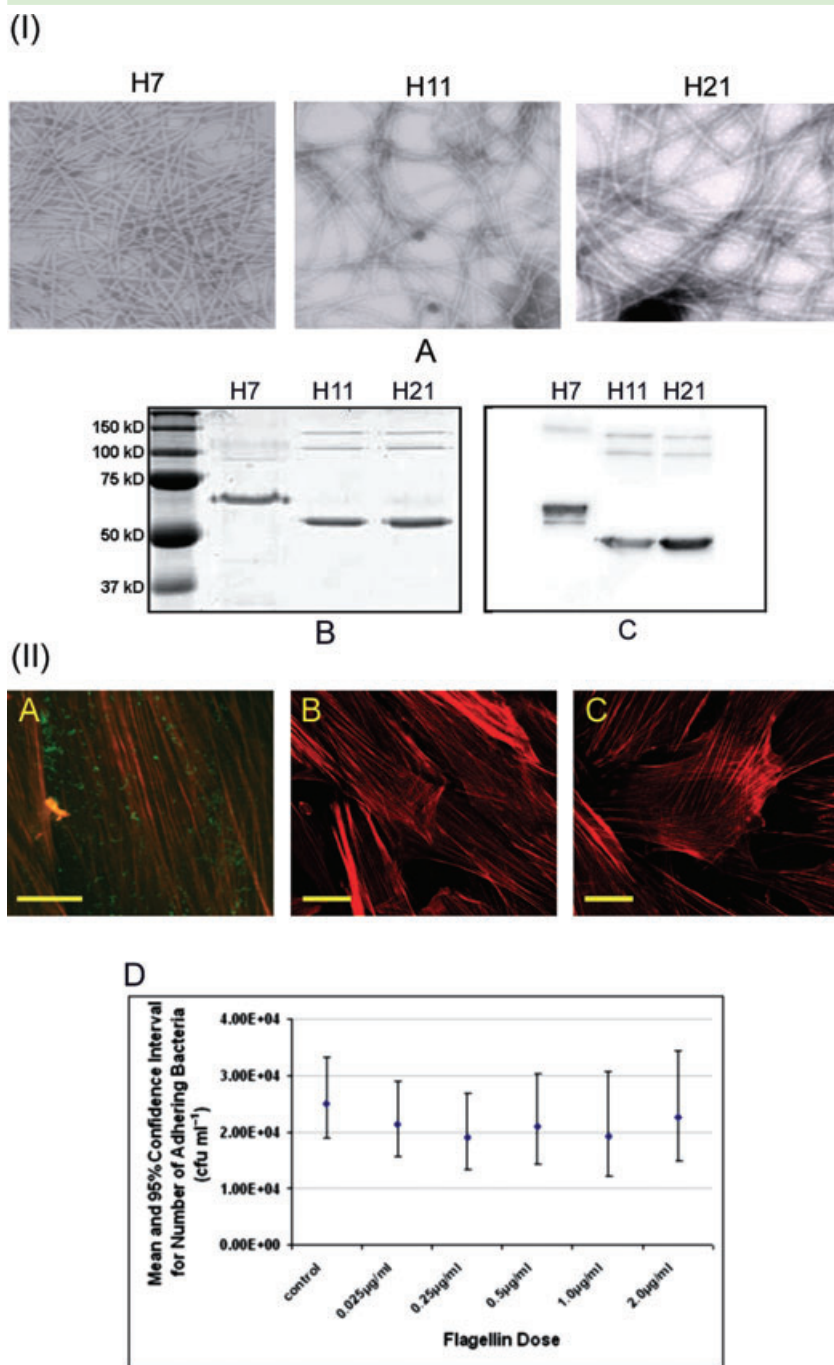


Fig. 3. Binding of purified flagella H7, H11 and H21 to bovine rectal primary epithelial cells.

I. Electron micrographs of mechanically sheared flagellin filaments from *E. coli* O157 : H7, *E. coli* O26 : H11 and *E. coli* 113 : H21 (A). The isolated flagella were resolved on a 12% polyacrylamide gel (B) transblotted to nitrocellulose membranes, stained with type-specific antisera (C) and detected with horseradish peroxidase-conjugated secondary antibody.

II. Epithelial monolayers were treated with purified flagella ($1 \mu\text{g ml}^{-1}$) at 37°C , 5% CO_2 for 3 h. After thorough washing, the flagella bound to cells were labelled with type-specific anti-H7, anti-H11 and anti-H21 flagellar antibodies and detected with secondary FITC (green)-conjugated antibody. Flagella fragments of H7 (A) but not of H11 (B) and H21 (C) are seen adhering to the cell monolayer, confirming the adhesive properties of H7 flagella. In adherence inhibition assay (D) the monolayers were pre-incubated (30 min; 37°C , 5% CO_2) with various levels of purified H7 flagella protein (0.025 , 0.25 , 0.5 , 1.0 and $2.0 \mu\text{g ml}^{-1}$) before infection with wild-type *E. coli* O157 : H7 for 60 min. Adherent bacteria were solubilized, serially diluted and plated to enumerate as colony forming units (cfu). The graph summarizes the mean adherence for each dose level. A comparison of the mean adherence at flagella concentrations of $0.25 \mu\text{g ml}^{-1}$ with that for the negative control showed that the adherence significantly decreased ($P = 0.007$). Mock flagella preparation from *fliC* mutant *E. coli* O157 strain was used as a control. The epithelial cells have been stained with actin-specific phalloidin-TRITC (red) and nuclear stain TO-PRO (blue). The images were captured using Leica TCS NT confocal microscope (63 \times objective lens). Image II (A) was digitally magnified by a factor of 2. Scale bar, $10 \mu\text{m}$.

flagella might vary during stages of infection. Expression of flagella by *E. coli* O157 : H7 strains MCI10, MCI24 and MCI66 was examined during the course of adherence to bovine rectal primary epithelial cells qualitatively and quantitatively using IF staining and in-cell Western assays respectively. After 1 h of infection the majority of individual adherent bacteria expressed flagella (Fig. 7IA and E); by 3 h of infection bacteria formed visible microcolonies and at this stage only few of the adherent bacteria in microcolonies expressed flagella (Fig. 7IB and F).

By 8 h post infection adherent bacteria formed typical A/E lesions characterized by subjacent actin-pedestals and absence of flagella (Fig. 7IC and D), whereas attached bacteria not associated with actin-pedestals remained flagellated.

In-cell Western assays were used to quantify the total bacterial adherence *vis a vis* flagellar expression during the course of *E. coli* O157 : H7 interaction with bovine rectal epithelial cells. The relationship between adherence and expression varied over time. For both strains (MCI10

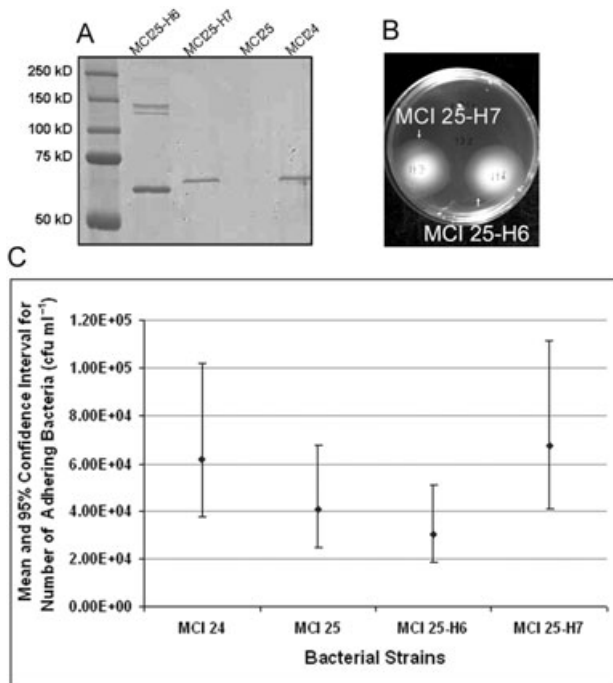


Fig. 4. Specificity of H7 flagella in binding to bovine rectal epithelial cells.

A and B. Flagella isolated from wild-type flagellate MCI24 (*E. coli* O157 : H7), the isogenic $\Delta fliC$ MCI25, the complemented $\Delta fliC$ MCI25-H6 (*fliC_{H6}*) and $\Delta fliC$ MCI25-H7 (*fliC_{H7}*) strains were resolved on 12% polyacrylamide gel and stained with coomassie blue (A). Expression of flagella by the $\Delta fliC$ MCI25-H6 (*fliC_{H6}*) and $\Delta fliC$ MCI25-H7 (*fliC_{H7}*) was assessed for their motility phenotype – visible as a diffused halo around the inoculated spot on the soft LB agar motility plates (B).

