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Role of Intimin-Tir Interactions and the Tir-Cytoskeleton Coupling Protein in the Colonization of Calves and Lambs by *Escherichia coli* O157:H7

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Intimin facilitates intestinal colonization by enterohemorrhagic *Escherichia coli* O157:H7; however, the importance of intimin binding to its translocated receptor (Tir) as opposed to cellular coreceptors is unknown. The intimin-Tir interaction is needed for optimal actin assembly under adherent bacteria in vitro, a process which requires the Tir-cytoskeleton coupling protein (TccP/EspF$_U$) in *E. coli* O157:H7. Here we report that *E. coli* O157:H7 *tir* mutants are at least as attenuated as isogenic *eae* mutants in calves and lambs, implying that the role of intimin in the colonization of reservoir hosts can be explained largely by its binding to Tir. Mutation of *tccP* uncoupled actin assembly from the intimin-Tir-mediated adherence of *E. coli* O157:H7 in vitro but did not impair intestinal colonization in calves and lambs, implying that pedestal formation may not be necessary for persistence. However, an *E. coli* O157:H7 *tccP* mutant induced typical attaching and effacing lesions in a bovine ligated ileal loop model of infection, suggesting that TccP-independent mechanisms of actin assembly may operate in vivo.

Enterohemorrhagic *Escherichia coli* (EHEC) is a zoonotic enteric pathogen of worldwide importance and may cause severe diarrheal disease, hemorrhagic colitis, and hemolytic-uremic syndrome in humans (27). Healthy ruminants are the primary reservoir of EHEC, and human infections are frequently associated with direct or indirect contact with ruminant feces (33, 38). EHEC is closely related to enteropathogenic *E. coli* (EPEC), which is a major cause of infantile diarrhea, and both pathovars have the ability to induce attaching and effacing (A/E) lesions on intestinal epithelia. A/E lesions are characterized by localized effacement of microvilli, intimate adherence of bacteria to the apical plasma membrane, and formation of filamentous (F)-actin-enriched pedestal structures beneath adherent bacteria.

The genes that determine the A/E phenotype are located within the locus of enterocyte effacement (LEE) (35) and encode a filamentous type III secretion system, which delivers bacterial effector proteins into host cells that subvert, inhibit, or activate cellular processes (21). Both EPEC and EHEC insert their own receptors into the target cell plasma membrane. This molecule (Tir; translocated intimin receptor) is inserted into the host cell membrane in a hairpin loop topology with the central loop of the molecule exposed to the host cell surface and accessible for interaction with an LEE-encoded bacterial outer membrane adhesin called intimin (11, 13, 29).

The intracellular amino and carboxyl termini of Tir interact with a number of focal adhesion and cytoskeletal proteins and contribute to pedestal formation (reviewed in reference 6). The mechanisms by which actin polymerization is stimulated by EPEC O127:H6 Tir and EHEC O157:H7 Tir are different. Actin assembly by EHEC O157:H7 Tir requires the type III secreted protein TccP/EspF$_U$, which couples Tir with the neural Wiskott-Aldrich syndrome protein (N-WASP), an activator of the cellular Arp2/3 complex that is required for pedestal formation (8, 22). Mutation of *tccP* prevents actin nucleation under adherent EHEC O157:H7 in vitro but not intimin-Tir-mediated adherence (8, 22); however, the consequences of this event in vivo are unknown. In contrast, actin nucleation by EPEC O127:H7 Tir involves tyrosine phosphorylation of a C-terminal 12-amino-acid motif that recruits the host adaptor protein Nck and in turn N-WASP (5, 7, 23). EHEC O157:H7 Tir lacks the critical tyrosine Y$_{474}$ residue, and the 12-amino-acid region required for Nck recruitment is not conserved (14). As a consequence, EHEC O157:H7 Tir is not tyrosine phosphorylated and cannot functionally substitute for Tir in EPEC O127:H6 or the A/E mouse pathogen *Citrobacter rodentium* (12, 28).

