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$EspF_U$, a type III-translocated effector of actin assembly, fosters epithelial association and late-stage intestinal colonization by *E. coli* O157:H7

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Abstract

Enterohemorrhagic *Escherichia coli* (EHEC) O157:H7 induces filamentous actin-rich "pedestals" on intestinal epithelial cells. Pedestal formation *in vitro* requires translocation of bacterial effectors into the host cell, including Tir, an EHEC receptor, and $EspF_U$, which increases the efficiency of actin assembly initiated by Tir. While inactivation of $espF_U$ does not alter colonization in two reservoir hosts, we utilized two disease models to explore the significance of $EspF_U$-promoted actin pedestal formation. EHEC $\Delta espF_U$ efficiently colonized the rabbit intestine during co-infection with wild type EHEC, but co-infection studies on cultured cells suggested that $EspF_U$ produced by wild type bacteria might have rescued the mutant. Significantly, EHEC$\Delta espF_U$ by itself was fully capable of establishing colonization at 2 days post-inoculation but unlike wild type, failed to expand in numbers in the cecum and colon by 7 days. In the gnotobiotic piglet model, an $espF_U$ deletion mutant appeared to generate actin pedestals with lower efficiency than wild type. Furthermore, aggregates of the mutant occupied a significantly smaller area of the intestinal epithelial surface than those of the wild type. Together, these findings suggest that, after initial EHEC colonization of the intestinal surface, $EspF_U$, may stabilize bacterial association with the epithelial cytoskeleton and promote expansion beyond initial sites of infection.

Keywords

EHEC; $EspF_U$; filamentous actin; intestinal colonization

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INTRODUCTION

Enterohemorrhagic *Escherichia coli* (EHEC) is a frequent cause of food-related outbreaks of diarrhea and hemorrhagic colitis in developed nations world-wide (reviewed in (Kaper *et al.*, 2004)). Although these pathogenic *E. coli* do not invade beyond the gastrointestinal tract, the intestinal absorption of Shiga toxin, an EHEC-produced AB_5-type toxin can result in serious systemic disease including the hemolytic uremic syndrome (Nataro and Kaper, 1998; Karmali *et al.*, 1983; Riley *et al.*, 1983). *E. coli* O157:H7 is the most common EHEC serotype associated with both sporadic infection and outbreaks in the United States, but other serogroups have been increasingly associated with human disease (Johnson *et al.*, 2006; Karch *et al.*, 2005).

EHEC, together with enteropathogenic *E. coli* (EPEC) and *Citrobacter rodentium*, are often referred to as “A/E pathogens” because all three of these enteric bacteria are able to induce distinctive ultrastructural changes, known as attaching and effacing (A/E) lesions, on intestinal epithelial cells (Moon *et al.*, 1983). The formation of A/E lesions by this group of extracellular pathogens is thought to promote colonization and damage of the intestinal epithelium because mutants that are incapable of generating A/E lesions are diminished in their capacity to colonize the intestine and cause disease in experimental animals and human volunteers (Deng *et al.*, 2003; Ritchie *et al.*, 2003; Marches *et al.*, 2000; Tacket *et al.*, 2000; Abe *et al.*, 1998; Tzipori *et al.*, 1995; Donnenberg *et al.*, 1993b; Donnenberg *et al.*, 1993a). Moon and co-workers first described the morphological features of these lesions, which include the localized effacement of the brush border microvilli, intimate bacterial attachment to the host epithelium and the assembly of electron-dense fibrillar structures underneath attached bacteria producing a “pedestal-like” protrusion from the cell (Moon *et al.*, 1983).

EHEC, EPEC and *C. rodentium* all share a homologous ~35 kb DNA region called the locus of enterocyte effacement (LEE) pathogenicity island that contains most or all of the genes necessary for A/E lesion formation (Elliott *et al.*, 1999; McDaniel *et al.*, 1995). The LEE encodes transcriptional regulators, the components of a type III secretion system, the outer membrane adhesin, intimin, and several effector proteins that are translocated directly into host cells by the type III apparatus (reviewed in (Garmendia *et al.*, 2005)). One of the translocated effectors essential for pedestal formation is Tir, which inserts into the host plasma membrane with a hairpin loop topology in which the central extracellular portion of Tir serves as a receptor for intimin (de Grado *et al.*, 1999; Hartland *et al.*, 1999; Kenny *et al.*, 1997). Tir-intimin interaction mediates the intimate attachment of bacteria to the epithelial cell surface, and mutants incapable of producing Tir or intimin are also incapable of colonization in experimental infections (Sheng *et al.*, 2006; Deng *et al.*, 2003; Ritchie *et al.*, 2003; Marches *et al.*, 2000).

Whereas the central region of Tir binds intimin, the amino and carboxyl termini are located in the host cytoplasm, where they interact with host proteins and induce remodelling of the cytoskeleton and pedestal formation (Hayward *et al.*, 2006; Goosney *et al.*, 2001). Studies of pedestal formation in cultured mammalian cells indicate that A/E pathogens can employ several pathways to induce actin assembly. In EPEC, at least three pathways have been distinguished, one mediated by phosphorylated Tir tyrosine 474 (Y474) and the host adaptor Nck, a second mediated by phosphorylated Y474 that functions independently of Nck, and a third mediated by Tir Y454, which also functions independently of Nck. In contrast to these EPEC pathways, canonical EHEC strains encode a Tir that lacks a Nck-binding sequence (Campellone *et al.*, 2002; Gruenheid *et al.*, 2001; DeVinney *et al.*, 1999), but contains Y458, the equivalent of EPEC Tir Y454 (Campellone *et al.*, 2006). Whereas this Nck-independent pathway leads to inefficient actin assembly in EPEC, EHEC encodes an additional type III-translocated effector, $EspF_U$ (also known as TccP) that binds and

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activates the host protein N-WASP (Campellone *et al.*, 2004; Garmendia *et al.*, 2004) to increase the efficiency of this pathway (Brady *et al.*, 2007). In the absence of $EspF_U$, EHEC binds to host cells at wild type efficiency, but induces only low (but detectable) levels of actin polymerization in cultured cells (Campellone *et al.*, 2004).