C. Adherence of wild-type *E. coli* O157 : H7 (MCI24), the isogenic $\Delta fliC$ MCI25, the complemented MCI25-H6 (*fliC_{H6}*) and MCI25-H7 (*fliC_{H7}*) strains to bovine rectal primary epithelial cells. Bacterial cultures in the mid-log phase of growth were used to infect bovine rectal epithelial cells at an approximate multiplicity of infection of 100 at 37°C, 5% CO₂ for 1 h. Adherent bacteria were solubilized with PBS-0.1% (v/v) Triton X-100, serially diluted and enumerated as colony forming units (cfu). MCI24 and MCI25-H7 adhered at statistically significant higher mean levels than MCI25 ($t_{5,8} = 2.47$; $P = 0.05$) ($t_{5,8} = 3.00$; $P = 0.025$) respectively. There was no significant difference in adherence of the MCI25 and the complemented MCI25-H6 ($t_{5,8} = 1.77$; $P = 0.13$).

and MCI24) the mean level of adherence was statistically significantly increased ($P < 0.001$) by 5 h while mean H7 expression was statistically significantly decreased ($P < 0.001$) (Fig. 7IIC and D). Analysis of the relationships between adherence and H7 expression showed that at time 1 h there was a statistically significant relationship between mean levels of bacterial adherence and expression of flagella ($P < 0.001$). At times 3 h and 5 h, the relationship between H7 expression and adherence was statistically significantly weaker ($P < 0.001$), and there was no evidence of any difference in the relationship at the two times ($P = 0.54$). At time 5 h, the overall mean level of H7 expression was depressed (Fig. 7F). There

was no statistically significant evidence of strain-specific effects on any of these relationships between H7 expression and adherence.

Discussion

We and others have previously reported the terminal rectum as a principal site of *E. coli* O157 : H7 colonization in cattle. To characterize factors contributing to such a tropism we developed a primary cell culture system for terminal rectum epithelium cells of cattle and have used it as an *in vitro* model to assess adherence (Low *et al.*, 2006; Dziva *et al.*, 2007). Initial immunofluorescent microscopic investigations of *E. coli* O157 : H7 adherence to rectal primary epithelium cells in culture, revealed abundant expression of H7 flagella on contact with epithelium at 1 h of *E. coli* O157 : H7 infection. Notably, there appeared to be patent contact points between flagella and epithelial cells, an observation supported by SEM that revealed the presence of flagella-like structures intercalating with the microvilli. Although flagella are primarily motility organelles, a role in adherence to epithelial cells has also been described for certain bacterial flagella and indeed for H6 flagella of enteropathogenic *E. coli* (Giron *et al.*, 2002). Although the latter publication ruled out a role for H7 flagella in attachment of *E. coli* O157 : H7, the model systems employed in that work were human epithelial HeLa and Hep-2 cells (neither of which are intestinal in origin), and hence may not apply to intestinal cells of bovine origin. Therefore, as a consequence of the observed intercalation of H7 flagella with microvilli of bovine enterocytes, we set out to test the postulate that H7 flagella may act as an adherence factor in attachment to bovine intestinal epithelial cells.

First, adherence of parental and flagellin (*fliC*) mutant *E. coli* O157 : H7 was compared using bovine rectum epithelial cell cultures. The *fliC* mutant strain showed significantly reduced adherence ($P < 0.001$) indicating that flagella do play a role in the binding of *E. coli* O157 : H7 to bovine rectal epithelial cells. The involvement of H7 in epithelial interactions was also indicated through pre-treatment of bacteria with rabbit anti-H7 polyclonal antiserum that significantly reduced the mean binding of *E. coli* O157 : H7 but not of its *fliC* mutant strain. Notably, bovine sera containing antibodies to H7 flagella also significantly inhibited average adherence of *E. coli* O157 : H7. Antibodies purified from mucosal extracts from cattle immunized with purified H7 (McNeilly *et al.*, 2007) also reduced mean bacterial attachment to primary bovine rectal epithelial cells (McNeilly *et al.*, 2008), thus further corroborating these findings. Flagella are potent immunogens and antibodies to H7 have also been detected in mucosal secretions from animals colonized with *E. coli* O157 : H7 (Fig. S1). To determine whether

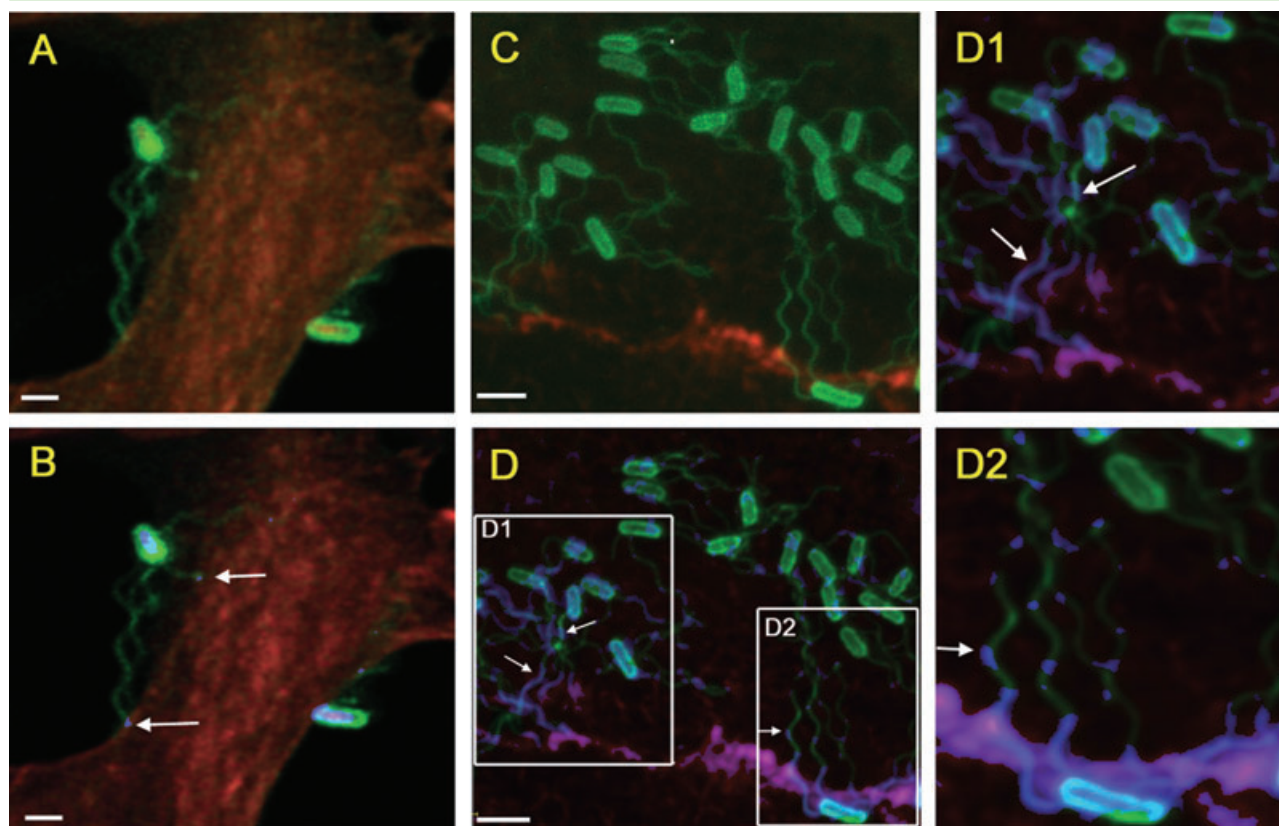


Fig. 5. Interaction of H7 flagella with bovine rectal epithelial cells. Confocal micrographs of MCI66 (*E. coli* O157 : H7, Walla 1) adhering to bovine rectal primary epithelial cells. The cells were infected at an approximate multiplicity of infection of 100 with bacterial cultures in the mid-log phase of growth at 37°C, 5% CO₂ for 1 h. Flagella are seen tethered to the epithelial membrane as an initial contact (A) – clear contact points seen as blue spots are marked with arrows (B). The adherent bacteria expressed flagella in abundance, seen as wavy filaments (C). Bacteria can be seen to bind to the cell surface via flagella (D) – contact points (arrows) seen in the inset images D1, D2 (digitally magnified by a factor of 6). The bacteria labelled with anti-O157 and anti-H7 antibodies were detected with secondary FITC (green)-conjugated antibody. The cells were stained with phalloidin-TRITC (red). The images were acquired using Leica TCS NT confocal microscope (63× objective lens) and colocalization studies were carried out using colocalization module of Imaris. The per cent of green channel volume colocalized with red was calculated in ImarisColoc (Bitplane AG) and the colocalization voxels were displayed in blue colour, as described in the *Experimental procedures*. Scale bar, 2.5 μm.

flagella filaments *per se* were required for adherence of *E. coli* O157 : H7 to epithelial cells, the motility defect of the *fliC* mutant was overcome through gentle centrifugation of bacteria onto the epithelial monolayer. As expected this procedure increased average adherence of both parent and *fliC* mutant strains although average adherence of the latter remained significantly lower than that of the parent strain. Thus, presence of intact flagella on the bacterial surface promotes adherence and is a further indication that flagella directly contribute to host cell attachment.

