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

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Received 12 February 2007; accepted 14 March 2007.

DOI:10.1111/j.1574-6968.2007.00724.x

Editor: Rob Delahay

Keywords
Escherichia coli; EspP; cattle; adherence; colonization.

Abstract

Enterohaemorrhagic *Escherichia coli* (EHEC) comprise a group of zoonotic diarrhoeal pathogens of worldwide importance. Cattle are a key reservoir; however the molecular mechanisms that promote persistent colonization of the bovine intestines by EHEC are ill-defined. The large plasmid of EHEC O157:H7 encodes several putative virulence factors. Here, it is reported that the pO157-encoded Type V-secreted serine protease EspP influences the intestinal colonization of calves. To dissect the basis of attenuation, a bovine primary rectal epithelial cell line was developed. Adherence of *E. coli* O157:H7 to such cells was significantly impaired by *espP* mutation but restored upon addition of highly purified exogenous EspP. Data of this study add to the growing body of evidence that cytotoxins facilitate intestinal colonization by EHEC.

Introduction

Enterohaemorrhagic *Escherichia coli* (EHEC) pose a significant public health threat worldwide. Ruminants are a key reservoir and humans are frequently infected following direct or indirect exposure to ruminant faeces. Strategies to reduce the carriage of EHEC in ruminants are expected to lower the incidence of human disease. However, cross-protective vaccines do not yet exist and the molecular mechanisms that influence the persistence of EHEC in the bovine intestines are incompletely understood. By allelic exchange and signature-tagged mutagenesis (STM), portfolios of bacterial genes that influence colonization of calves by EHEC O157:H7 and O26:H-have been identified, including conserved and serotype-specific factors (Dziva *et al.*, 2004; van Diemen *et al.*, 2005). Among the EHEC O26:H- genes putatively required for the intestinal colonization of calves is *pss*A (van Diemen *et al.*, 2005). PssA (*p*rotein *s*ecreted by *S*higa toxin-producing *E. coli*) was first described in EHEC O26 strains and is highly related to a family of serine protease autotransporters of *Enterobacteriaceae* (SPATEs; Djafari *et al.*, 1997). Parallel studies on EHEC O157:H7 identified a pO157-encoded secreted serine protease (EspP) which cleaves human coagulation factor V (Brunder *et al.*, 1997). Through hybridization and immunoblot assays, EspP was shown to be widespread in EHEC O157:H7 strains and to exist in EHEC O26 strains (Brunder *et al.*, 1997) and it is now clear that PssA and EspP are 99% identical at the amino acid level. Despite this close homology, PssA has been reported to cleave casein and exhibit cytopathic activity for Vero cells, phenotypes that have not been reported with EspP (Djafari *et al.*, 1997; Dutta *et al.*, 2002).

Several SPATEs have been extensively characterized *in vitro*, including EspC of enteropathogenic *E. coli* (EPEC; Stein *et al.*, 1996; Navarro-Garcia *et al.*, 2004), Pet of enteroaggregative *E. coli* (EAEC; Eslava *et al.*, 1998), EatA

of enterotoxigenic *E. coli* (ETEC; Patel *et al.*, 2004), SigA of *Shigella flexneri* (Al-Hasani *et al.*, 2000), and the uropathogenic *E. coli* (UPEC) proteins Sat (Guyer *et al.*, 2000) and PicU (Parham *et al.*, 2004). In rabbit ileal loop models, mutation of *sat* (of diffusely adhering *E. coli*), ETEC *eatA* and *Shigella* SigA impaired the induction of intestinal inflammatory and secretory responses (Al-Hasani *et al.*, 2000; Patel *et al.*, 2004; Taddei *et al.*, 2005). Furthermore, EAEC Pet causes damage to human intestinal explants cultured *ex vivo* (Henderson *et al.*, 1999) and EPEC EspC is toxic to rat jenunal explants (Mellies *et al.*, 2001). Taken together, these observations indicate that SPATEs may play key roles in host–pathogen interactions at epithelial surfaces (reviewed in Henderson *et al.*, 2004).