E. coli O157:H7 intimin is required for bacterial adherence to cultured cells and actin assembly under adherent bacteria in vitro (17, 24, 51). In vivo studies using different animal models have demonstrated that in comparison to the wild type, intimin-deficient EHEC O157:H7 is defective in intestinal colonization and, in some models, in the induction of enteritis (9, 10, 15, 25, 26, 36, 42, 47, 50). Intimin exists in at least six phylogenetically distinct subtypes which exhibit marked se-

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Description	Reference(s)
Strains		
85-170 Nal^r	EHEC O157:H7 spontaneous stx_1 and stx_2 mutant, nalidixic acid resistant	46
85-170 Nal^r Δeae (ICC170)	85-170 Nal^r lacking eae	18
85-170 Nal^r Δtir	85-170 Nal^r lacking tir	46
85-170 Nal^r Δeae Δtir	85-170 Nal^r lacking eae and tir	49
ICC203	85-170 Nal^r Δ$tccP$::Kan^r	This study
NCTC12900 Nal^r	EHEC O157:H7 naturally stx_1 and stx_2 mutant, Nal^r	31, 50
NCTC12900 Nal^r eae::Cam^r	NCTC12900 Nal^r lacking eae	31
NCTC12900 Nal^r tir::Str^r	NCTC12900 Nal^r lacking tir	This study
Plasmids		
pLMF1	pCR2.1 derivative containing a 1,317-bp tir amplicon from NCTC12900	This study
pLMF2	pCR2.1 derivative containing a 1,317-bp tir amplicon from NCTC12900 Nal^r, disrupted by the streptomycin resistance cassette from p723	This study
pLMF3	pERFORMK derivative containing the tir::str^r construct from pLMF2	This study

quence divergence in their carboxyl-terminal cell-binding domains (1, 18, 19, 39, 40, 47). The carboxyl-terminal domain of intimin mediates the interaction with Tir; however, interactions between intimin and cellular coreceptors, including β1-integrins and surface-localized nucleolin, have been described previously (20, 43). β1-Integrins are not essential for the adherence of EPEC and actin nucleation in vitro (32). Intimin subtypes α, β, and γ, which confer a tropism for distinct intestinal sites, bind to nucleolin with comparable affinities but with lower avidity than to Tir (44). The relative importance of the intimin-Tir interaction as opposed to interactions with cellular coreceptors in vivo has so far received little attention.

The aim of this study was to assess the importance of the intimin-Tir interaction in colonization of reservoir hosts and to elucidate the function of TccP/EspF$_U$, and consequently of actin pedestal formation, in intestinal colonization and A/E lesion formation.

In this study, the importance of the intimin-Tir interaction was studied using two different *E. coli* O157:H7 strains in two reservoir hosts. Animal procedures have been described in detail previously (46) and were performed in accordance with the Animals (Scientific Procedures) Act of 1986 and approved by the local Ethical Review Committee. Fecal shedding data were statistically analyzed for the effect of mutation by means of an F test, with age as a cofactor and data taken as repeated measurements (Proc Mixed, Statistical Analysis System, 1995; SAS Institute, North Carolina). P values of <0.05 were taken to be significant.

Calf studies were performed using single and double non-polar *eae* and *tir* deletion mutants of the *stx*-negative *E. coli* O157:H7 strain 85-170 Nal^r (Table 1). The 85-170 Nal^r Δ*eae* mutant has been described previously (4, 18). The 85-170 Nal^r Δ*tir* and 85-170 Nal^r Δ*eae* Δ*tir* mutant strains were constructed by Stevens et al. (46) and Vlisidou et al. (49), respectively. Studies of lambs were performed with the *stx*-negative strain NCTC12900 Nal^r, an isogenic NCTC12900 Nal^r *eae*::Cam^r mutant (31), and with NCTC12900 Nal^r *tir*::Str^r. The *tir* mutant of NCTC12900 Nal^r was made by the insertion of a nonpolar streptomycin resistance cassette into *tir* followed by allelic exchange using a positive-selection suicide vector. Primers TirF (5′-CTGGCGGCGTCTGAGATAACACT-3′) and TirR (5′-CCCCGCACTTGGATTTC-3′) were used to amplify an