In assessing the capacity of purified flagella of different EHEC serotypes O157 : H7, O26 : H11 and O103 : H21 to adhere, only isolated H7 flagella bound to bovine rectal primary epithelial cells at detectable levels, confirming that these flagella *per se* can confer attachment presumably via a specific ligand–receptor interaction. One of the hallmark features of ligand–receptor interaction is that

purified ligand can competitively inhibit binding to its cognate receptor in dose-dependent manner. Indeed, purified H7 flagella tended to inhibit *E. coli* O157 : H7 binding to bovine rectal primary epithelial cells, showing a statistically significant decrease in average adherence at a flagella concentration of 0.25 μg ml⁻¹. These data support our contention that H7 flagella perform an initiating role in attachment to rectal epithelium. The specificity of H7 binding was further confirmed when flagella mutant O157 strain complemented with *fliC_{H7}* regained adherence levels similar to the wild type where complementation with *fliC_{H6}* had no effect on the adherence. A comparison of the H7 FliC sequence with that of H6, H11 and H21 demonstrated heterogeneity in the central variable region that may confer differential H7 binding (Giron *et al.*, 2002; Erdem *et al.*, 2007a).

The molecular basis of H7 flagella binding remains elusive; however, it is conceivable that H7 flagella

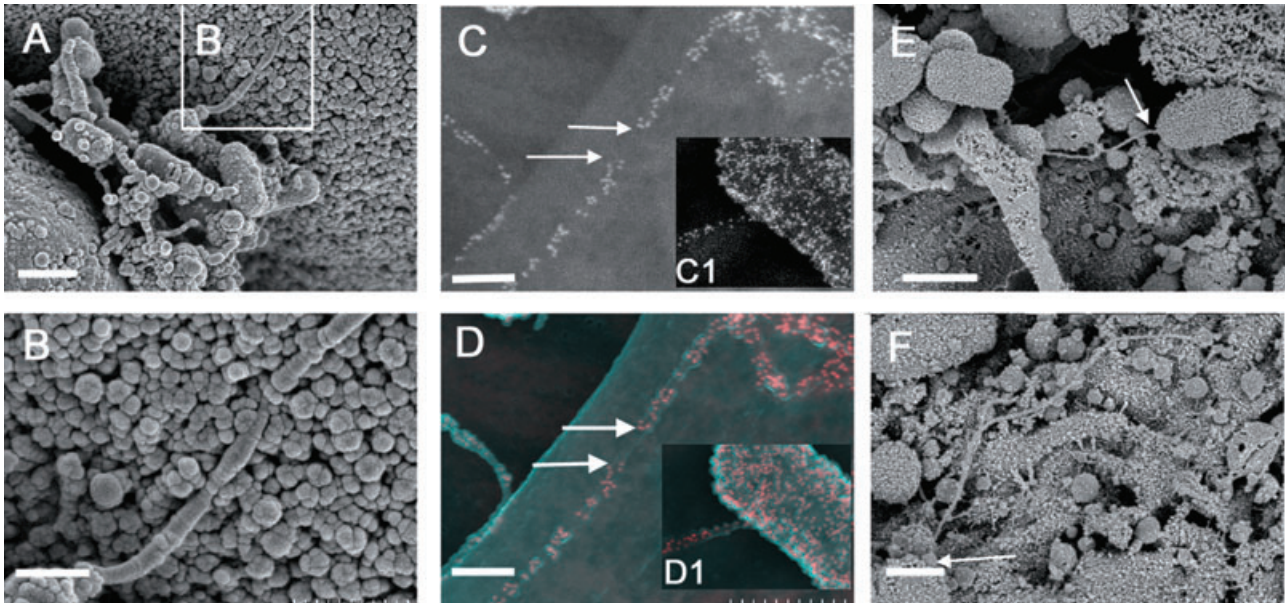


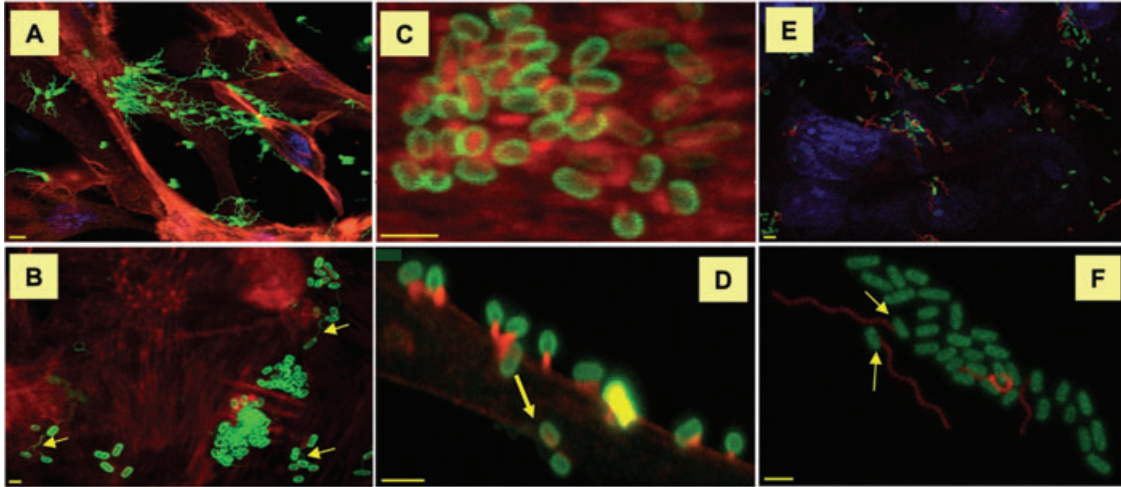
Fig. 6. High-resolution scanning electron micrographs of *E. coli* O157 : H7 (MCI66) adhering to bovine rectal epithelium. Filamentous structures resembling typical flagella can be seen intercalating with the microvilli on the apical surface of the cell (A and B). Immunogold (IG) labelling of bacteria with both anti-O157 and anti-H7 antibodies (C1, D1) demonstrated the aggregation of 10 nm gold particles to the 50-nm-wide wavy filament. The flagellum is uniformly labelled with gold particles except where it interacts with the epithelial cell and thus is not available for IG binding (arrows) (C) as also shown in the combined back scattered and secondary electron image with pseudocoloured gold particles (D). Adherent bacterium expressing filamentous structure (arrow) resembling typical polar flagellum could be seen interacting with bovine rectal epithelium of a calf experimentally infected with *E. coli* O157 : H7 (E and F). The specimens were examined using secondary and back scattered electron detection system in Hitachi 4700 Field Emission Scanning Electron Microscope. Scale bar, 2.5 μm .

possess domains that confer attachment. We propose that TLR5 is unlikely to be the initial receptor for flagella as the flagellin domain that binds TLR5 forms part of the core of the flagellar filament (Eaves-Pyles *et al.*, 2001; Smith *et al.*, 2003), and thus is not exposed in the native structure and TLR5 is usually located on the basolateral surface of enterocytes (Gewirtz *et al.*, 2001). Other epithelial receptors or coreceptors for flagella have been proposed, including TLR2 (Adamo *et al.*, 2004), gangliosides (Feldman *et al.*, 1998; McNamara *et al.*, 2001; Ogushi *et al.*, 2004) and mucin (Lillehoj *et al.*, 2002). Recently it has been shown that H7 flagella bind mucus potentially via Muc2 (Erdem *et al.*, 2007b). It is currently being investigated whether this is also expressed as a non-secreted cell surface component that could act as a potential receptor on rectal epithelial cells.