The majority of SPATEs are encoded on large plasmids. In addition to EspP, the large plasmid of EHEC O157:H7 (pO157) encodes enterohaemolysin (EhxA), catalase-peroxidase (KatP), a large clostridial toxin homologue (ToxB), a metalloprotease that cleaves C1 esterase inhibitor (StcE, initially identified as TagA) and the *ecf* operon (Burland *et al.*, 1998; Makino *et al.*, 1998). It has been reported that pO157 is required for full adherence to epithelial cells (Toth *et al.*, 1990) and ToxB and StcE have since been implicated in this process (Tastuno *et al.*, 2001; Stevens *et al.*, 2004; Grys *et al.*, 2005). Although it was initially reported that pO157 does not influence *E. coli* O157:H7-induced enteritis and adherence in gnotobiotic piglets (Tzipori *et al.*, 1987), it was recently reported that pO157 influences colonization of the bovine terminal rectum (Sheng *et al.*, 2006) and that the *ecf* operon is required for persistence in the bovine intestines (Yoon *et al.*, 2005). With the exception of *toxB*, which is not required for colonization of young calves (Stevens *et al.*, 2004), the role of other pO157-encoded genes *in vivo* has not been defined. Evidence for expression during *E. coli* O157:H7 infection of humans has been obtained for EspP (Brunder *et al.*, 1997), EhxA (Schmidt *et al.*, 1995), StcE (Paton & Paton, 2002) and a pO157-encoded inner membrane acyltransferase (MsbB2; John *et al.*, 2005).

Here, it is shown that EspP contributes to adherence to bovine primary rectal cells and colonization of the bovine intestines by the predominant EHEC serotype affecting humans in Europe and North America.

Materials and methods

Bacterial strains and media

Strain 85-170nal^R is a nalidixic acid-resistant nontoxigenic *E. coli* O157:H7 strain that reliably colonizes the intestines of calves and induces AE lesions *in vivo* (Stevens *et al.*, 2004; Vlisidou *et al.*, 2006a). The laboratory *E. coli* strain HB101 was transformed with plasmid pB9-5espP (kindly supplied by Anthony Scott-Tucker, University of Birmingham) for expression and purification of EspP. Bacterial strains were grown in Luria–Bertani (LB) broth or agar supplemented with ampicillin (Amp, 100 μg mL^{-1}), kanamycin (Kan, 50 μg mL^{-1}) and nalidixic acid (Nal, 25 μg mL^{-1}) as appropriate. For adherence assays, bacteria were grown in minimum essential medium buffered with HEPES (MEM-HEPES; Sigma, St Louis).

Construction and characterization of an *E. coli* O157:H7 ΔespP::kan^R mutant

The *espP* gene was replaced by a kanamycin resistance cassette by integration of a linear PCR product upon transient expression of λRed recombinase, essentially as described (Datsenko & Wanner, 2000). The amplicon was generated using Vent^{TM} proofreading DNA polymerase (New England Biolabs, Hitchin, UK) and primers *espP*1F-FRT (5′-GATAAACATACTGTGTTTGTTATCCGTATGATA ACAAACACATAAAGGAG**GTGTAGGCTGGAGCTGCTTC** -3′), and *espP*2-FRT (5′-CGGCAGGCACTGAGGGTAAAG GGCCCGCAGGCCCTTTTGAATACGGAGTA**CATATGAA TATCCTCCTTAG**-3′) which contain 50 nucleotide homology extensions corresponding to the regions immediately 5′ and 3′ of *espP* and priming sequences for the kan^R cassette of pKD4 (bold; Datsenko & Wanner, 2000). The PCR product was *DpnI* digested to remove residual template, gel purified and electroporated into 85-170nal^R carrying the temperature-sensitive λRed helper plasmid pKD46 following induction of λRed recombinase with 10 mM L-arabinose at 30 °C. A recombinant (85-170nal^R Δ*espP*::kan^R) was selected on LB agar containing nalidixic acid and kanamycin and cured of pKD46 by growth at 42 °C. The insertion was confirmed by PCR using the *espP* flanking primers *esp*P-F (5′-CTCCTTTA CTGTATCCCTCAT-3′) and *esp*P-R (5′-AGGCACTGAGGG TAAAGG-3′).