internal 1,317-bp fragment of *tir* from NCTC12900 Nal^r. The amplicon was purified and ligated into the multiple cloning site of the cloning vector pCR2.1 (Invitrogen, Paisley, United Kingdom), producing pLMF1. A blunt-ended 1.2-kb PvuII fragment from plasmid p723 (2) containing a nonpolar streptomycin resistance cassette was cloned into a unique HpaI site within the *tir* gene, yielding pLMF2. A SpeI fragment and a NotI fragment of pLMF2 carrying *tir*::Str^r was then subcloned into the SrfI site of pERFORMK (3), and the resulting plasmid (pLMF3) was introduced into NCTC12900 Nal^r by conjugation from *E. coli* K-12 S17-1λ*pir* with selection for streptomycin and nalidixic acid resistance. Colonies from filter matings were tested for loss of the antibiotic conferred by the suicide vector by replica plating onto minimal medium supplemented with the antibiotic conferred by the suicide vector. All *eae* and *tir* mutants were verified by PCR analysis using primers flanking the targeted genes or by Southern hybridization. Western blotting using specific antisera confirmed the absence of intimin and Tir in the respective mutants, and no polar effects on the expression of *eae* or *tir* were detected by analysis of total or secreted proteins (data not shown). Fluorescent actin staining tests confirmed that each mutant was unable to nucleate actin at sites of attachment to HeLa cells, and introduction of the cloned *eae* or *tir* gene restored fluorescent actin staining reactivity to the respective mutants (data not shown).