In assessing *E. coli* O157 : H7 attachment to intestinal epithelial cells, it was evident that flagella were expressed in a time-dependent manner: at early time points post infection flagella were expressed extensively by attaching bacterial cells whereas by later time points flagella expression was apparent only by isolated bacterial cells. Notably, flagella expression diminished as bacterial microcolonies were formed and as aggregation of actin became apparent beneath attached bacteria. This observation suggests that H7 flagella are important in early stages of interaction of *E. coli* O157 : H7 with rectal epithelium while

LEE-dependent factors assume greater significance following initial attachment, taking on the role as major adhesive factors and modifying host cellular function. Another potentially significant consequence of flagella downregulation following attachment is evasion of host inflammatory responses. Flagella, including H7, are recognized as potent inflammatory ligands, and indeed they can evoke inflammatory responses through engagement of each of the receptors mentioned above (Berin *et al.*, 2002; Zhou *et al.*, 2003). Thus, downregulation of this potent pro-inflammatory signal after initiating attachment, and while other contact-dependent factors assume their roles in adherence, represents a further stratagem to evade activation of immune responses. A recent study (Dobbin *et al.*, 2006) has examined the contribution of flagella to colonization of cattle experimentally challenged either orally or rectally. Interpretation is confounded through use by those authors of a cochallenge approach using parental *E. coli* O157 : H7 with a *fliCH7* mutant giving rise to possible trans-complementation effects. Despite this, those authors showed a trend towards greater colonization by the *fliC* mutant strain, which showed longer persistence than the parental strain, particularly in orally challenged animals. We have shown a similar pattern of colonization and persistence in challenges of sheep with the *E. coli* O157 : H7 and *fliC* mutant strains used in the present investigations (R.M. La Ragione, A. Best and M.J.

(I)



(II)

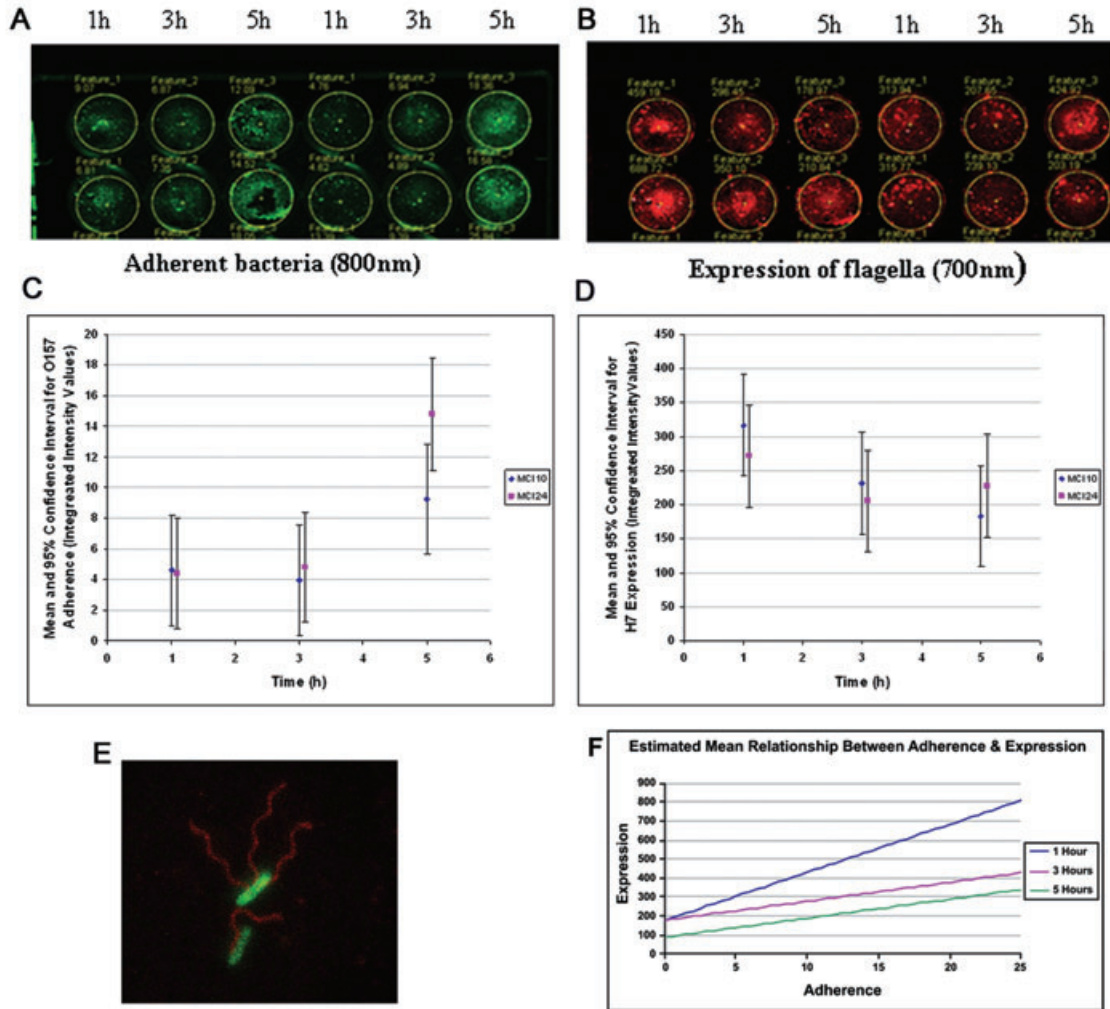


Fig. 7. I. Temporal expression of flagella by different wild-type *E. coli* O157 : H7 on binding to bovine rectal primary epithelial cells. The cells were infected at approximate multiplicity of infection 100 with *E. coli* O157 : H7 strains MCI66 (A–D) and MCI24 (E and F) strains in mid-log phase of growth at 37°C, 5% CO₂. At 1 h post infection most of the adherent bacteria expressed flagella (A and E) and at 3 h the flagellar expression was limited to only few adherent bacteria (arrow) but rarely by those in microcolonies (B and F). At 8 h post infection the bacteria associated with actin-pedestals forming A/E lesion did not show expression of flagella (C and D), although the bacterium associated with cells, but not with actin-pedestals, was still flagellated (arrow) (D). Bacteria labelled with anti-O157 and anti-H7 antibodies were detected with secondary FITC (green) (A–D) or TRITC (red) (E and F) conjugated antibody. The cells were stained with phalloidin-TRITC (red) (A–D) and nuclear stain TO-PRO (blue). The images were captured using a Leica TCS NT confocal microscope (63× objective lens). Scale bar, 2.5 µm. II. Quantification of flagella *vis a vis* adherence of *E. coli* O157 : H7 strains during binding to bovine rectal primary epithelial cells. The cells were infected at approximate multiplicity of infection 1:100 with *E. coli* O157 : H7 strains (MCI10 and MCI 24) culture in mid-log phase of growth at 37°C, 5% CO₂ for 1, 3 and 5 h. For in-cell Western assays, images of adherent bacteria (anti-O157/Alexa Fluor 780) (A), and flagella (anti-H7/Alexa Fluor 647) (B), were scanned with Odyssey Infrared Imaging System. The images represent a 24-well two-colour in-cell Western assay detecting total adherent bacteria (at 800 nm) (A) and expression of flagella (at 700 nm) (B). The graph represents normalized quantitative integrated intensity values demonstrating total mean bacterial adherence (C) and total mean flagellar expression (D). A representative immuno-stained image of *E. coli* O157 : H7 demonstrating the specificity of anti-O157 (green) and anti-H7 (red) antibodies (E). Relationship (for both MCI10 and MCI24) between mean flagella expression and adherence for different time intervals (F).

Woodward, unpublished). One interpretation from these experimental challenges is that *E. coli* O157 : H7 expresses multiple adherence factors and, therefore, both parental and mutant strains can colonize; lack of flagella, however, removes the major pro-inflammatory ligand, thus reducing inflammatory responses to mutant strains and facilitating their persistence. This complex interplay among multiple adhesins of *E. coli* O157 : H7 and host epithelium was beyond the scope of the current study; however, it does demonstrate that the contribution of H7 flagella to pathogenesis of *E. coli* O157 : H7 is not facile. In a recent study (Bretschneider *et al.*, 2007) investigating the role of tir- in colonization of cattle, some of the O157 strains inadvertently did not express flagellin and were found to colonize poorly. Further, calves inoculated with a non-flagellated variant of *E. coli* O157 : H7 colonized higher following second challenge with motile *E. coli* O157 : H7 than those originally inoculated with motile strains and thus role of flagella in colonization of cattle was hypothesized.