To verify the mutant, 85-170nal^R and 85-170nal^R Δ*espP*::-kan^R were cultivated in Dulbecco's Modified Eagles Medium (DMEM) to induce protein secretion and secreted proteins were prepared by trichloroacetic acid precipitation (Stevens *et al.*, 2004). Secreted proteins were analysed by 4–15% gradient sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and visualized following staining with GelCode Blue reagent (Perbio Science UK Ltd, Cheshire, UK).

Bovine primary cell culture

Bovine primary epithelial cells were derived from the region 0 to 5 cm proximal to the rectal-anal junction of cattle up to 30 months old using a modification of the method described for colonic epithelial cultures (Hoey *et al.*, 2003). Unless stated otherwise, reagents for the procedure were obtained from Sigma-Aldrich. Briefly, mucosal scrapings from the terminal rectum were digested in DMEM containing 1% (v/v) foetal

calf serum (FCS), 100 U mL^{-1} penicillin, 30 μg mL^{-1} streptomycin, 25 μg mL^{-1} gentamicin, 75 U mL^{-1} collagenase and 20 μg mL^{-1} dispase (Roche, Rockford) with gentle shaking at 37 °C until isolated crypts could be observed microscopically. A series of differential centrifugation steps with DMEM containing 2% (w/v) sorbitol was used to enrich the isolated crypts from undigested material, endogenous microbial communities and single cells including fibroblasts (Booth *et al.*, 1995). The crypt cell pellet was resuspended in cell culture medium [DMEM, 2.5% (v/v) FCS, 0.25 U mL^{-1} insulin, 10 ng mL^{-1} epidermal growth factor (EGF) and 30 μg mL^{-1} gentamicin]. Approximately 400–600 crypts were seeded per well into 24-well Costar culture plates (Corning, Cambridge) precoated with collagen (Vitrogen Collagen, Nutacon, Netherlands). To inhibit fibroblast growth, the medium was replaced with MEM D-Valine medium containing 10% (v/v) batch-tested FCS, 0.25 U mL^{-1} insulin, 10 ng mL^{-1} EGF and 30 μg mL^{-1} gentamicin after 24 h as described previously (Hoey *et al.*, 2003). The cells were grown to confluence (*c.* 3×10^5 cells per well, typically achieved 10–14 days after isolation). The cultured cells were confirmed by immunocytochemistry to possess epithelial cell-specific cytokeratins (CK4, 5, 6, 8, 10, 13 and 18) and lack a marker for fibroblasts as described (Hoey *et al.*, 2003).

Purification of exogenous EspP

Purified recombinant EspP was prepared essentially as described (Navarro-Garcia *et al.*, 2004). Briefly, the EspP-overproducing strain HB101(pB9-5 espP) was grown in LB overnight at 37 °C and the supernatant harvested following centrifugation. The culture supernatant was filtered through a 0.22 μm pore-size low-protein-binding membrane, then concentrated by centrifugation in against a Centricon filter with a 100 kDa cut-off (Millipore, Bedford). The retentate was reconstituted in MEM-HEPES, checked for purity by SDS-PAGE and confirmed to contain EspP by Western blotting. The amount of protein was estimated using the standard BCA method (Pierce Biotechnology Inc, Rockford) and stored at − 20 °C until required.