Recent studies have shown that mutations in one or more of these actin assembly pathways does not necessarily diminish the ability of these mutants to colonize and form A/E lesions *in vivo*. For example, a *C. rodentium* Tir mutant lacking the equivalent of Y474 did not undergo efficient tyrosine phosphorylation or induce actin assembly *in vitro*, yet still colonized mice, forming A/E lesions detectable by electron microscopy (Deng *et al.*, 2003). Similarly, an EPEC strain expressing a Tir derivative lacking both Y474 and Y454 was unable to recruit actin *in vitro* but was still capable of generating A/E lesions on human intestinal explants (Schuller *et al.*, 2007). Finally, an EHEC $espF_U$ mutant, which inefficiently triggers actin assembly on cultured monolayers, colonized the intestine of infected calves and lambs indistinguishably from wild type and formed A/E lesions in bovine ligated loops (Vlisidou *et al.*, 2006).

Whereas calves and lambs serve as models to investigate factors that promote EHEC persistence in reservoir hosts, in the current study, we investigated the role of $EspF_U$ in colonization of the intestines of infant rabbits and gnotobiotic piglets, two disease models that might provide insight into EHEC colonization of the human intestine. We found that an $espF_U$ mutant colonized rabbits with wild type efficiency at an early time point, but unlike the wild type, failed to increase in numbers in the cecum and large intestine as infection progressed. By adapting a high resolution, *in situ* fluorescence-based imaging technique to directly visualize EHEC cells bound to the luminal surfaces of the piglet intestinal epithelium, we found that the $espF_U$ mutant formed smaller bacterial aggregates on the mucosal surface than the wild type. Taken together our observations suggest that following initial EHEC colonization, $EspF_U$ may stabilize bacterial association with the epithelial cytoskeleton and promote expansion beyond initial sites of infection.

RESULTS

An EHEC *espFU* mutant shows no colonization defect in co-infection experiments

EHEC colonizes the infant rabbit ileum, cecum and large bowel and causes diarrhea and intestinal inflammation in an intimin- and Tir-dependent manner (Ritchie *et al.*, 2003). To examine the potential role of $EspF_U$ during colonization in this disease model, we generated an $espF_U$ deletion mutant in EHEC strain 905, a clinical *E. coli* O157:H7 isolate that has been used previously in the rabbit model (Ritchie and Waldor, 2005; Ritchie *et al.*, 2003). We initially carried out co-infection (competition) experiments between the wild type strain and this isogenic $espF_U$ mutant. The readout for co-infection studies is the competitive index (C.I.), i.e., the ratio of mutant to wild type CFU in infected tissues divided by the ratio of mutant to wild type CFU in the inoculum. This experimental design limits the effects of animal-to-animal variation since the wild type strain serves as an internal control in every animal and often provides a more sensitive way to detect colonization defects than single infection experiments (Logsdon and Mecsas, 2003). As a control to validate the competition format for these experiments, we performed a competition experiment between isogenic kanamycin-resistant and kanamycin-sensitive EHEC strains. In the kanamycin-resistant strain, we replaced *lacZ* (a gene not required for EHEC intestinal colonization in single infection rabbit experiments; J.M.R. and M.K.W. unpublished observations) with a kanamycin-resistance gene. At day 7 after oro-gastric inoculation with $5x10^8$ bacteria per 90 gram rabbit, we found approximately equal numbers of 905 and 905Δ*lacZ::kan* in homogenates of different regions of the intestine, yielding a C.I. of approximately 1.0 (Table 1), validating the competition format in this animal model. Co-infection experiments using

905 and 905Δ*tir* provided additional validation for the competition format. The 905Δ*tir* mutant, which is highly attenuated in single infection experiments (Ritchie *et al.*, 2003), was also markedly attenuated in co-infection experiments, where C.I. values ≤ 0.0003 were found in all sampled regions (Table 1).

When we co-inoculated 3-day old infant rabbits with approximately equal numbers of 905 and 905Δ$espF_U$, the $espF_U$ mutant did not exhibit a colonization defect. Approximately equal numbers of 905 and 905Δ$espF_U$ CFU were recovered in samples from the ileum, cecum, mid-colon and stool at 7 days post-inoculation resulting in a C.I. of ~1 (Table 1, experiment I). Similar experiments examining colonization 2 days after inocula of $5x10^6$ or $5x10^4$ per 90 g rabbit weight yielded similar findings, as all C.I.s did not significantly differ from 1 (Table 1, experiment II). The latter dose is apparently near the ID_{50} for EHEC in this model, because 12 out of 31 (39%) rabbits did not become infected and 9 of the 31 (29%) became infected almost exclusively with one strain (Table 1 legend). In these 9 rabbits, 5 became predominantly infected with wild type 905 and 4 with the $espF_U$ mutant, indicating equal propensity for establishing infection. Thus, co-infection experiments did not provide evidence for a role for $espF_U$ in initiating intestinal colonization.

The F-actin assembly defect of an *espFU* mutant can be *trans*-complemented by a co-infecting bacterium

The lack of a discernible colonization defect by the $espF_U$ mutant in these competition assays might result from the ability of 905Δ$espF_U$ to take advantage of $EspF_U$ injected into epithelial cells by wild type 905. In fact, before the identification of $EspF_U$, DeVinney and coworkers showed that whereas an EPEC strain expressing EHEC Tir could not generate actin pedestals *in vitro*, co-infection with an EHEC strain competent for type III secretion "*trans*-complemented" this defect (DeVinney *et al.*, 2001). To investigate whether such *trans*-complementation is due to $EspF_U$, we co-infected HeLa cells with strains that were competent or incompetent for $EspF_U$ translocation. In these experiments and in the piglet infection studies described below, we utilized TUV93-0, a Shiga toxin-deficient derivative of the prototype sequenced O157:H7 strain EDL933, in order to analyze EHEC association with intestinal epithelia while avoiding the neurological complications that result from Shiga toxin expression (Campellone *et al.*, 2007). To allow the two strains to be distinguished using anti-O157 antiserum, we infected cells with TUV93-0, an (O157-positive) EHEC strain competent for $EspF_U$ delivery, and KC12, an EPEC strain (O157-negative) that does not express $EspF_U$ and in which the endogeneous EPEC *tir* is replaced with EHEC *tir* (Campellone *et al.*, 2002). KC12 harboring p$espF_U$, an $EspF_U$–encoding plasmid, generates pedestals in a fashion mechanistically identical to canonical EHEC strains ((Campellone *et al.*, 2002); Figure 1, row 2), but in the absence of $EspF_U$, KC12 is incapable of efficient pedestal formation ((Campellone *et al.*, 2004); Figure 1, row 3). However, KC12 was able to form pedestals when co-infected with TUV93-0 (Figure 1, row 4). Interestingly, pedestal formation by TUV93-0 appeared diminished upon co-infection with KC12, suggesting that the co-infecting KC12 might be effectively sequestering translocated $EspF_U$ in this *in vitro* system (Figure 1, row 4 arrowhead). *Trans*-complementation was dependent upon co-infection of the same cell by both KC12 and TUV93-0 (data not shown), and on $EspF_U$, because no KC12-associated pedestals were observed upon co-infection with TUV93-0Δ$espF_U$ (Figure 1, row 5). These observations strongly suggest that $EspF_U$, translocated into HeLa cells by TUV93-0, can promote pedestal formation by $EspF_U$-deficient KC12.