Specifically, we have demonstrated that H7 flagella facilitate attachment of *E. coli* O157 : H7 to bovine intestinal (rectal) epithelial cells. The role of H7 flagella is twofold, first through its role as the organelle of motility and second by conferring adherence to bovine rectal epithelial cells in culture. Thus, H7 flagella may be employed by *E. coli* O157 : H7 to 'browse' the epithelial surface, thence tethering the organism to accessible receptor(s), potentially mucin but perhaps other glycoconjugates. Having performed this role in initiating contact and establishing a nidus of infection, other adhesins can engage with their receptors and the contribution of flagella to this process is superseded. Colonization of epithelium then progresses into a secondary phase, corresponding to decreased expression of flagella and increased expression of LEE and associated factors. This regulatory and functional switch promotes persistence resulting from intimate attachment and modulation of inflammatory responses. Cross-regulation between type III secretion and flagella has been demonstrated in EHEC O157

(lyoda *et al.*, 2006). Although H7 flagella are not the sole factors responsible for conferring epithelial tropism, their involvement offers potential as a further candidate to target in intervention approaches and experiments are under way to assess H7 in immunological strategies to reduce *E. coli* O157 in cattle. Overall, this study re-emphasizes the multifactorial nature of infection caused by *E. coli* O157 : H7 in the reservoir host and further demonstrates the complex, multiphasic basis of bacterium–epithelium interaction during colonization.

Experimental procedures

Bacterial strains, flagella preparation and antibodies

EHEC strains: MCI24 (Stx-negative *E. coli* O157 : H7 strain NCTC 12900) and MCI25 (*fliC*-isogenic mutant derived from strain NCTC 12900 (Best *et al.*, 2005); MCI10 (Stx-negative *E. coli* O157 : H7) and MCI66 (Stx2-positive *E. coli* O157 : H7) (Ostroff *et al.*, 1990); ZAP 116 (*E. coli* O26 : H11) (Whittam *et al.*, 1993) and ZAP 244 (*E. coli* O113 : H21) (Luck *et al.*, 2006). Flagella from MCI24 (O157 : H7), ZAP116 (O26 : H11) and ZAP244 (O113 : H21) were isolated as described (DePamphilis and Adler, 1971). Antibodies used in this study were rabbit anti-H7, -H11, -H21, -O157 polyclonal antibody (Mast Diagnostics), anti-rabbit IgG FITC/TRITC-conjugated antibodies (Sigma), 10 nm gold-labelled anti-rabbit IgG (British Biocell International), horseradish peroxidase-conjugated goat anti-rabbit IgG (Dako A/S, Denmark).

Complementation of *E. coli* O157 Δ*fliC* with *pFliC_{H6}* or *pFliC_{H7}*

fliC_{H7} was amplified from *E. coli* O157 : H7 Walla3 (MCI0) using primers Nt-*fliC*-BamHI (AAAAAGGATCCCGATGAAATACTTGC CATGC) and Ct-*fliC*-EcoRI (AAAAAGAATTCGGCAATTTG GCGTTGCCG). *fliC_{H7}* PCR products were cloned into pACCNOT (pWSK29 ΔAccl). *fliC_{H6}* was amplified from *E. coli* O127 : H6 E2348/69 (ZAP286) using the primers JTFliC.F+.BamHI (CCG GATCCGAACTTAAATCCAGACCTGA) and JTFliC.R.BamHI (CCGGATCCCTAACCCCTGCAGACAGACAG). PCR products were A-tailed and cloned into pGEM T-Easy as per manufacture's instructions (Promega). The presence of *fliC_{H6}* with upstream

promoter elements was confirmed by sequencing. Constructs were transferred into *E. coli* O157 $\Delta fliC$ (MCI25). Functional expression and export of FliC_{H6} and FliC_{H7} were confirmed by restoration of motility and isolation of flagella. To assess restoration of motility of complemented strains MCI25-H6 (fliC_{H6}) and MCI25-H7 (fliC_{H7}), LB agar 0.25% (w/v) plates were stab-inoculated and incubated for 16–18 h at 37°C. The motility was visualized as a circular halo around the inoculation spot on the LB agar plates.

Flagella isolation and purification

Flagella from MCI24 (O157 : H7), $\Delta fliC$ MCI25(O157 : H7), MCI25-H6 (fliC_{H6}), MCI25-H7 (fliC_{H7}), ZAP116 (O26 : H11) and ZAP244 (O113 : H21) were isolated as described (DePamphilis and Adler, 1971). Briefly, bacterial lawns on LB agar plates were suspended in formyl saline (v/v, 0.4%) and mechanically sheared. Following differential centrifugation the flagellin preparation was further purified on a caesium chloride gradient (Tackett *et al.*, 1987). The presence of flagella was confirmed by electron microscopy as described above. Protein concentrations were determined using DC Protein Assay kit Bradford as per the manufacturer's instructions (Bio-Rad, Richmond, CA). To confirm purity of the isolated flagellin the samples were subjected to SDS-PAGE (Laemmli, 1970) and immuno-blotted using H type-specific antibodies (Mast Diagnostics) (DePamphilis and Adler, 1971). The protein band of interest was excised and subjected to N-terminal sequence analysis (Proteomics Facility, Moredun Research Institute). A mock flagella preparation was prepared in an identical manner using MCI25 (fliC mutant) as a control. Protein concentrations were determined using DC Protein Assay kit Bradford as per the manufacturer's instructions, using bovine serum albumin as standard (Bio-Rad, Richmond, CA).

Bovine primary epithelial cell culture

Bovine primary epithelial cells were derived from 0 to 5 cm proximal to rectal-anal junction as described (Dziva *et al.*, 2007). Briefly, the mucosal scrapings from terminal rectum of adult cattle from a local abattoir were digested in DMEM [1% (v/v) fetal calf serum (FCS), 100 U ml⁻¹ penicillin, 30 µg ml⁻¹ streptomycin, 25 µg ml⁻¹ gentamicin] containing 75 U ml⁻¹ collagenase and 20 µg ml⁻¹ dispase (Roche) with gentle shaking at 37°C until isolated crypts could be observed microscopically. Crypt enrichment from undigested material, contaminating gut microflora and single cells including fibroblasts, was performed using a series of differential centrifugation steps with DMEM containing 2% sorbitol (Booth *et al.*, 1995). The crypt cell pellet was re-suspended in cell culture medium [DMEM, 2.5% (v/v) FCS, 0.25 U ml⁻¹ insulin, 10 ng ml⁻¹ epidermal growth factor and 30 mg ml⁻¹ gentamicin]. Approximately 400–600 crypts were seeded per well into four-well chamber slides (Costar, Corning USA) or Thermanox coverslips (LABTEK, Nalge Nunc International) pre-coated with collagen (Vitrogen Collagen, Nutacon, Netherlands). The cells were grown to a stage of confluence (approximately 3×10^5 cells per well, typically at 10–14 days following initial primary epithelial cell culture). To inhibit the growth of fibroblasts, the culture medium was replaced with MEM D-valine (Servichem GmbH) containing 2.5% (v/v) FCS, 0.25 U insulin, 10 ng ml⁻¹ epidermal growth factor and 25 mg ml⁻¹ gentamicin, 24 h after initial seeding of crypts and culture was continued in this medium until

cells were used. Epithelial origin of the cells was confirmed by immunocytochemistry for cytokeratin (CK4, 5, 6, 8, 10, 13 and 18) (Sigma) and lack of a fibroblast-specific marker as described (Hoey *et al.*, 2003). Electron microscopic investigations revealed a number of ultrastructural and morphological features typical of intestinal epithelial cells *in vivo* such as apical microvilli associated with a glycocalyx and tight junctions (Hoey *et al.*, 2003).

Interaction with primary bovine rectal epithelial cells

Bovine rectal primary epithelial cells cultured from terminal rectum were used for the adherence, immunofluorescent and ultrastructural studies. The bacterial adherence and the adherence inhibition experiments were done essentially as described previously (Giron *et al.*, 2002; Dziva *et al.*, 2007). Briefly, the stationary phase bacterial cultures of wild-type and mutant bacteria grown in MEM/Hepes with appropriate antibiotics were diluted 1:10 in MEM-Hepes and incubated at 37°C with shaking (200 r.p.m.) to an OD at 600 nm of 0.3–0.4. The bacterial cultures prior to infection at a multiplicity 1:100 were pre-treated for 30 min at room temperature (RT) with rabbit anti-H7 polyclonal antibody (1:10) in MEM-Hepes or antiserum from cattle immunized with purified H7-flagellin (McNeilly *et al.*, 2007). Serum samples from the same cattle prior to immunization were used as control. In ligand inhibition studies prior to addition of bacteria, the primary epithelial cells were pre-incubated with various levels of purified H7 flagellin as potential binding inhibitors as described (Lillehoj *et al.*, 2002). To control for other factors in the flagella preparation a mock preparation from fliC-deficient MCI25 strain was used as a control. To examine the effect of motility on adherence immediately upon bacterial inoculation, mild centrifugation (1000 r.p.m. for 3 min) was applied to infected cells in 24-well tissue culture plates as described (La Ragione *et al.*, 2003). The infected cells were washed three times with phosphate-buffered saline (PBS) to remove the nonadherent bacteria; the adherent bacteria were solubilized with PBS-0.1% (v/v) Triton X-100, serially diluted and plated onto LB agar to determine the number of bacteria adhering to the cells in culture as colony forming units.