Adherence assays

Stationary-phase LB cultures of wild-type and the 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. Confluent bovine rectal primary epithelial cells (above) were separately infected in duplicate with each of the strains at a multiplicity of infection of *c.* 100 in MEM-HEPES and incubated for 1 h at 37 °C in a humidified 5% CO_2 atmosphere. Purified recombinant EspP was supplemented to a final concentration of 178.8 ng mL^{-1} of protein where appropriate. Nonadherent bacteria were removed by washing three times with phosphate-buffered saline (PBS) and adherent viable bacteria were enumerated following dispersion with PBS-0.1% (v/v) Triton X-100, serial 10-fold dilution and plating onto selective media. The assays were carried out independently on three occasions and the data analysed with a generalized linear mixed model (GLMM) assuming a Poisson response variable (McCullagh & Nelder, 1989). The canonical link function was used. The experimental replicate was fitted as a random effect. Strain and the presence/absence of exogenous EspP were fitted as fixed effects. Evaluation of the significance of fixed effects was carried out using the Chi-squared approximation for the Wald test.

Oral infection of calves with *E. coli* O157:H7

Procedures for oral inoculation of calves with *E. coli* O157:H7 have been described (Stevens *et al.*, 2004) and comply with the Animals (Scientific Procedures) Act 1986 (licence 30/2009). Three 12-day-old Friesian bull calves were coinfected with *c.* 1×10^{10} CFU each of 85-170nal^R and 85-170nal^R Δ*espP*::kan^R grown to stationary phase in brain heart infusion broth in a 1 : 1 ratio. The magnitude and duration of faecal excretion of the bacteria were followed at least once daily for 12 days by plating of triplicate serial dilutions of fresh faeces collected by rectal palpation to sorbitol MacConkey agar (Oxoid, Basingstoke, UK) supplemented with 2.5 μg mL^{-1} potassium tellurite and 20 μg mL^{-1} nalidixic acid (T–SMAC–Nal) and T–SMAC–Nal containing 50 μg mL^{-1} kanamycin (T–SMAC–Nal–Kan). The number of wild-type bacteria was calculated by subtracting the viable count on T–SMAC–Nal–Kan from that obtained using T–SMAC–Nal as previously described (Dziva *et al.*, 2004). Recoveries of wild-type and the mutant bacteria were confirmed by PCR from selected colonies using *espP*-flanking primers. The faecal shedding data were analysed for the effect of mutation by means of an F-test, with the data taken as repeated measurements and the animal as a covariant (Proc Mixed, Statistical Analysis System 1995, SAS Institute, Cary). *P* values < 0.05 were taken to be significant.

Results and discussion

Characterization of an *E. coli* O157:H7 espP::kan^R mutant

A *espP*::kan^R mutant of *E. coli* O157:H7 strain 85-170nal^R was constructed by λRed-mediated linear recombination and verified by PCR with *espP*-flanking primers (data not shown) and SDS-PAGE analysis of proteins secreted by the wild-type and mutant strains. While both the mutant and parent strains secreted comparable quantities of the Type III secreted proteins EspA, -B, -D and Tir, a protein of the

expected size of EspP was absent in the secreted fraction of the mutant (Fig. 1). The protein present in the secreted proteome of the wild-type at this position has been independently confirmed to be EspP by tandem MS (Roe *et al.*, 2007). The growth rate of the 85-170nal^R *espP*::kan^R mutant was confirmed to be identical to that of the parent strain in both rich and minimal media by real-time spectrophotometry (data not shown).

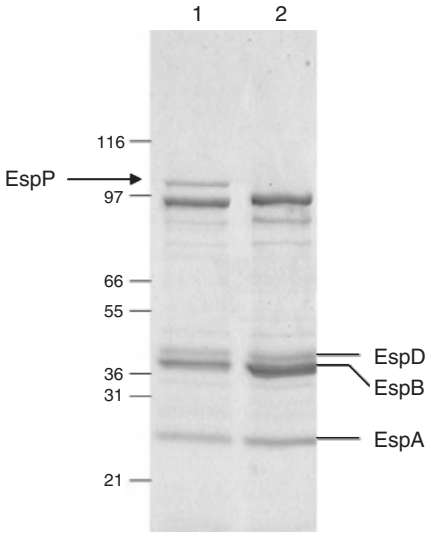

Fig. 1. Characterization of a Δ*esp*P::kan^R mutant of *Escherichia coli* O157:H7 strain 85-170nal^R. Precipitated secreted proteins from the parent (lane 1) and Δ*esp*P::kan^R mutant (lane 2) strains were analysed by SDS-PAGE.

