Role of intimin-Tir interactions and the Tir-cytoskeleton coupling protein in the colonization of calves and lambs by Escherichia coli O157 : H7

Citation for published version:

Digital Object Identifier (DOI):
10.1128/IAI.74.1.758-764.2006

Link:
Link to publication record in Edinburgh Research Explorer

Document Version:
Publisher's PDF, also known as Version of record

Published In:
Infection and Immunity

Publisher Rights Statement:
Copyright © 2006, American Society for Microbiology

General rights
Copyright for the publications made accessible via the Edinburgh Research Explorer is retained by the author(s) and / or other copyright owners and it is a condition of accessing these publications that users recognise and abide by the legal requirements associated with these rights.

Take down policy
The University of Edinburgh has made every reasonable effort to ensure that Edinburgh Research Explorer content complies with UK legislation. If you believe that the public display of this file breaches copyright please contact openaccess@ed.ac.uk providing details, and we will remove access to the work immediately and investigate your claim.
Role of Intimin-Tir Interactions and the Tir-Cytoskeleton Coupling Protein in the Colonization of Calves and Lambs by *Escherichia coli* O157:H7

Isabella Vlisidou,1 Francis Dziva,1 Roberto M. La Ragione,2 Angus Best,2 Junkal Garmendia,3 Pippa Hawes,4 Paul Monaghan,4 Shaun A. Cawthraw,2 Gad Frankel,3 Martin J. Woodward,2 and Mark P. Stevens1*

Division of Microbiology, Institute for Animal Health, High Street, Compton, Berkshire RG20 7NN, United Kingdom1; Department of Food & Environmental Safety, Veterinary Laboratories Agency (Defra), Woodham Lane, Addlestone, Surrey KT15 3NB, United Kingdom2; Centre for Molecular Microbiology and Infection, Division of Cell and Molecular Biology, Imperial College London, London SW7 2AZ, United Kingdom3; and Bioimaging Department, Institute for Animal Health, Ash Road, Pirbright, Surrey GU24 0NF, United Kingdom4

Received 9 August 2005/Returned for modification 15 September 2005/Accepted 8 October 2005

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<sub>1</sub>) 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 pathogens 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<sub>1</sub>, 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<sub>474</sub> 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-
sequence divergence in their carboxy-terminal cell-binding do-

TABLE 1. Bacterial strains and plasmids

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Description</th>
<th>Reference(s)</th>
</tr>
</thead>
</table>
| Strains
85-170 Nal' | EHEC O157:H7 spontaneous stx1 and stx2 mutant, nalidixic acid resistant | 46 |
85-170 Nal' Δeae (ICC170) | 85-170 Nal' lacking eae | 18 |
85-170 Nal' Δtir | 85-170 Nal' lacking tir | 46 |
85-170 Nal' Δeae Δtir | 85-170 Nal' lacking eae and tir | 49 |
ICC203 | 85-170 Nal' Δtir::CamR | This study |
NCTC12900 Nal' | EHEC O157:H7 naturally stx1 and stx2 mutant, Nal' | 31, 50 |
NCTC12900 Nal' eae::CamR | NCTC12900 Nal' lacking eae | 31 |
NCTC12900 Nal' tir::StrR | NCTC12900 Nal' 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', disrupted by the streptomycin resistance cassette from p723 | This study |
pLMF3 | pPERFORMK derivative containing the tir::strR construct from pLMF2 | This study |

The aim of this study was to assess the importance of the intimin-Tir interaction as opposed to interactions with cellular coreceptors in vivo and to elucidate the function of TccP/EspFU, and consequently of the intimin-Tir interaction in colonization of reservoir hosts and to study the antibiotic conferred by the suicide vector.

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' (Table 1). The 85-170 Nal' Δeae mutant has been described previously (4, 18). The 85-170 Nal' Δtir and 85-170 Nal' Δeae Δtir 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', an isogenic NCTC12900 Nal' eae::CamR mutant (31), and with NCTC12900 Nal' tir::StrR. The tir mutant of NCTC12900 Nal' 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’-CTGGCGCGGCTTCAGATAAACACT-3’) and TirR (5’-CCCCGCACCTGAGATTTC-3’) were used to amplify an internal 1,317-bp fragment of tir from NCTC12900 Nal'. 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 Hpal site within the tir gene, yielding pLMF2. A SpeI fragment and a NotI fragment of pLMF2 carrying tir::StrR was then subcloned into the SrfI site of pPERFORMK (3), and the resulting plasmid (pLMF3) was introduced into NCTC12900 Nal' by conjugation from E. coli K-12 S17-1pir 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 activity 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 × 10^10 ± 0.9 × 10^10 CFU (mean ± standard error of the mean) of strain 85-170 Nal' Δeae (n = 4), 1.17 × 10^10 ± 0.55 × 10^10 CFU of 85-170 Nal' Δtir (n = 4), 5.00 × 10^10 ± 0.93 × 10^10 CFU of 85-170 Nal' Δeae Δtir (n = 5), or 2.60 × 10^10 ± 0.42 × 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' was detected (P > 0.05); therefore, statistical analysis was performed taking into account only the effect of mutation. All 85-170 Nal' mutants were found to be defective in colonization compared to that of the wild type (Fig. 1A). The 85-170 Nal' Δtir and Δeae Δtir 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' Δtir mutant followed a trend similar to that of the double mutant, indicating that the deletion of eae did not cause further...
attenuation. Both the 85-170 Nal r tir mutants and 85-170 Nal r eae mutants were eliminated by day 10 postinoculation. The 85-170 Nal r 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 eae mutant was recovered in significantly higher numbers than the isogenic tir and Δeae Δtir 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' ΔtccP::Kan' (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' 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).
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

FIG. 3. TccP is not essential for the induction of A/E lesions in calves. HeLa cells were infected with 85-170 NaI' (A) and ICC203 (ΔtccP::Kan' 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-Alexa568 (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 NaI' wild type (E) and strain ICC203 (F) in bovine ileal loops in the presence of 5 mM NE are also shown.
colonization in calves and sheep, tccP was replaced in strain 85-170 Nal 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 ΔtccP::Kan′ 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 × 10¹⁰ ± 0.21 × 10¹⁰ and 8.00 × 10⁹ ± 0.35 × 10⁹ CFU of the 85-170 Nal 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 were enumerated by plating serial dilutions of feces onto ICC203, respectively, and the course of fecal excretion of the lambs were separately inoculated with 85-170 Nalr or ICC203. However, to address this possibility, two groups of three 12-week-old cattle and six 6-week-old lambs were inoculated with the NE-treated 85-170 Nalr wild type, the isogenic eae mutant (strain ICC203) was co-incubated in adult cattle (10, 37), we have observed 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 Δtir mutant expressing a Y77F 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.

We gratefully acknowledge Lisa Faulkner. We also acknowledge the support of the Department for the Environment, Food and Rural Affairs (grant OZ0707) and the Wellcome Trust.

REFERENCES


Editor: J. T. Barbieri