In vitro organ culture assay

Tissue specimens obtained from adult cattle at a local abattoir were transported in cold HBSS (Gibco BRL). The lymphoid-dense terminal rectal mucosa 3 cm proximal to recto-anal junction was carefully excised, washed in cold PBS, cut into 1 cm squares with a thickness of 2 mm and placed in tissue culture medium RPMI 1640 (Gibco Brl). Tissue explants were prepared for *ex vivo* binding studies as described previously (Baehler and Moxley, 2000). Briefly, the tissue explants were infected with cultures of MCI24 or MCI25 strains (100 µl) in mid-log phase at an OD₆₀₀ 0.3–0.4 (as grown for the adherence assay) for 8 h at 37°C, 5% CO₂, 95% air in a humidified atmosphere. After 2 h of infection the medium was replaced at every 1 h interval. The infected tissue explants were given three washes in PBS, fixed and permeabilized overnight (4°C) in 4% (w/v) PFA/0.2% (v/v) Triton X-100, processed as whole mounts for IF studies.

In-cell Western assays

To quantify adherent bacteria and *in situ* expression of flagella during course of *E. coli* O157 : H7 (MCI10, MCI24) interaction to

bovine rectal primary epithelial cells in-cell Western assays were done as described (Arredondo *et al.*, 2005). The infected cells were washed three times with PBS, fixed/permeabilized in 2% (v/v) formalin-0.2% (v/v) Triton X-100 for 20 min. at RT. The fixed cells were washed three times in PBS and adherent bacteria were stained with rabbit anti-O157 (Mast Diagnostics; diluted 1:500 in PBS) followed by secondary antibody conjugated with Alexa Fluor 647 (Molecular Probes) was used at RT for 30 min to detect the bacteria. Flagella were stained with anti-H7 flagellar type-specific polyclonal rabbit antiserum (Mast Diagnostics; diluted 1:500 in PBS) conjugated with Alexa Fluor 780 (Molecular Probes, Eugene, OR, with USA) at RT for 30 min. Images were captured using Odyssey Infrared Imaging System at appropriate wavelengths (700 and 800 nm). The number of attached bacteria and the level of expression of flagella were estimated from the scanned data as integrated intensity values (in quadruplicate for each time point). The values for each well were background-corrected using integrated intensity data for control wells with non-infected cells.

Fluorescence microscopy

Immunofluorescence microscopy was used to detect expression of flagella during adherence of *E. coli* O157 strains to bovine rectal primary epithelial cells, as described (Giron *et al.*, 2002). Briefly, the infected cells were washed three times with PBS, fixed/permeabilized in 2% (v/v) formalin-0.2% (v/v) Triton X-100 for 20 min. at RT. The fixed cells were washed twice in PBS. The adherent bacteria were stained with rabbit anti-O157 and anti-flagellar type-specific polyclonal antiserum (Mast Diagnostics; diluted 1:500 in PBS) at RT for 30 min, washed then incubated with FITC-labelled secondary antibody (Sigma anti-rabbit; diluted 1:1000). Cells were then treated for 20 min. at RT with TRITC-Phalloidin (5 µg ml⁻¹; Sigma), To-Pro (1 µg ml⁻¹; Molecular Probes), washed twice with PBS and mounted in fluorescent mounting medium (DAKO). The specimens were visualized with a Leica DMLB epifluorescence microscope and images were analysed using different microscopy softwares.

To examine the binding of purified flagella, bovine rectal primary epithelial cells were washed three times with pre-warmed MEM-Hepes and incubated with isolated flagella 1 µg ml⁻¹ (H7, H11 or H21) for 3 h at 37°C, 5% CO₂. The samples were processed for IF as above.

Image acquisition procedures in confocal microscopy

Images were acquired using a Leica TCS NT confocal system (Leica Microsystems, GmbH, Heidelberg, Germany), equipped with an Argon/Krypton mixed gas laser, allowing three-channel detection of fluorophores. Multi-channel images were acquired either simultaneously, or sequentially. A 63× Plan Apo oil immersion lens (NA 1.32) were routinely used, often in conjunction with the digital zoom capability of the confocal system.

As the size of bacteria is close to the limits of resolution of imaging systems (approximately 300 nm), image restoration techniques were employed to achieve optimal results. For this, data were acquired at Nyquist sampling rates, which is the minimum sampling distance required to ensure all information present in the sample is collected. These data sets were then deconvolved, using Huygens Professional (Scientific Volume

Imaging b.v., Hilversum, the Netherlands), running on a Silicon Graphics computer. This restoration of acquired data was recommended before further analysis of data sets, in particular, for any colocalization analysis.

Information was required on possible colocalization between the bacterial flagella and cell surface. Deconvolution was routinely carried out on the experimental data sets acquired for this, before colocalization analysis, using the colocalization module of Imaris (Bitplane AG, Zurich, Switzerland). This program analysed the complete 3D data set, and reported the number of voxels colocalized and percentages of colocalization between each channel in the data set. The per cent of green channel volume colocalized with red was calculated in ImarisColoc (Bitplane AG) and the colocalization voxels were displayed in blue colour.

Ultrastructural studies

The ultrastructural details of flagella interacting with bovine rectal epithelium during *E. coli* O157 : H7 adherence to primary rectal epithelial cells or colonization at terminal rectum of experimentally infected calves were visualized using Hitachi 4700 Field Emission Scanning Electron. Bovine rectal tissue from a calf experimentally infected with *E. coli* O157 : H7 strain (MC110) were collected following necropsy 4 weeks post challenge (Naylor *et al.*, 2003). The tissue specimens were fixed in 3% glutaraldehyde and processed for SEM as mentioned above.

Detection of H7-specific mucosal immune responses

Rectal mucosal samples from calf experimentally infected with *E. coli* O157 : H7 were collected at necropsy as described (Naylor *et al.*, 2003). The mucosal scrapings (approximately 50 mg) were suspended in 5 ml of ice-cold PBS (pH 7.2) plus protease inhibitor cocktail (Complete-Mini, Roche, Mannheim, Germany), vortexed and clarified by centrifugation at 16 000 g and 4°C for 15 min. The supernatant was used to detect H7-specific mucosal immune responses as described (McNeilly *et al.*, 2007). Briefly, purified H7 and H21 flagellin proteins were resolved by SDS-PAGE, trans-blotted and incubated with clarified scraping suspension [diluted in PBS containing 0.5 M NaCl and 0.5% Tween 80 (PBS/NaCl/T)] for 1 h at 37°C and detected using mouse anti-bovine IgA mAb (K84.2F9, Serotec) followed by secondary peroxidase conjugate (Dako). The incubation steps were performed for 60 min at RT with three washes between each step. Peroxidase activity was revealed by chemiluminescence using ECL reagent (Amersham Life Sciences, Bucks, UK).

Statistical analysis

Count data were analysed using generalized linear mixed models (Brown and Prescott, 1999), modelling the response as a Poisson variable, using a logarithmic link function and fitting the log of assay dilution as an offset. Experimental replicate and other experimental blocking factors were fitted as random effects where appropriate, and other terms as fixed effects. Where possible, residual variability was modelled as a random effect, otherwise a dispersion parameter was estimated. The statistical significance of fixed effects were evaluated using approximate *F*-tests (Kenward and Roger, 1997). In-cell Western assays were analysed as continuous variables using linear mixed models

(Brown and Prescott, 1999), fitting adherence as an explanatory variable, expression as a normally distributed response variable and time and strain as factors. Where multiple comparisons were made between mean values from a single analysis, the false discovery rate was controlled using the method described (Benjamini, 1995).