EspP plays a role in the intestinal colonization of calves by *E. coli* O157:H7

The role of EspP in the intestinal colonization of calves was determined using coinfection experiments, using a model that has reliably identified attenuating defects previously (Stevens *et al.*, 2002; Dziva *et al.*, 2004; Vlisidou *et al.*, 2006a, b). From day 5 postoral inoculation, the 85-170nal^R Δ*espP*::kan^R mutant was shed in the faeces in lower numbers than the parent strain and the reduction became statistically significant from day 9 onwards (P values < 0.05; Fig. 2). Although the role of secreted serine protease in persistence of the bovine intestines was first suggested by the isolation of two independent signature-tagged mini-Tn*5*Km2 mutants of EHEC O26:H- with insertions in *pssA* (van Diemen *et al.*, 2005), it has been observed that some genes identified by STM are not required for persistence when defined mutants are constructed and screened in isolation or by coinfection in the same model (*map*; Dziva *et al.*, 2004; *nleD*; Marchés *et al.*, 2005). Thus, the data herein provide novel independent validation of the role of EspP in intestinal colonization. The extent of attenuation caused by the *espP* mutation was slight compared with the effect of intimin and Tir mutations in the same strain in calves of the same age (Vlisidou *et al.*, 2006a), but is significant when compared with an 85-170nal^R mutant lacking the putative cytotoxin/adhesin ToxB (Stevens *et al.*, 2004).

Although it has been shown that EspP is required for intestinal colonization of cattle by *E. coli* O157:H7, it is noteworthy that sorbitol-fermenting EHEC O157:H-strains isolated from cattle lack *esp*P (Brunder *et al.*, 1999; Karch &

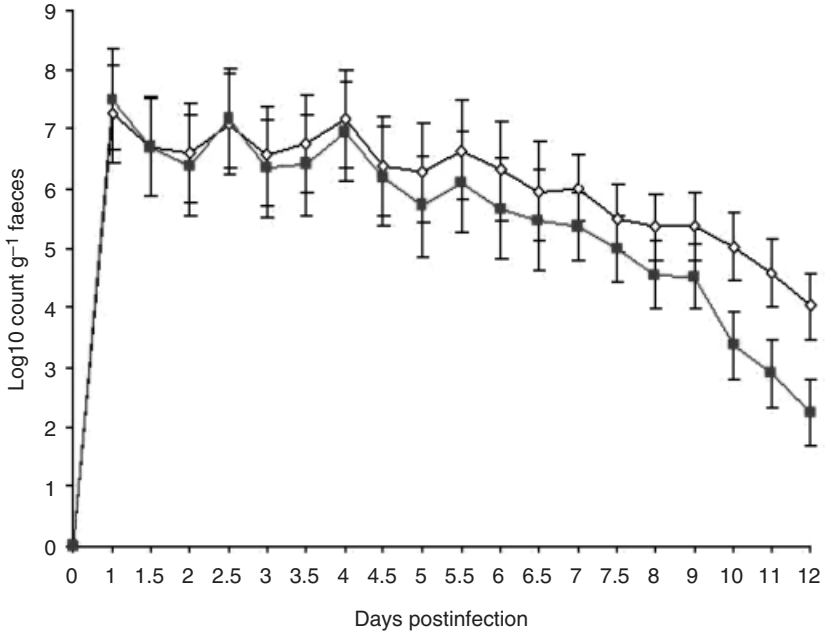

Fig. 2. Course of faecal excretion of *Escherichia coli* O157:H7 strain 85-170nal^R following coinfection of calves ($n = 3$) with the parent strain (-◇-) and an isogenic Δ*esp*P::kan^R mutant (-■-). Data represent the mean daily faecal count ± SE of the mean.