Conventional Friesian bull calves 11 to 16 days old were individually challenged by the oral route with $2.44 \times 10^{10} \pm 0.9 \times 10^{10}$ CFU (mean ± standard error of the mean) of strain 85-170 Nal^r Δ*eae* ($n = 4$), $1.17 \times 10^{10} \pm 0.55 \times 10^{10}$ CFU of 85-170 Nal^r Δ*tir* ($n = 4$), $5.00 \times 10^{10} \pm 0.93 \times 10^{10}$ CFU of 85-170 Nal^r Δ*eae* Δtir ($n = 5$), or $2.60 \times 10^{10} \pm 0.42 \times 10^{10}$ CFU of the wild-type strain ($n = 7$) as described previously (46). No significant effect of age on the course of fecal excretion of 85-170 Nal^r was detected ($P > 0.05$); therefore, statistical analysis was performed taking into account only the effect of mutation. All 85-170 Nal^r mutants were found to be defective in colonization compared to that of the wild type (Fig. 1A). The 85-170 Nal^r Δ*tir* and Δ*eae* Δ*tir* mutants were shed in significantly lower numbers than the wild type from day 2 postinoculation ($P < 0.05$). The excretion of the 85-170 Nal^r Δ*tir* mutant followed a trend similar to that of the double mutant, indicating that the deletion of *eae* did not cause further
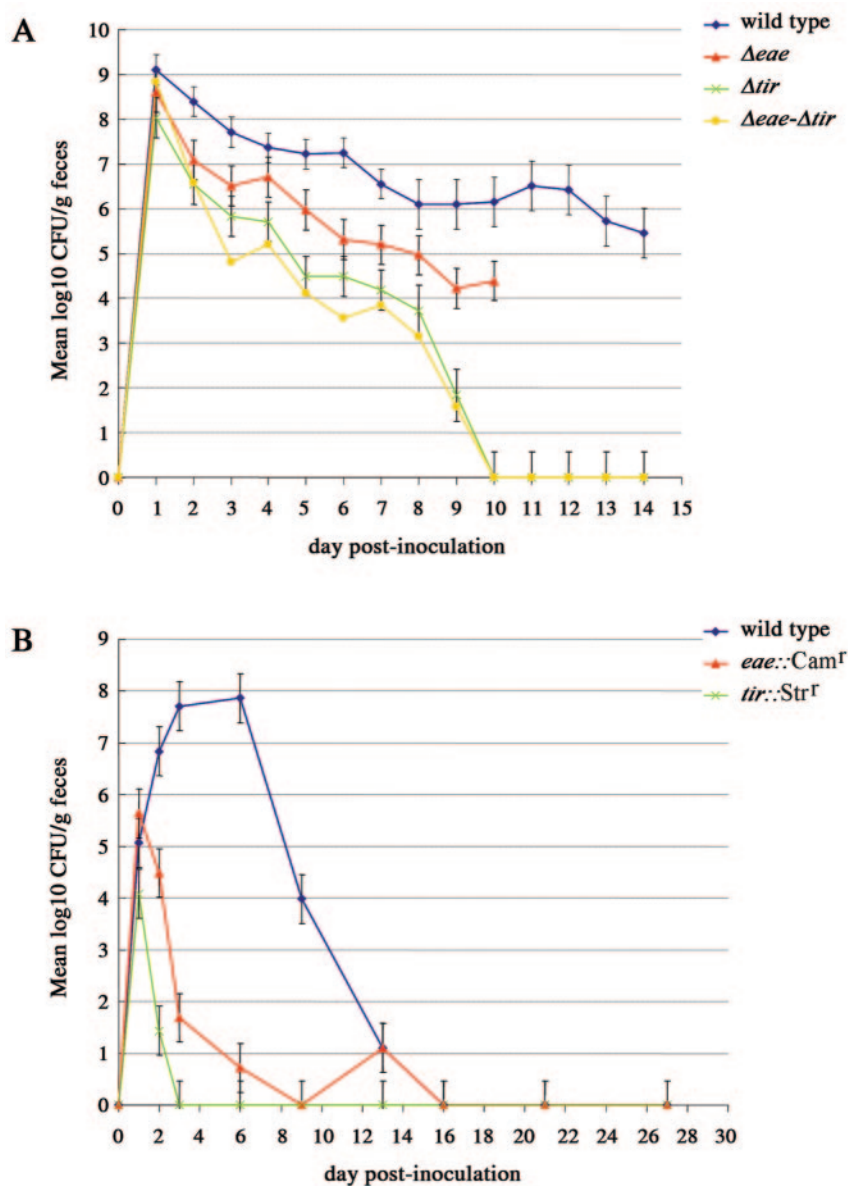
FIG. 1. Course of fecal excretion of *E. coli* O157:H7 *eae* and *tir* mutants in 14-day-old calves (A) and six-week-old lambs (B). The level of colonization is indicated by the mean log-transformed viable count (CFU/g) of *E. coli* O157:H7 in fecal samples taken twice daily for 12 days (A) or at intervals for 27 days (B). The data for the 85-170 Nal^r Δ*tir* single mutant have been described previously (16) and are shown for the purpose of comparison. The error bars indicate the standard errors of the means.

attenuation. Both the 85-170 Nal^r Δ*tir* and 85-170 Nal^r Δ*eae* Δ*tir* mutants were eliminated by day 10 postinoculation. The 85-170 Nal^r Δ*eae* mutant was shed in significantly lower numbers than the wild type from day 5 postinoculation, with the number of bacteria consistently recovered in the feces being at least 1 order of magnitude lower ($P < 0.05$), except on day 8 postinoculation, when no statistically significant difference was observed ($P = 0.1136$). The 85-170 Nal^r Δ*eae* mutant was recovered in significantly higher numbers than the isogenic Δ*tir* and Δ*eae* Δ*tir* mutants on days 9 and 3 postinoculation, respectively ($P < 0.05$).