Acknowledgements

We thank Mrs Linda Wilson and John Findlay for their assistance in confocal and electron microscopy and the staff of the Clinical Department at the Moredun Research Institute for providing expert animal care and assistance. We also wish to thank the staff of Linlithgow abattoir and Hugh Currie for sourcing mucosal samples. We acknowledge the support of the Darwin Trust of Edinburgh for A.M.'s postgraduate studentship and Genesis-Faraday Partnerships for supporting S.M. We are also grateful to Department for Environment, Food and Rural Affairs of the UK, including funding under VTRI Programme (VT0102) and the Scottish Executive for funding this research.

References

- Adamo, R., Sokol, S., Soong, G., Gomez, M.I., and Prince, A. (2004) *Pseudomonas aeruginosa* flagella activate airway epithelial cells through asialoGM1 and toll-like receptor 2 as well as toll-like receptor 5. *Am J Respir Cell Mol Biol* **30**: 627–634.
- Arora, S.K., Ritchings, B.W., Almira, E.C., Lory, S., and Ramphal, R. (1998) The *Pseudomonas aeruginosa* flagellar cap protein, FliD, is responsible for mucin adhesion. *Infect Immun* **66**: 1000–1007.
- Arredondo, J., Chernyavsky, A.I., Webber, R.J., and Grando, S.A. (2005) Biological effects of SLURP-1 on human keratinocytes. *J Invest Dermatol* **125**: 1236–1241.
- Baehler, A.A., and Moxley, R.A. (2000) *Escherichia coli* O157:H7 induces attaching-effacing lesions in large intestinal mucosal explants from adult cattle. *FEMS Microbiol Lett* **185**: 239–242.
- Benjamini, Y.A.H.Y. (1995) Controlling the false discovery rate: a practical and powerful approach to multiple testing. *J R Stat Soc B* **57**: 289–300.
- Berin, M.C., rfeuille-Michaud, A., Egan, L.J., Miyamoto, Y., and Kagnoff, M.F. (2002) Role of EHEC O157:H7 virulence factors in the activation of intestinal epithelial cell NF-kappaB and MAP kinase pathways and the upregulated expression of interleukin 8. *Cell Microbiol* **4**: 635–648.
- Best, A., La Ragione, R.M., Sayers, A.R., and Woodward, M.J. (2005) Role for flagella but not intimin in the persistent infection of the gastrointestinal tissues of specific-pathogen-free chicks by shiga toxin-negative *Escherichia coli* O157:H7. *Infect Immun* **73**: 1836–1846.
- Bitzan, M., Ludwig, K., Klemm, M., Konig, H., Buren, J., and Muller-Wiefel, D.E. (1993) The role of *Escherichia coli* O157 infections in the classical (enteropathic) haemolytic uraemic syndrome: results of a Central European, multi-centre study. *Epidemiol Infect* **110**: 183–196.
- Booth, C., Patel, S., Bennion, G.R., and Potten, C.S. (1995) The isolation and culture of adult mouse colonic epithelium. *Epithelial Cell Biol* **4**: 76–86.
- Borczyk, A.A., Karmali, M.A., Lior, H., and Duncan, L.M. (1987) Bovine reservoir for verotoxin-producing *Escherichia coli* O157:H7. *Lancet* **1**: 98.
- Bretschneider, G., Berberov, E.M., and Moxley, R.A. (2007) Reduced intestinal colonization of adult beef cattle by *Escherichia coli* O157:H7 tir deletion and nalidixic-acid-resistant mutants lacking flagellar expression. *Vet Microbiol* **125**: 381–386.
- Brown, H., and Prescott, R. (1999) *Applied Mixed Models in Medicine*. Chichester: John Wiley and Sons.
- Cobbold, R.N., Hancock, D.D., Rice, D.H., Berg, J., Stilborn, R., Hovde, C.J., et al. (2007) Rectoanal junction colonization of feedlot cattle by *Escherichia coli* O157:H7 and its association with supershedders and excretion dynamics. *Appl Environ Microbiol* **73**: 1563–1568.
- Cornick, N.A., Booher, S.L., and Moon, H.W. (2002) Intimin facilitates colonization by *Escherichia coli* O157:H7 in adult ruminants. *Infect Immun* **70**: 2704–2707.
- Dean-Nystrom, E.A., Bosworth, B.T., Moon, H.W., and O'Brien, A.D. (1998) *Escherichia coli* O157:H7 requires intimin for enteropathogenicity in calves. *Infect Immun* **66**: 4560–4563.
- DePamphilis, M.L., and Adler, J. (1971) Purification of intact flagella from *Escherichia coli* and *Bacillus subtilis*. *J Bacteriol* **105**: 376–383.
- Dobbin, H.S., Hovde, C.J., Williams, C.J., and Minnich, S.A. (2006) The *Escherichia coli* O157 flagellar regulatory gene flhC and not the flagellin gene flhC impacts colonization of cattle. *Infect Immun* **74**: 2894–2905.
- Donnenberg, M.S., Tzipori, S., McKee, M.L., O'Brien, A.D., Alroy, J., and Kaper, J.B. (1993) The role of the eae gene of enterohemorrhagic *Escherichia coli* in intimate attachment *in vitro* and in a porcine model. *J Clin Invest* **92**: 1418–1424.
- Dons, L., Eriksson, E., Jin, Y., Rottenberg, M.E., Kristensson, K., Larsen, C.N., et al. (2004) Role of flagellin and the two-component CheA/CheY system of *Listeria monocytogenes* in host cell invasion and virulence. *Infect Immun* **72**: 3237–3244.
- Dziva, F., van Diemen, P.M., Stevens, M.P., Smith, A.J., and 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., et al. (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.
- Eaves-Pyles, T.D., Wong, H.R., Odoms, K., and Pyles, R.B. (2001) Salmonella flagellin-dependent proinflammatory responses are localized to the conserved amino and carboxyl regions of the protein. *J Immunol* **167**: 7009–7016.
- Elder, R.O., Keen, J.E., Siragusa, G.R., Barkocy-Gallagher, G.A., Koohmaraie, M., and Laegreid, W.W. (2000) Correlation of enterohemorrhagic *Escherichia coli* O157 prevalence in feces, hides, and carcasses of beef cattle during processing. *Proc Natl Acad Sci USA* **97**: 2999–3003.
- Erdem, A.L., Avelino, F., Xicohtencatl-Cortes, J., and Giron,