In contrast to the EHEC $espF_U$ mutant, the EHEC *tir* mutant demonstrated a severe colonization defect in the competition assay in rabbits, suggesting that a *tir* mutant cannot be efficiently *trans*-complemented by Tir translocated by a co-infecting strain. To test this, we utilized KC12Δ*tir*/p$espF_U$, a strain that cannot generate pedestals due to the lack of Tir

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(Figure 1, row 6). Although co-infection with TUV93-0 resulted in detectable Tir-dependent localized actin assembly beneath KC12Δ*tir*/p$espF_U$ (Figure 1, rows 7 and 8), indicating some degree of Tir *trans*-complementation, the intensity of F-actin assembly was significantly lower than that observed with *trans*-complementation by $EspF_U$ (compare Figure 1, rows 4 and 7, arrows). Whereas co-infection of TUV93-0 with KC12 appeared to diminish pedestal formation by TUV93-0, co-infection with KC12Δ*tir*/p$espF_U$ did not, suggesting that KC12Δ*tir*/p$espF_U$ did not sequester Tir away from TUV93-0 to an extent sufficient to inhibit pedestal formation (Figure 1, row 7, arrowhead). These results are consistent with a hypothesis that the absence of a colonization defect of the $espF_U$ mutant in the *in vivo* competition experiments described above results from efficient *in vivo trans*-complementation of this $espF_U$ mutant by wild type bacteria also present in the rabbit intestine. In contrast, the very poor *trans*-complementation of *tir in vitro* is consistent with the severe colonization defect of the *tir* mutant observed in the *in vivo*competition experiments.

In single infection experiments the *espFU* mutant is defective in intestinal colonization

To assess the role of $espF_U$ during animal infection without the potentially confounding effect of *trans*-complementation, we performed single infection experiments with the 905Δ$espF_U$ mutant. In these experiments, 3 day-old rabbits were orogastrically inoculated with $5x10^8$ CFU per 90g rabbit weight of either 905 or 905Δ$espF_U$. Regardless of the infecting strain, all rabbits developed severe diarrhea and exhibited some degree of mucosal damage with acute diffuse suppurative colitis, indistinguishable from that previously described (Ritchie *et al.*, 2003) (data not shown), suggesting that $espF_U$ is not essential for the development of disease in this model. Furthermore, two days post-inoculation, there were no differences in the number of 905 or 905Δ$espF_U$ CFU recovered from ileal, cecal or mid-colon tissue sections or in the stool of 905-or 905Δ$espF_U$-infected rabbits (Figure 2). However, while the numbers of 905 CFU in the ileum, cecum, and mid-colon were 5- to 30-fold ($P<0.01$) greater by day 7 than day 2, a significant increase in the number of 905Δ$espF_U$ CFU was observed only in the ileum. Thus, at this time point, colonization by 905Δ$espF_U$ was significantly impaired compared to 905 in the cecum (4-fold reduction, $P\leq0.05$), mid-colon (6-fold reduction, $P\leq0.001$), and stool (2-fold reduction, $P\leq0.05$). Together these observations suggest that $EspF_U$ promotes robust EHEC colonization in the cecum and colon after the first 48 hours of infection.

EspFU may increase the efficiency of, but is not absolutely required for, A/E lesion formation during piglet infection

Gnotobiotic piglets are not a useful model to quantify EHEC intestinal colonization by viable counts because EHEC mutants defective for the expression of intimin, a known colonization factor, or for type III secretion, are still present at levels comparable to the wild type in the piglet intestine ((Tzipori *et al.*, 1995); MB, ST, unpublished observations). However, the absence of competing intestinal microflora in this animal model allows EHEC to be readily visualized within the intestine, making this a particularly attractive system in which to examine the interaction between EHEC and the intestinal epithelium. Therefore, to examine whether EHEC mutants deficient in $EspF_U$ interact with the intestinal epithelium *in vivo*, TUV93-0 or isogenic derivatives lacking $espF_U$ or *tir* (Campellone *et al.*, 2002) were orally inoculated into 1-day old gnotobiotic piglets. One day later, a time point known to yield numerous A/E lesions ((Campellone *et al.*, 2007); data not shown), intestinal tissue samples were removed and assessed for bacterial attachment by staining sections with hematoxylin and eosin (H&E). As previously observed with other EHEC strains (Tzipori *et al.*, 1986), in much of the small intestine and throughout the large intestine, TUV93-0 was closely associated with the host epithelium, appearing to disrupt the regular brush border observed in uninfected tissue (data not shown). In marked contrast, TUV93-0Δ*tir* was not

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observed in close association with the epithelium (data not shown), as has been reported in previous studies using *tir*-deficient mutants in different experimental infection models (Sheng *et al.*, 2006; Deng *et al.*, 2003; Ritchie *et al.*, 2003; Marches *et al.*, 2000). Bacterial attachment in tissue samples from piglets infected with the $espF_U$ mutant appeared indistinguishable from that seen in samples taken from piglets infected with TUV93-0; thus, H&E staining of cross-sections of piglet intestinal tissue did not reveal a gross defect in the ability of the $espF_U$ mutant to associate with epithelial cells *in vivo* at this time point.