Similar trends were observed following the inoculation of groups of six 6-week-old lambs with NCTC12900 Nal^r,

NCTC12900 Nal^r *eae*::Cam^r, or NCTC12900 Nal^r *tir*::Str^r (Fig. 1B). Significant reductions in the fecal shedding of both mutants relative to that of the wild type were detected from day 2 postinoculation (P values of 0.0006 and <0.0001, respectively). Consistent with the results obtained using the calf model, the NCTC12900 Nal^r *tir*::Str^r mutant was shed in lower numbers and for a shorter duration than the isogenic *eae* mutant. Indeed, the NCTC12900 Nal^r *tir*::Str^r mutant could not be detected even after enrichment from day 3 postinoculation, whereas the NCTC12900 Nal^r *eae*::Cam^r mutant was eliminated on day 16 postinoculation.

While differences in the kinetics of fecal excretion of the *eae* and *tir* mutants were detected between the calf and lamb mod-
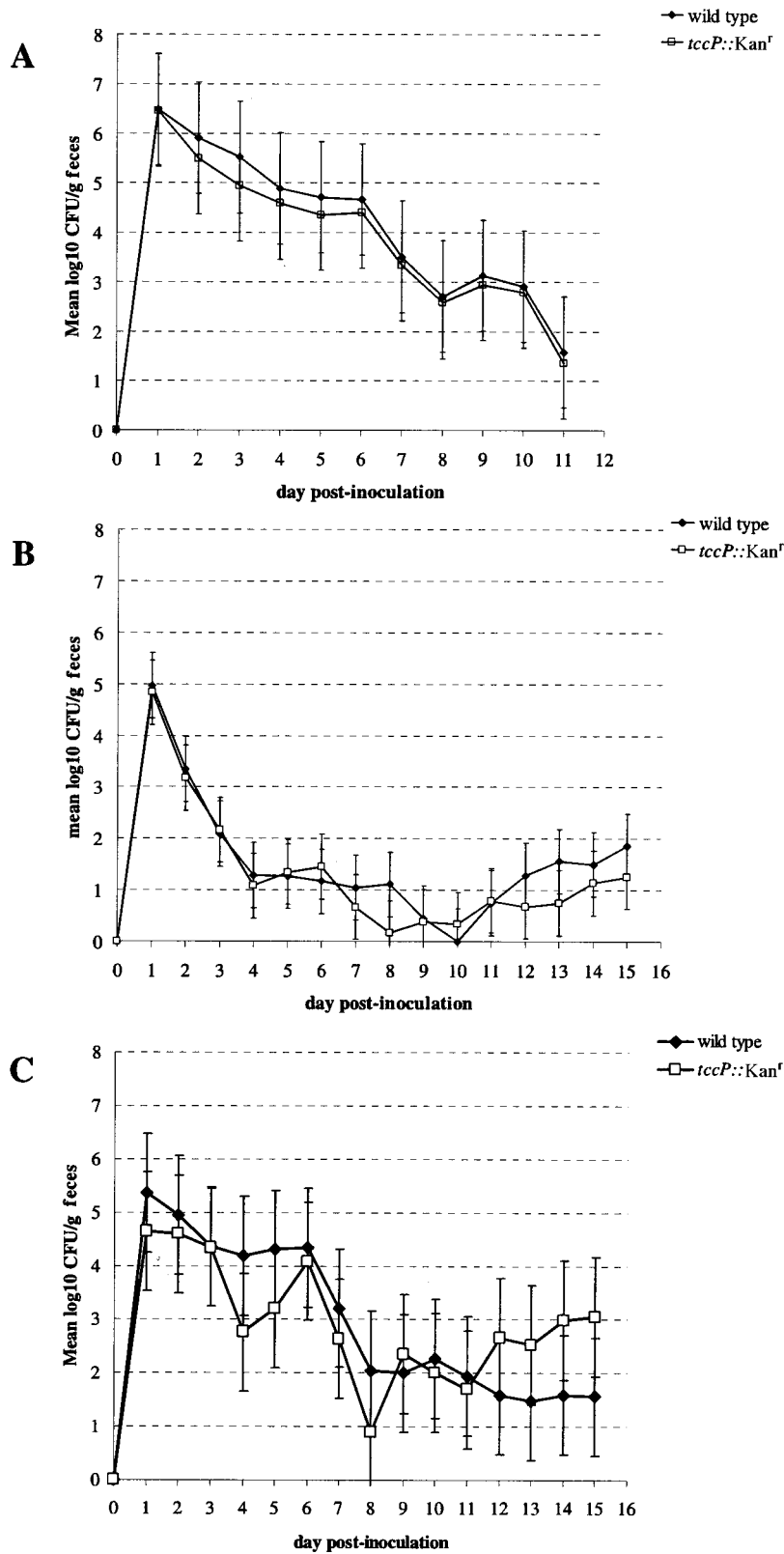
FIG. 2. Colonization of the bovine and ovine intestine by 85-170 Nal^r $\Delta tccP::$Kan^r (ICC203) in mixed-infection experiments with the wild-type strain in 14-day-old calves (A) and 6-week-old lambs (B). The level of colonization is indicated by the mean log-transformed viable count (CFU/g) of fecal samples taken once daily for 11 days postinoculation. The