- J.A. (2007a) Host protein binding and adhesive properties of H6 and H7 flagella of attaching and effacing *Escherichia coli*. *J Bacteriol* **189**: 7426–7435.
- Erdem, A.L., Avelino, F., Xicohtencatl-Cortes, J., and Giron, J.A. (2007b) Host protein binding and adhesive properties of H6 and H7 flagella of attaching and effacing *Escherichia coli*. *J Bacteriol* **189**: 7426–7435.
- Feldman, M., Bryan, R., Rajan, S., Scheffler, L., Brunnert, S., Tang, H., *et al.* (1998) Role of flagella in pathogenesis of *Pseudomonas aeruginosa* pulmonary infection. *Infect Immun* **66**: 43–51.
- Frankel, G., Phillips, A.D., Rosenshine, I., Dougan, G., Kaper, J.B., and Knutton, S. (1998) Enteropathogenic and enterohaemorrhagic *Escherichia coli*: more subversive elements. *Mol Microbiol* **30**: 911–921.
- Gewirtz, A.T., Navas, T.A., Lyons, S., Godowski, P.J., and Madara, J.L. (2001) Cutting edge: bacterial flagellin activates basolaterally expressed TLR5 to induce epithelial proinflammatory gene expression. *J Immunol* **167**: 1882–1885.
- Giron, J.A., Torres, A.G., Freer, E., and Kaper, J.B. (2002) The flagella of enteropathogenic *Escherichia coli* mediate adherence to epithelial cells. *Mol Microbiol* **44**: 361–379.
- Grys, T.E., Siegel, M.B., Lathem, W.W., and Welch, R.A. (2005) The StcE protease contributes to intimate adherence of enterohaemorrhagic *Escherichia coli* O157:H7 to host cells. *Infect Immun* **73**: 1295–1303.
- Hoey, D.E., Sharp, L., Currie, C., Lingwood, C.A., Gally, D.L., and Smith, D.G. (2003) Verotoxin 1 binding to intestinal crypt epithelial cells results in localization to lysosomes and abrogation of toxicity. *Cell Microbiol* **5**: 85–97.
- Hussein, H.S. (2007) Prevalence and pathogenicity of Shiga toxin-producing *Escherichia coli* in beef cattle and their products. *J Anim Sci* **85**: E63–E72.
- Inglis, T.J., Robertson, T., Woods, D.E., Dutton, N., and Chang, B.J. (2003) Flagellum-mediated adherence by *Burkholderia pseudomallei* precedes invasion of *Acanthamoeba astronyxis*. *Infect Immun* **71**: 2280–2282.
- Iyoda, S., Koizumi, N., Satou, H., Lu, Y., Saitoh, T., Ohnishi, M., *et al.* (2006) The GrlR-GrlA regulatory system coordinately controls the expression of flagellar and LEE-encoded type III protein secretion systems in enterohaemorrhagic *Escherichia coli*. *J Bacteriol* **188**: 5682–5692.
- Jordan, D.M., McEwen, S.A., Lammerding, A.M., McNab, W.B., and Wilson, J.B. (1999) Pre-slaughter control of *Escherichia coli* O157 in beef cattle: a simulation study. *Prev Vet Med* **41**: 55–74.
- Jordan, D.M., Cornick, N., Torres, A.G., an-Nystrom, E.A., Kaper, J.B., and Moon, H.W. (2004) Long polar fimbriae contribute to colonization by *Escherichia coli* O157:H7 *in vivo*. *Infect Immun* **72**: 6168–6171.
- Karmali, M.A., Steele, B.T., Petric, M., and Lim, C. (1983a) Sporadic cases of haemolytic-uraemic syndrome associated with faecal cytotoxin and cytotoxin-producing *Escherichia coli* in stools. *Lancet* **1**: 619–620.
- Karmali, M.A., Petric, M., Lim, C., Fleming, P.C., and Steele, B.T. (1983b) *Escherichia coli* cytotoxin, haemolytic-uraemic syndrome, and haemorrhagic colitis. *Lancet* **2**: 1299–1300.
- Kenward, M.G., and Roger, J.H. (1997) Small sample inference for fixed effects from restricted maximum likelihood. *Biometrics* **53**: 983–997.
- Kim, S.H., and Kim, Y.H. (2004) *Escherichia coli* O157:H7 adherence to HEp-2 cells is implicated with curli expression and outer membrane integrity. *J Vet Sci* **5**: 119–124.
- Kirov, S.M., Castrisios, M., and Shaw, J.G. (2004) Aeromonas flagella (polar and lateral) are enterocyte adhesins that contribute to biofilm formation on surfaces. *Infect Immun* **72**: 1939–1945.
- La Ragione, R.M., Cooley, W.A., Velge, P., Jepson, M.A., and Woodward, M.J. (2003) Membrane ruffling and invasion of human and avian cell lines is reduced for aflagellate mutants of *Salmonella enterica* serotype Enteritidis. *Int J Med Microbiol* **293**: 261–272.
- Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**: 680–685.
- Lillehoj, E.P., Kim, B.T., and Kim, K.C. (2002) Identification of *Pseudomonas aeruginosa* flagellin as an adhesin for Muc1 mucin. *Am J Physiol Lung Cell Mol Physiol* **282**: L751–L756.
- Locking, M.E., O'Brien, S.J., Reilly, W.J., Wright, E.M., Campbell, D.M., Coia, J.E., *et al.* (2001) Risk factors for sporadic cases of *Escherichia coli* O157 infection: the importance of contact with animal excreta. *Epidemiol Infect* **127**: 215–220.
- Low, A.S., Dziva, F., Torres, A.G., Martinez, J.L., Rosser, T., Naylor, S., *et al.* (2006) Cloning, expression, and characterization of fimbrial operon F9 from enterohaemorrhagic *Escherichia coli* O157:H7. *Infect Immun* **74**: 2233–2244.
- Low, J.C., McKendrick, I.J., McKechnie, C., Fenlon, D., Naylor, S.W., Currie, C., *et al.* (2005) Rectal carriage of enterohaemorrhagic *Escherichia coli* O157 in slaughtered cattle. *Appl Environ Microbiol* **71**: 93–97.
- Luck, S.N., Badea, L., nett-Wood, V., Robins-Browne, R., and Hartland, E.L. (2006) Contribution of FliC to epithelial cell invasion by enterohaemorrhagic *Escherichia coli* O113:H21. *Infect Immun* **74**: 6999–7004.
- McDaniel, T.K., and Kaper, J.B. (1997) A cloned pathogenicity island from enteropathogenic *Escherichia coli* confers the attaching and effacing phenotype on *E. coli* K-12. *Mol Microbiol* **23**: 399–407.
- McKee, M.L., Melton-Celsa, A.R., Moxley, R.A., Francis, D.H., and O'Brien, A.D. (1995) Enterohaemorrhagic *Escherichia coli* O157:H7 requires intimin to colonize the gnotobiotic pig intestine and to adhere to HEp-2 cells. *Infect Immun* **63**: 3739–3744.
- McNamara, N., Khong, A., McKemy, D., Caterina, M., Boyer, J., Julius, D., *et al.* (2001) ATP transduces signals from ASGM1, a glycolipid that functions as a bacterial receptor. *Proc Natl Acad Sci USA* **98**: 9086–9091.
- McNeilly, T.N., Naylor, S.W., Mitchell, M.C., McAteer, S., Mahajan, A., Smith, D.G., *et al.* (2007) Simple methods for measurement of bovine mucosal antibody responses *in vivo*. *Vet Immunol Immunopathol* **118**: 160–167.
- McNeilly, T.N., Naylor, S.W., Mahajan, A., Mitchell, M.C., McAteer, S., Deane, D., *et al.* (2008) *Escherichia coli* O157:H7 colonization in cattle following systemic and mucosal immunization with purified H7 flagellin. *Infect Immun* **76**: 2594–2602.

Supporting information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Adherence of wild-type *E. coli* O157 : H7 strain MCI24 and the isogenic *fliC* mutant MCI25 to the tissue explants from terminal rectum of cattle. Tissue explants were infected with bacterial cultures in mid-log phase of growth at 37°C, 5% CO₂ and 95% air in a humidified chamber. The infected tissue explants were fixed/permeabilized in 4% (w/v) PFA/0.2%(v/v) Triton X-100 for 30 min at RT and stained with Phalloidin-TRITC (red) overnight at 4°C. The bacteria labelled with anti-O157 and anti-H7 antibodies were detected with secondary FITC (green) conjugated antibody. The tissue explants were microscopically examined as whole tissue mounts using a Leica TCS NT confocal system (63× objective). At 8 h post infection the wild-type MCI24 formed compact microcolonies. Some of the bacteria could be seen expressing flagella (arrow) (A). MCI25 (*fliC*-) exhibited sparse binding (B). Scale bars = 5 µm.

Fig. S2. Immunoblot showing anti-H7 mucosal immune response in a calf experimentally infected with *E. coli* O157 : H7 (MCI10). Purified flagellin proteins H7 and H21 were resolved on a 12% polyacrylamide gel and stained with colloidal blue (A). The transblotted proteins were incubated with rectal mucosa secretions from a calf experimentally colonized with *E. coli* O157 : H7 and detected using mouse anti-bovine IgA mAb (K84.2F9,

Serotec) followed by secondary peroxidase conjugate (Dako). IgA antibodies specific to purified flagellar proteins H7 (lane 1) and not H21 (lane 2) were detected.

Fig. S3. Multiple sequence alignment for H6, H7, H11 and H21 variants of FliC. FliC sequences from O157 : H7 EDL933 (NP_288384), O157 : H7 Sakai (NP_310689), O127 : H6 E2348/69 (http://www.sanger.ac.uk/Projects/Escherichia_Shigella/), O26 : H11 CL5 (partial CDS, AAW84044), O157 : H11 strain 111 (AAQ22673), O113 : H21 (ABI23966) and *Salmonella typhimurium* SL1344 (NP_456520) were aligned using ClustalW and shaded using BoxShade (<http://www.angis.org.au>). Differing residues are shaded grey and similar residues are shaded grey with white text. Domains D0–D3 are indicated for *S. typhimurium* SL1344 FliC as described by Samatey *et al.* (2001). Variation is illustrated in domains D2 and D3 of FliC_{H7} that has previously been described (Wang *et al.*, 2003). Residues that have been shown to be important for TLR5 binding in SL1344 FliC are indicated with asterisks (Smith *et al.*, 2003; Andersen-Nissen *et al.*, 2005). The residues in domains D0 and D1 appear to be highly conserved between FliC_{H7} of EDL933 and Sakai O157 strains but domains D2 and D3 vary from H serotypes FliC_{H6}, FliC_{H21}, FliC_{H11} and FliC_{SL1344}.

Please note: Wiley-Blackwell are not responsible for the content or functionality of any supporting materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.