Bielaszewska, 2001) and a mutated form of *esp*P was reported in five of 30 bovine *E. coli* O157:H7 isolates (McNally *et al.*, 2001). However, the possibility of redundancy in the repertoire of secreted proteases in these strains cannot be precluded. Data of this study add to the growing number of secreted cytotoxins that play a role in EHEC-intestinal interactions. Shiga toxin was recently observed to influence intestinal colonization of mice by *E. coli* O157:H7, possibly by modulating the surface expression of intimin coreceptors (Robinson *et al.*, 2006). Furthermore, an inhibitor of lymphocyte proliferation and cytokine synthesis (LifA, lymphostatin) influences intestinal colonisation in calves by EHEC O5:H- and O111:H- (Stevens *et al.*, 2002) and persistence and the induction of colonic hyperplasia by *Citrobacter rodentium* in mice (Klapproth *et al.*, 2005). However, *lifA* mutations also impair adherence (Stevens *et al.*, 2002) and the contribution of direct cytotoxicity to colonization is not fully understood. Enterohaemolysin was also suggested to be important in colonization of calves by EHEC O26:H- by STM and the gene is highly conserved among EHEC (van Diemen *et al.*, 2005). It is possible that such cytotoxins act in concert to promote intestinal colonization.

EspP influences adherence of *E. coli* O157:H7 to bovine primary rectal epithelial cells

To assess the basis of attenuation, bovine primary rectal epithelial cells were isolated for *in vitro* adherence assays. Epithelial cells were obtained from the terminal rectum of cattle *c.* 0–5 cm proximal to the rectal–anal junction. The relevance of cells derived from this site is suggested by the finding that colonization of cattle can be established by the direct administration of *E. coli* O157:H7 to terminal rectal mucosa (Sheng *et al.*, 2004) in a manner dependent on pO157 (Sheng *et al.*, 2006). The cultured cells were polarized and differentiated, expressed the epithelial cell-specific cytokeratins and resembled those seen previously with bovine colonic primary epithelial cell cultures (Hoey *et al.*, 2003).

To examine the impact of secreted EspP on adherence, EspP were overexpressed in *E. coli* strain HB101 and the protein from the supernatant was purified. The purity of the protein preparation was confirmed by SDS-PAGE (Fig. 3a) and the identity of the protein species verified by Western blotting (data not shown). The purification method has previously been used to prepare homogenous catalytically active EspC from EPEC (Mellies *et al.*, 2001; Navarro-Garcia *et al.*, 2004).

Mutant and parent strains were allowed to interact with primary rectal epithelial cells in the presence or absence of purified exogenous EspP. The mean adherence data indicated that the 85-170nal^R Δ*espP*::kan^R mutant was significantly ($P < 0.001$) impaired in its ability to adhere to bovine rectal primary epithelial cells compared with the parent strain (Fig. 3b). The cells showed no evidence on cytotoxicity or cytoskeletal changes (data not shown). The adherence defect of the mutant was rescued by addition of highly