error bars indicate the standard errors of the means. To assess the possibility of cross-complementation of the $tccP$ defect, 85-170 Nal^r and ICC203 were fed separately to groups of three lambs (C). No statistically significant difference was observed between colonization in lambs infected with ICC203 and that in the wild-type control (C).
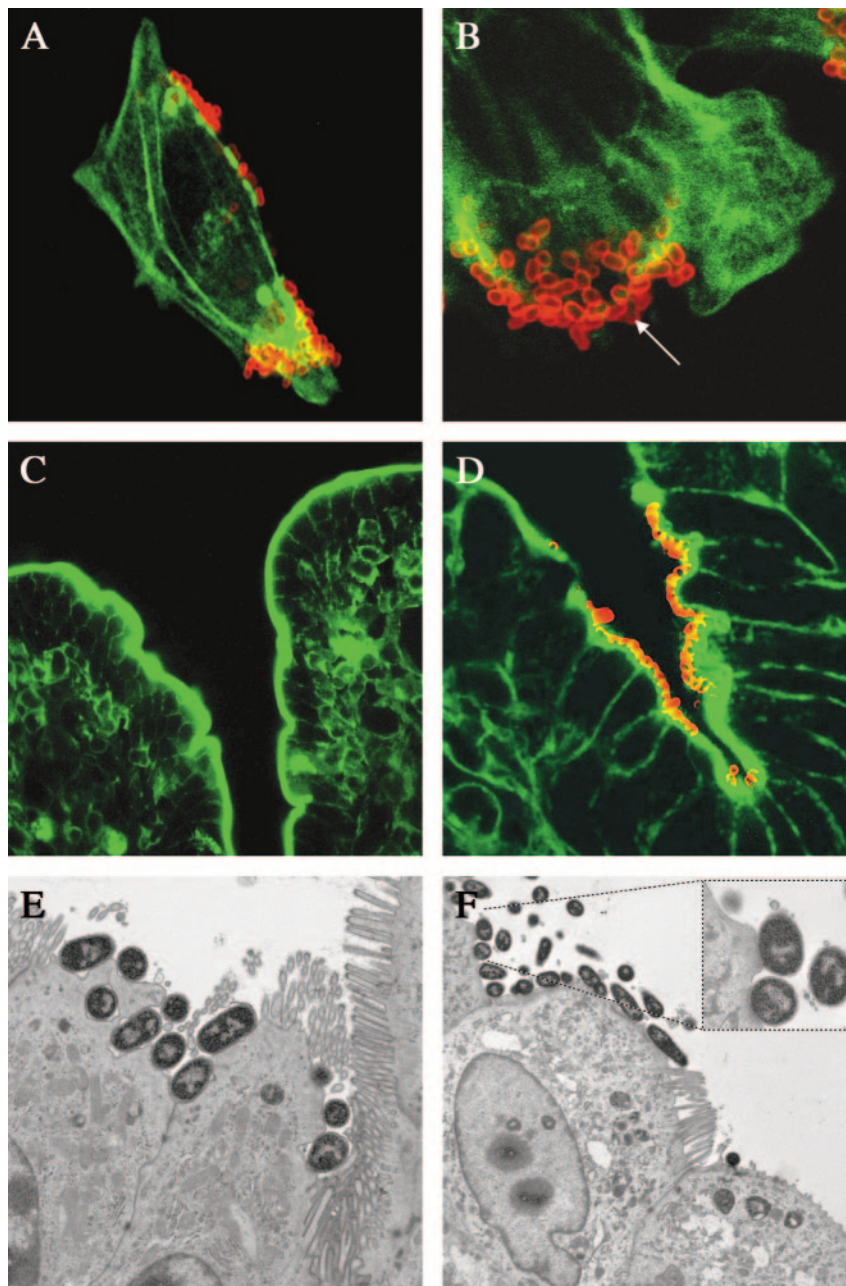
FIG. 3. TccP is not essential for the induction of A/E lesions in calves. HeLa cells were infected with 85-170 Nal[r] (A) and ICC203 ($\Delta tccP$::Kan[r] mutant) (B) for 8 h. The arrow shows an absence of F-actin nucleation under adherent ICC203, consistent with previous observations (8, 22). Fifty-micrometer-thick vibrating-microtome sections of bovine ileum 12 h after inoculation with sterile LB medium (C) and ICC203 (D) in the presence of 5 mM NE are shown. Bacteria were detected with rabbit anti-O157 lipopolysaccharide typing serum and anti-rabbit immunoglobulin-Alexa[568] (red) as described previously (45). Filamentous actin was stained with fluorescein isothiocyanate phalloidin (green). Magnification, ×630. Transmission electron micrographs showing A/E lesions induced by the 85-170 Nal[r] wild type (E) and strain ICC203 (F) in bovine ileal loops in the presence of 5 mM NE are also shown.