TUV93-0Δ$espF_U$ retains the ability to induce actin assembly on cultured Hela cells but with significantly reduced efficiency compared with TUV93-0 (Campellone *et al.*, 2004; Garmendia *et al.*, 2004). To assess the ability of TUV93-0Δ$espF_U$ to generate A/E lesions *in vivo*, samples from infected piglets were analyzed using transmission electron microscopy. In ileal samples, TUV93-0 was frequently associated with raised pedestals under which electron-dense material could be observed (Figure 3A). Ileal samples from piglets infected with TUV93-0Δ$espF_U$ also revealed some pedestals, albeit at apparently lower frequency than observed with TUV93-0. Many TUV93-0Δ$espF_U$ bacteria in these images appeared to be in close contact with the epithelium in the absence of regions of electron-dense staining characteristic of localized actin assembly (Figure 3B). Infection with TUV93-0Δ$espF_U$ harboring a complementing $EspF_U$-encoding plasmid resulted in restoration of high frequency pedestal formation (Figure 3C). Although there are clearly limitations inherent in the analysis of electron micrographs, our observations suggest that TUV93-0Δ$espF_U$ has a diminished capacity to generate actin pedestals *in vivo*; however since some pedestals were observed, our findings also indicate that $EspF_U$ is not absolutely essential for pedestal formation *in vivo*.

EspFU promotes EHEC association with the intestinal epithelium *in vivo*

Because sampling variability in electron micrographic analysis of cross-sections of intestinal tissue limits measurement of bacterial attachment to the host epithelium, we adapted a high resolution, *in situ* fluorescence-based imaging technique originally developed for *en face* viewing of the vascular endothelium (Herman *et al.*, 1987; Wong *et al.*, 1983; Herman *et al.*, 1982) as an alternative method. We applied fluorescent anti-O157 antiserum to directly visualize the distribution of attached bacteria over relatively large (0.5cm by 0.5cm) mucosal segments. Pilot experiments revealed that the cecum was the easiest intestinal segment to assess attached TUV93-0 (data not shown). In these tissue samples, non-adherent bacteria could readily be distinguished from adherent bacteria by virtue of the fact that they were usually observed as individual cells and located out of the plane of focus, apparently suspended in the mucus layer. Consistent with this classification, TUV93-0Δ*tir* was generally seen as isolated bacteria that were almost never observed in close apposition to the epithelium (data not shown). In contrast, adherent TUV93-0 was routinely observed in aggregates in the same focal plane as the surface of the epithelial cells (Figure 4A).

Comparison of cecal sections from piglets inoculated with TUV93-0 or TUV93-0Δ$espF_U$ revealed that there were much larger clusters of TUV93-0 present on the cecal epithelium (compare Figure 4A and 4B). In many sections, clusters of TUV93-0 cells covered most of the visible cecal surface, whereas TUV93-0Δ$espF_U$ cells were only observed as relatively small discrete foci covering less of the cecal surface. These qualitative observations were corroborated by more quantitative analyses of these micrographs, in which the percentage of the cecal epithelial surface covered with bacteria was scored in a blinded manner (see methods). In piglets from at least 2 independent experiments and in multiple cecal segments, the area of the cecum covered by TUV93-0 was significantly ($P \leq 0.01$) larger than that covered by the $espF_U$ mutant (Figure 4D). This defect in epithelial colonization by the $espF_U$ mutant could be restored to wild-type levels by the introduction of a plasmid-borne

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copy of *espF*$_U$ into TUV93-0Δ*espF*$_U$ (Figure 4C and 4D). These observations suggest that *espF*$_U$ promotes EHEC association at the intestinal epithelial surface.

DISCUSSION

Intimate attachment of EHEC to the intestinal epithelium is well established as a requirement for EHEC pathogenesis, since mutations of intimin or Tir preclude EHEC colonization in experimental models of disease (Ritchie *et al.*, 2003; Tzipori *et al.*, 1995; Donnenberg *et al.*, 1993a). However**,** uncovering the significance of actin assembly during pedestal formation has been elusive. By deleting *espF*$_U$ from EHEC, the role of intimate adherence mediated by intimin and Tir can be partially uncoupled from the function of robust actin polymerization, since EHECΔ*espF*$_U$ binds to cultured host cells at normal levels but exhibits a ~20-fold lower frequency of actin assembly into pedestals (Campellone *et al.*, 2004). While an EHEC *espF*$_U$ mutant does not exhibit a colonization defect in calves or lambs, both normal animal reservoirs (Vlisidou *et al.*, 2006), we examined the role of EspF$_U$ during infection using two complementary animal models for EHEC disease, rabbits and piglets. We found that EspF$_U$ had a significant influence on intestinal colonization in both systems suggesting a link between actin assembly and pathogenesis by these extracellular bacteria.

No defect in colonization by an EHEC *espF*$_U$ mutant was detectable when infant rabbits were co-infected with wild type EHEC (Table 1). However, this observation could reflect *trans*-complementation by EspF$_U$ injected into epithelial cells by the wild type strain during co-colonization. Indeed, we demonstrated highly efficient EspF$_U$ *trans*-complementation in cultured cells (Figure 1). Such *trans*-complementation in the host cell does not appear to be a universal feature of EHEC type III secreted effectors, since Tir from wild type EHEC is incapable of efficient *trans*-complementation during co-infection of rabbits or cultured epithelial cells (Table 1 and Figure 1), and strains lacking the type III secreted effectors Map or NleD were not *trans*-complemented during co-infection experiments in calves (van Diemen *et al.*, 2005;Dziva *et al.*, 2004).

Importantly, when rabbits were singly infected with wild type or EHECΔ*espF*$_U$, the mutant showed a moderate but significant colonization defect at day seven. No defect was observed in any segment of the intestine at day two, suggesting that EspF$_U$ does not play an important role in the establishment of infection in this model. Consistent with this, during the competition experiments, when rabbits were co-infected with wild type and mutant at a dose close to the apparent ID_{50}, the number of rabbits that became predominately infected with the mutant was similar to the number that became predominately infected with the wild type strain. Between day 2 and day 7, however, the wild type increased significantly in numbers in all segments of the intestine, whereas the mutant increased only in the ileum, resulting in a 4- to 6-fold colonization defect at this time point. Interestingly, a *Citrobacter rodentium* strain harbouring a point mutation mutation in *tir* that specifically diminishes actin pedestal formation also showed a small (~5-fold) colonization defect at 10 days post-infection, but the difference did not reach statistical significance (Deng *et al.*, 2003) The late-stage defect associated with deletion of *espF*$_U$ in EHEC contrasts with lack of a colonization defect previously observed using a different EHECΔ*espF*$_U$ strain in calves and lambs (Vlisidou *et al.*, 2006). In this earlier study, neither single nor dual infection experiments of 11 to 15 days duration revealed a colonization phenotype for EHECΔ*espF*$_U$ at any time point, as reflected in the number of EHEC CFU found in stool. These disparate findings may be due to the differences in animal host species, the EHEC strain backgrounds, or experimental methodologies. For example, the calf and lamb study monitored colonization by stool CFU. In rabbits, both in previous studies (Ritchie and Waldor, 2005), and in the current study, we

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have found that colonization defects are usually more pronounced in tissue homogenates than in fecal material.