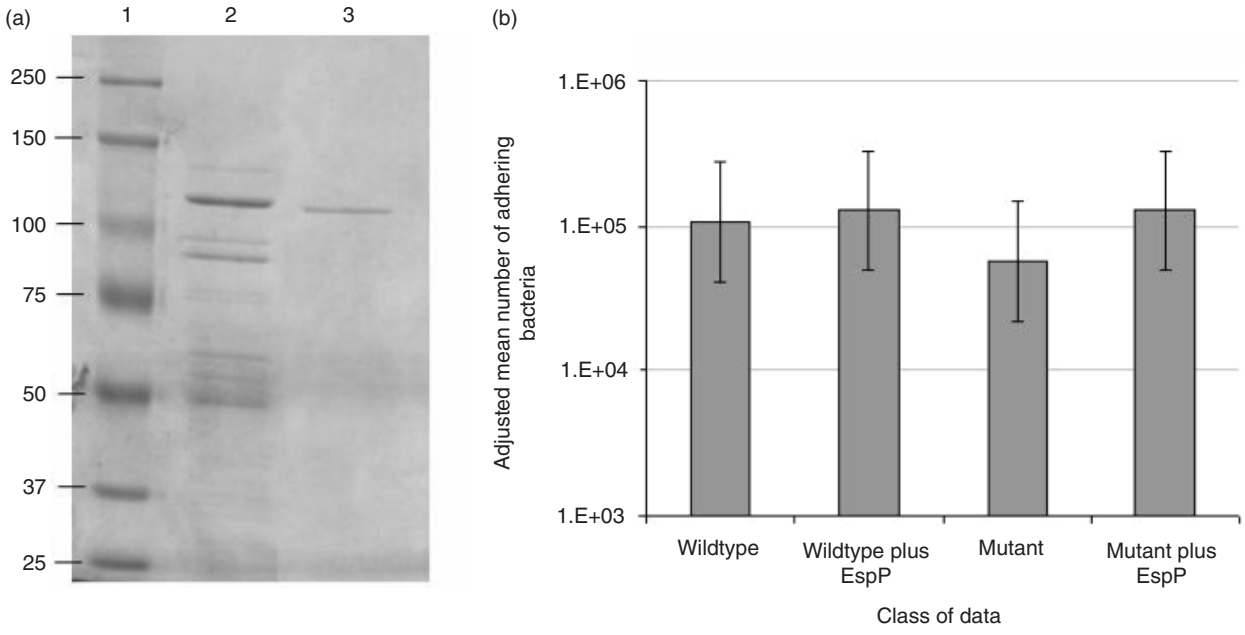

Fig. 3. Adherence of *Escherichia coli* strain 85-170nal^R and its Δ*esp*P::kan^R mutant to bovine primary rectal epithelial cells in the presence or absence of purified exogenous EspP. The purity of exogenous EspP was assessed by SDS-PAGE (a). Lane 1, standard molecular weight marker (kDa); lane 2, whole cell lysate of strain HB101 (pB9-5espP) overproducing EspP; lane 3, purified EspP following ultrafiltration with a 100 kDa cut-off centricon. Adherence data represent the mean from three independent assays (b).

purified recombinant EspP, suggesting that the phenotype of the mutant was due to loss of EspP rather than due to second-site or polar effects. Adherence of the wild-type strain was increased by the addition of exogenous EspP, but not significantly so ($P = 0.03$).

Although the mechanism by which EspP affects intestinal colonization and adherence to cultured cells is unknown, a previous study indicated that it cleaves pepsin A and human coagulation factor V (Brunder *et al.*, 1997). Degradation of factor V is a shared feature among SPATES from other *E. coli* pathotypes (Dutta *et al.*, 2002) and this has been suggested to contribute to mucosal haemorrhage in humans (Brunder *et al.*, 1997). Some SPATEs possess mucinase activity (reviewed in Henderson *et al.*, 2004); however, it has been reported that EspP is unable to cleave bovine submaxilliary mucus *in vitro* (Dutta *et al.*, 2002). Type V-secreted proteins typically possess a β-domain that remains anchored on the bacterial outer membrane and a passenger domain that is often cleaved and released in the milieu. Recently, it has been reported that some autotransported proteins are capable of mediating adhesion directly (Fink *et al.*, 2003) or by acting as scaffolds for bacterial adhesins (Veiga *et al.*, 2003) and it remains possible that surface-anchored EspP may act in this way. The contribution of the secreted and surface-associated portions of EspP and other EHEC cytotoxins in colonization and pathogenesis merits further investigation.

Acknowledgements

The authors gratefully acknowledge the support of the Department for the Environment, Food and Rural Affairs (project no. OZ0707), the support from DEFRA VTRI (project no. VT0102) and the Scottish Executive Environment and Rural Affairs Department (WP2.2-22050).

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