els, the data imply that the role of intimin in the colonization of the bovine and ovine intestines can be explained largely by its binding to Tir as opposed to cellular coreceptors. If interactions with cellular coreceptors had been of key importance, then it may have been anticipated that *eae* mutants would be more attenuated than those lacking *tir*. The role of Tir in intestinal colonization by rabbit EPEC and EHEC O157:H7 in infant rabbits (34, 42) as well as *C. rodentium* in mice (12) has

been confirmed. In addition, a vaccine containing secreted proteins from *E. coli* O157:H7 lacking *tir* was not as efficient in cattle as was an identical formulation from an isogenic wild-type isolate (41). However, this is the first report of the relative attenuation of isogenic *eae* and *tir* mutants of *E. coli* O157:H7 strains in reservoir hosts.

To determine whether actin assembly dependent on the Tir-cytoskeleton coupling protein is necessary for intestinal

colonization in calves and sheep, *tccP* was replaced in strain 85-170 Nal[r] by a λRed-mediated insertion of a nonpolar kanamycin resistance cassette using primers tccP-flank-f1, tccP-flank-r1, tccP-flank-f2, and tccP-flank-r2, as described previously (22). The Δ*tccP*::Kan[r] mutant (strain ICC203) was confirmed to adhere to HeLa cells but to be unable to nucleate F-actin under sites of attachment (Fig. 3B). Initially, mixed-oral-infection experiments were performed. Groups of three 14-day-old Friesian bull calves and six 6-week-old lambs were orally coinoculated with $1.41 \times 10^{10} \pm 0.21 \times 10^{10}$ and $8.00 \times 10^9 \pm 0.35 \times 10^9$ CFU of the 85-170 Nal[r] wild type and strain ICC203, respectively, and the course of fecal excretion of the bacteria was followed for 2 weeks. Wild-type and mutant bacteria were enumerated by plating serial dilutions of feces onto sorbitol MacConkey agar supplemented with potassium tellurite and with either nalidixic acid only or both nalidixic acid and kanamycin as described previously (16). The *tccP* mutant competed equally with the wild-type strain in both animal models, and no statistically significant differences in fecal excretion of the two strains could be detected at any time postinoculation (Fig. 2A and B). Cross-complementation of the *tccP* defect by wild-type bacteria is considered unlikely, since EHEC mutants deficient in type III secretion or in the production of effector proteins (e.g., Map or NleD) were found to be attenuated by screening pools of 95 signature-tagged transposon mutants in which the vast majority of bacteria were wild-type strains with respect to LEE function (16, 48). However, to address this possibility, two groups of three 6-week-old lambs were separately inoculated with 85-170 Nal[r] or ICC203. Consistent with the phenotype in coinfection experiments, the Δ*tccP*::Kan[r] mutation had no significant effect on the fecal excretion of EHEC O157:H7 following a single infection of lambs (Fig. 2C). These data suggest that it may be possible to uncouple a pedestal formation from intimin-Tir-mediated colonization; therefore, it was necessary to confirm that actin assembly by the *E. coli* O157:H7 Δ*tccP*::Kan[r] mutant had been abolished in vivo.