Although the gnotobiotic piglet model is very different from the infant rabbit model and cannot be easily adapted for quantitative kinetic studies of infection, robust A/E lesions form on the intestinal epithelium one day after high dose oral inoculation of gnotobiotic piglets (Tzipori *et al.*, 1995; Tzipori *et al.*, 1986). Thus, this model provided an attractive means to analyze the role of $EspF_U$ in epithelial attachment and colonization. Electron microscopic analysis of tissues from infected piglets revealed that EHECΔ$espF_U$ were intimately attached to the intestinal epithelium, but appeared to generate typical A/E lesions at a diminished frequency relative to wild type. This observation is consistent with studies indicating that $EspF_U$ dramatically increases the efficiency of pedestal formation on cultured cells (Brady *et al.*, 2007). Nevertheless, the mutant was still occasionally associated with A/E lesions in piglets, consistent with the previous observation that an EHECΔ$espF_U$ mutant generated A/E lesions in bovine ligated ileal loops (Vlisidou *et al.*, 2006) and with the relatively mild colonization defect of the $espF_U$ mutant in rabbits. EHECΔ*tir*, which is completely incapable of generating A/E lesions, is severely defective for colonization. That A/E lesions are associated with EHECΔ$espF_U$ during piglet infection reinforces the finding that mutants of A/E pathogens that are severely defective for pedestal formation *in vitro* may retain a significant ability to generate such lesions in animal models (Schuller *et al.*, 2007; Deng *et al.*, 2003).

Our findings suggest that $EspF_U$ facilitates EHEC's capacity to establish a larger niche in the rabbit intestine after initial steps in colonization have been achieved, since we observed that the EHECΔ$espF_U$ colonization defect was detectable after seven (but not two) days of infection. We adapted an *in situ* fluorescence-based imaging technique originally developed for *en face* analysis of the vascular endothelium to investigate colonization of the lumenal surface of the piglet intestine to gain insight into how $EspF_U$ promotes bacterial colonization. Interestingly, we found that the 4-6-fold colonization defect in rabbits was similar to the 5-6-fold reduction in the area of cecal epithelium covered by discreet masses of attached bacteria in piglets (Figure 4). $EspF_U$ appears to have several activities, as its homolog EspF harbors a mitochrondrial localization signal (Nagai *et al.*, 2005) and triggers apoptosis of cultured mammalian cells (Nougayrede and Donnenberg, 2004;Crane *et al.*, 2001), and both EspF and $EspF_U$ can promote disruption of tight junctions (Viswanathan *et al.*, 2004;McNamara *et al.*, 2001). Nevertheless, an EPEC strain expressing the Tir Y474F mutant that is diminished in its ability to stimulate actin assembly also appeared to generate smaller bacterial aggregates on human explants (Schuller *et al.*, 2007). Thus, it is tempting to speculate that actin assembly promoted by $EspF_U$ facilitates an expansion of primary colonization foci, perhaps by 'anchoring' bacteria to the epithelial cytoskeleton or promoting EHEC motility and spread across the intestinal epithelium, similar to the spread of some viral pathogens (Goldberg, 2001).. In fact, EPEC (Sanger *et al.*, 1996), and to a lesser degree EHEC (Shaner *et al.*, 2005), move along the surface of cultured epithelial cells in an actin polymerization-dependent fashion. By combining *in vitro* analyses of EHEC pedestal formation and motility with the animal-based techniques described in this study, we can test these and other models of processes that contribute to intestinal colonization by A/E.

MATERIALS AND METHODS

Bacterial strains, complementing plasmids and cell culture

Strain 905 is a Stx2-producing *E. coli* O157:H7 clinical isolate (Ritchie *et al.*, 2003). Deletion of $espF_U$ in 905 was preformed using a one-step PCR-based gene inactivation protocol (Datsenko and Wanner, 2000). Briefly, PCR-generated substrates containing the kanamycin resistance gene flanked by 36 nucleotides of $espF_U$ targeting sequences were

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electroporated into 905 containing the lambda-red plasmid, pKD46. Replacement of codons 63 -1116 of $espF_U$ with the kanamycin resistance gene was confirmed by PCR. Strain 905Δ*tir* has been previously described (Ritchie *et al.*, 2003). Strain 905Δ*lacZ* contains a deletion of *lacZ* codons 36 - 3036 and was made using the one-step PCR-based gene inactivation protocol. TUV93-0 is a Shigatoxin deficient form of the prototype sequenced *E. coli* O157:H7 strain EDL933, and the isogenic *tir* and $espF_U$ mutants have been described (Campellone *et al.*, 2004; Campellone *et al.*, 2002). EPEC KC12, an O127:H6 strain in which EPEC *tir-cesT-eae* is replaced with EHEC *tir-cesT-eae*, and the isogenic *tir* derivative have been described (Campellone *et al.*, 2002). The complementing plasmid p$espF_U$ is based on a medium copy vector pKC321 and was previously described (Campellone *et al.*, 2004).

For routine passage, *E. coli* strains were cultured on or in LB at 37°C. EHEC or EPEC mutants containing antibiotic resistance genes were grown in media containing the appropriate antibiotic [kanamycin 50 μg ml^{-1}, chloramiphenicol 12.5 μg ml^{-1}]. None of the mutants appeared to have any obvious growth defects when cultured in LB at 37°C (data not shown).