While EHEC O157:H7 has been reported to form A/E lesions in neonatal colostrum-deprived calves and at the terminal part of the rectum in adult cattle (10, 37), we have observed, by confocal microscopy, only sparse microcolonies of adherent 85-170 Nal[r] at distal intestinal sites in calves of the age used and have so far failed to detect A/E lesions by transmission electron microscopy (data not shown). However, we recently reported that 85-170 Nal[r] can be induced to form extensive A/E lesions on the ileal mucosa of 28-day-old conventional calves in the presence of the neuroendocrine hormone norepinephrine (NE) (49). Following these observations, we used the bovine ligated intestinal loop assay to assess the role of TccP in the ability of EHEC O157:H7 to adhere to ileal mucosa and to induce the formation of A/E lesions in the presence of 5 mM NE. Mid-ileal loops were constructed in a 28-day-old conventional Friesian bull calf, and the loops were inoculated with the NE-treated 85-170 Nal[r] wild type, the isogenic *tccP* mutant ICC203, or sterile medium as a control. Tissue sections were excised 12 h after loop inoculation and analyzed by confocal laser scanning microscopy and transmission electron microscopy as described previously (45). ICC203 was able to form microcolonies and disrupt the rim of F-actin staining around villi, consistent with microvillus effacement

(Fig. 3D). Using transmission electron microscopy, characteristic A/E lesions were observed on the epithelial surfaces of loops infected with the NE-treated 85-170 Nal[r] wild type (Fig. 3E) and strain ICC203 (Fig. 3F). In both cases, relatively few bacteria were observed on raised electron-dense pedestals; rather, EHEC O157:H7 often adhered to the surfaces of enterocytes in cup-shaped depressions. No endogenous EHEC O157:H7 was detected in control loops filled with sterile medium (Fig. 3C). The finding that at least some *tccP*-deficient bacteria induced apparently normal pedestal structures implies that actin assembly may occur via TccP-independent mechanisms in vivo. This notion is supported by the finding that a *C. rodentium* Δ*tir* mutant expressing a $Y_{471}F$ variant of Tir, unable to assemble actin under adherent bacteria in vitro, colonized the intestines of mice efficiently and was observed to form normal A/E lesions on the colonic mucosa (12).

In summary, we report that Tir is an essential colonization factor for *E. coli* O157:H7 in calves and sheep and mediates bacterial attachment to intestinal mucosa by serving as the primary receptor for intimin. The finding that *tir* mutants are more attenuated than isogenic *eae* mutants implies that Tir may facilitate intestinal colonization in other ways. We have also shown that the Tir-cytoskeleton coupling protein (TccP) is not required by *E. coli* O157:H7 to colonize the intestines of calves and sheep and induce attaching and effacing lesions, suggesting that pedestal formation in vitro and A/E lesion formation in vivo may occur by distinct mechanisms. Bacterial factors mediating actin assembly in a continuous mammalian cell line may be redundant or functionally replaced by bacterial or host factors in vivo, and this study underlines the importance of studying bacterial gene function in target animal hosts wherever possible.

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