Mammalian culture and *trans*-complementation assays

HeLa cells were routinely cultured in DMEM plus 10% fetal bovine serum. For *trans*-complementation experiments, HeLa cell monolayers were co-infected with equal numbers (~10^6 colony forming units (CFU) / strain) of EHEC and KC12 or derivatives for 6 hours, fixed and permeabilized as previously described (Campellone *et al.*, 2002). For microscopic examination, fixed monolayers were treated with rabbit anti-O157 antisera (1:500 in PBS; Difco) for 30 minutes prior to washing and addition of Alexa488 goat anti-rabbit antisera (1:200; Molecular Probes) to detect EHEC. KC12 and F-actin were detected as described previously (Campellone *et al.*, 2002).

Infant rabbit experiments

Infant rabbit experiments were carried out as has been previously described (Ho and Waldor, 2007; Ritchie *et al.*, 2003). Briefly, 3-day infant rabbits were orogastrically inoculated with 905 and/or isogenic derivatives with deletions of $espF_U$, *tir*, or *lacZ* at a total dose equivalent to $5x10^8$ CFU per 90g rabbit weight. Diarrhea was scored as follows: none, no diarrhea; mild, diarrhea consisting of a mix of soft unformed and formed pellets, resulting in light staining of the hind legs; or severe, diarrhea consisting of unformed or liquid stool, resulting in significant staining of the perineum and hind legs. At 2 and 7 days post-infection, infected rabbits were euthanized and their intestines removed. Various intestinal sections were weighed and homogenized before the determination of bacterial numbers by serial dilution and plating on sorbitol MacConkey (SMAC) agar plates. Colonic sections were also evaluated for histologic changes including assessment for infiltration of heterophils, mononuclear cells, the extent of edema, congestion and mucosal damage, and numbers of globlet cells by a comparative pathologist blinded to the sample identity. For co-infection experiments, the SMAC plates were replica plated onto antibiotic containing SMAC plates to allow enumeration of antibiotic-resistant EHEC cells (representing either the $espF_U$, *tir* or *lacZ* mutants). Data were expressed as competitive indices (C.I.), the ratio of the number of mutant to wild type bacteria post-infection divided by the number of mutant to wild type bacteria in the inoculum. All single infection and co-infection experiments were repeated in at least two different rabbit litters.

Gnotobiotic piglet experiments

Gnotobiotic piglet experiments were carried out as has been previously described (Tzipori *et al.*, 1992). Briefly, piglets derived by caesarean section and maintained in microbiological

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isolation, were orally infected with 5 x 10^9 CFU TUV93-0 or one of its derivatives. After euthanasia at 24hr post-infection, sections of the intestine were removed and fixed in 10% neutral buffered formalin. Bacterial attachment was determined in intestinal cross sections stained with hematoxylin and eosin (H&E) and in unstained segments as described below. Processing of intestinal samples for electron microscopic analysis was performed as described previously (Donnenberg *et al.*, 1993a).

In situ localization and quantitative analysis of EHEC association with the piglet intestinal epithelium

To visualize and quantify bacterial attachment in infected gnotobiotic piglets, we adapted a fluorescence light microscopic *in situ* imaging technique originally developed for the quantitative analysis of vascular endothelial cytoskeletal arrays (Berceli *et al.*, 1991; Herman *et al.*, 1987; Wong *et al.*, 1983; Herman *et al.*, 1982). In brief, approximately 0.5cm long segments of the intestine were slit length-wise to expose the luminal surface and rinsed 3x in PBS-azide (PBS containing 0.02% Na-azide) for 5 minutes each at room temperature. The tissue samples were then incubated for 90 sec at room temperature in 0.1% Triton-X100, 40mM HEPES (pH 7.1), 1mM MgCl2, 0.5mM EGTA, 50mM PIPES (pH 6.9) and 75mM NaCl, before being rinsed twice in PBS-azide for 5 minutes. The samples were then stained with *E. coli* O157 antiserum [1:500 in PBS; Difco] for 30 minutes at room temperature. After further rinsing in PBS-azide (2 times), the samples were incubated for 30 minutes with Alexa488 conjugated anti-rabbit IgG (1:500 in PBS; Invitrogen) to enable detection of EHEC and Alexa546-phalloidin (1:50 in PBS; Invitrogen) for visualization of the tissue architecture in control and EHEC-infected intestinal segments. The samples were finally rinsed in PBS-azide before being flattened and mounted under cover slips, which were subsequently anchored to the slide surface to permit *en face* mucosal surface viewing. The samples were examined using a Zeiss Axiovert 200M Digital light microscope image acquisition system (Molecular Devices Corporation, CA, USA). To quantitatively assess the attachment of EHEC to the intestine, coded cecal samples were scanned for attached bacteria and representative images captured. In each experiment for each bacterial strain, five images from 2 - 3 cecal segments were examined for bacterial attachment. Each experiment was performed on two separate occasions. The area of EHEC attachment (visualized using anti-O157 antibody) was calculated as a percentage of the epithelial surface in the field of view (visualized using fluorescent phalloidin) by an observer who was blinded to the coding system.

Statistical analysis

Bacterial counts from single infection experiments were log transformed and analyzed using the Student's t-test (two-way), comparing each mutant to the wild type. C.I. values obtained from competition experiments between wild type and the $espF_U$ or *tir* mutants were compared using the Student's t-test (two-way) to C.I. values obtained from competition experiments between wild type and a *lacZ* mutant. Differences in the percentages of wild type and mutant bacteria attached to intestinal tissue were also analyzed using the Student's t-test (two-way).

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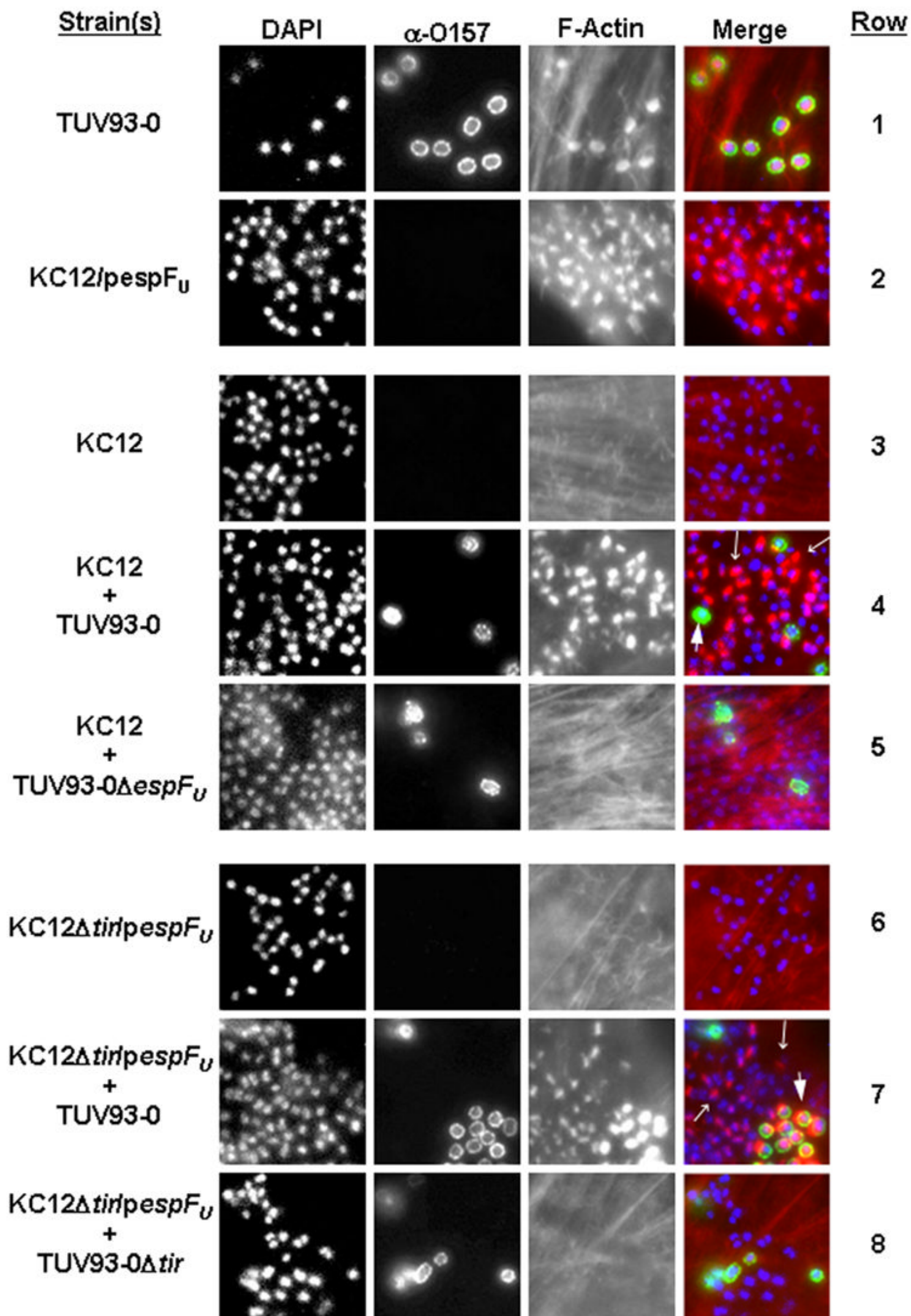

Figure 1. Efficient *trans*-complementation by EspF$_U$ but not Tir during mixed infection on HeLa cells

Infected monolayers were examined microscopically after staining with DAPI to visualize bacteria, anti-O157 antiserum to specifically detect EHEC strains and fluorescent phalloidin to detect F-actin. For merged images, DAPI staining is shown in blue, bacteria in green, F-actin in red, and foci of co-localized bacteria and F-actin in yellow. HeLa cells were infected singly or in combination with the indicated strains. In rows 4 or 7, large arrowheads on merged images indicate TUV93-0 or its derivatives; arrows indicate efficient (row 4) or inefficient (row 7) *trans*-complementation, i.e., sites of actin assembly associated with KC12 (anti-O157 negative bacteria).

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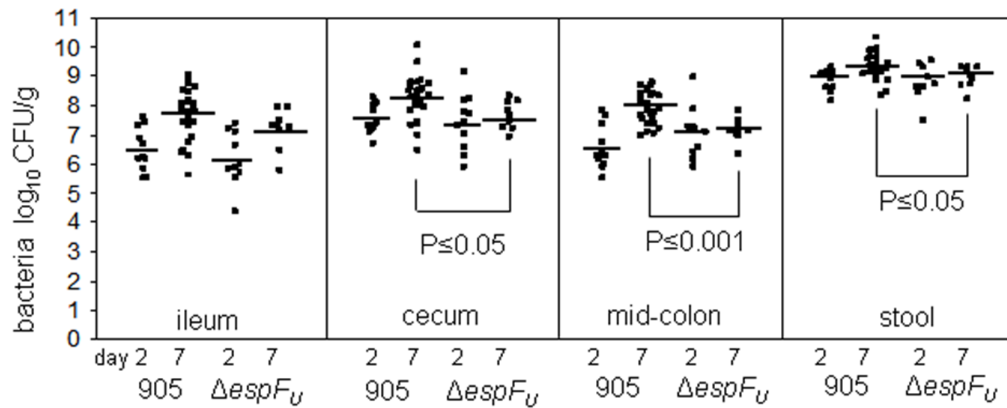

Figure 2. EspF$_U$ is associated with increased efficiency of colonization at day 7 post-infection of infant rabbits

905 or 905Δ*espF$_U$* were recovered from intestinal segments or stool samples from infected rabbits at 2 and 7 days post-inoculation. Each point represents an individual rabbit and bars represent the geometric mean for each strain. The level of wild type colonization differed significantly between day 2 and day 7 in the ileum ($P \leq 0.01$), cecum ($P \leq 0.01$), mid colon ($P \leq 0.0001$) and stool ($P \leq 0.01$), whereas the level of mutant colonization only differed significantly ($P \leq 0.05$) in the ileum, when analyzed by the Student's t-test (two-way).

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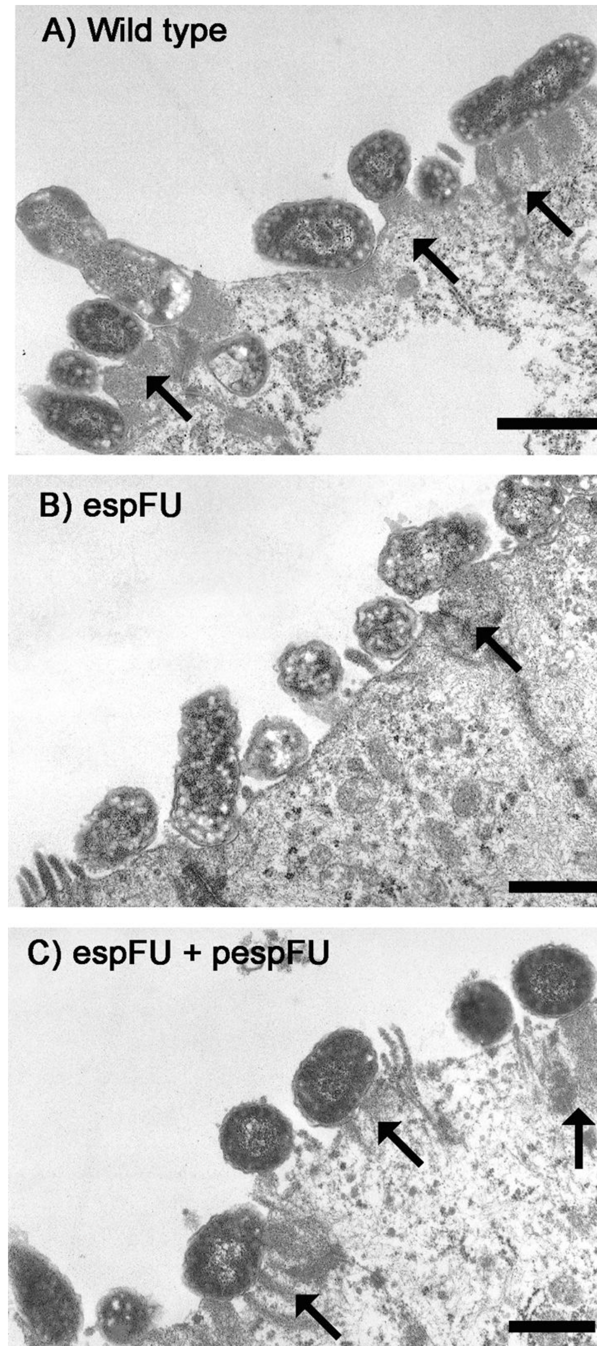
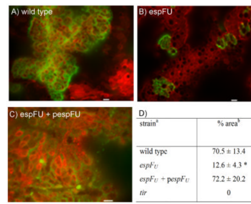


Figure 3. $EspF_U$ may increase the efficiency of, but is not absolutely required for A/E lesion formation during piglet infection

Electron micrographs of the ileum of piglets infected with wild type (A), $\Delta espF_U$ (B) or$\Delta espF_U$ / p$espF_U$ (C). Arrows show areas of electron-dense material. Bars equal 1μm.



strain[a]	% area[b]
wild type	70.5 ± 13.4
espF$_U$	12.6 ± 4.3 *
espF$_U$ + p*espF*$_U$	72.2 ± 20.2
tir	0

Figure 4. EspF$_U$ is associated with a larger size of clusters of attached bacteria

O157-labeled bacteria adherent to the cecum of piglets infected with wild type (A), Δ*espF*$_U$ (B), and Δ*espF*$_U$ / p*espF*$_U$ (C) –infected piglets. Bacteria are stained green and F-actin is stained red. Note the relatively small clusters of attached bacteria in (B). Scale bars are 10μm in each panel. Panel (D): Area of tissue colonized by O157-labeled bacteria divided by area visible with phalloidin staining expressed as a percentage. [a]Strains are derivatives of TUV93-0. [b]Values are mean ± standard deviation. Data were compared using the Student's t-test (two-way) and considered significantly different (*) at P≤0.05.

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Table 1

Co-infection of an $espF_U$ mutant with wild type EHEC does not reveal a role for $espF_U$ in intestinal colonization.

Expt	Post- infection day	Dose[a]	strain	C.I. values[b] in intestinal homogenates or stool samples:			
				ileum	cecum	mid-colon	stool
I [c]	7	$5x10^8$	Δ*lacZ*	1.13 (0.56 – 2.30)	0.99 (0.55 – 1.76)	1.34 (0.65 – 2.74)	1.10 (0.57 – 2.14)
			Δ*tir*	$2 x 10^{-4}$ [c,*] ($7x10^{-5}$–$6x10^{-4}$)	$3 x 10^{-5}$ * ($1x10^{-5}$–$7x10^{-5}$)	$3x 10^{-4}$ * ($1x10^{-4}$–$9x10^{-4}$)	$1x10^{-6}$ * ($6x10^{-7}$–$2x10^{-6}$)
			Δ*espFU*	1.48 (0.97 – 2.26)	1.41 (1.08 – 1.86)	1.17 (0.70 – 1.96)	1.38 (1.05 – 1.82)
II [d]	2	$5 x 10^6$	Δ*espFU*	1.20 (0.95 – 1.53)	1.25 (0.99 – 1.58)	1.25 (0.99 – 1.57)	1.27 (0.94 – 1.70)
		$5 x 10^4$	Δ*espFU*	0.64 (0.35 – 1.16)	0.54 (0.28 – 1.06)	0.59 (0.35 – 1.00)	0.76 (0.33 – 1.76)

[a] Total dose of 905 and its derivatives (*i.e.* $2.5 x 10^8$ CFU per strain), adjusted to 90g rabbit weight.

[b] C.I. (competitive index) is the ratio of mutant to wild type CFU recovered from indicated intestinal site following infection divided by the ratio of mutant to wild type CFU in the inoculum. Values marked with "*" were significantly ($P \leq 0.05$) different from those obtained from Δ*lacZ* co-infection in corresponding tissue segments, calculated using the Student's t-test (two-way).

[c] In experiment I, values are geometric mean (95% confidence interval) of 6 rabbits for Δ*lacZ*, 11 rabbits for Δ*tir* and 11 rabbits for $\Delta espF_U$ co-infection experiments.

[d] In experiment II, 7 rabbits were tested at a dose of $5 x 10^6$. 31 rabbits were tested at a dose of $5 x 10^4$; of these, 12 yielded neither wild type nor mutant, 5 yielded almost exclusively wild type, and 4 yielded almost exclusively $\Delta espF_U$. In rabbits that yielded almost exclusively either one or the other strain, the very small number of colonies from the less frequently detected strain precluded an accurate calculation of the C.I., so the C.I.s in the table were calculated from the remaining 10 rabbits that showed significant colonization by both strains.