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Dissecting the role of the Tir:Nck and Tir:IRTKS/IRSp53 signalling pathways in vivo

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Summary

Attaching and effacing (A/E) lesions and actin polymerization, the hallmark of enteropathogenic Escherichia coli (EPEC), enterohemorrhagic E. coli (EHEC) and Citrobacter rodentium (CR) infections, are dependent on the effector Tir. Phosphorylation of TirEPEC/CR Y474 leads to recruitment of Nck and neural Wiskott–Aldrich syndrome protein (N-WASP) and strong actin polymerization in cultured cells. TirEPEC/CR also contains an Asn-Pro-Tyr (NPY454) motif, which triggers weak actin polymerization. In EHEC the NPY458 actin polymerization pathway is amplified by TccP/EspFU, which is recruited to Tir via IRSp53 and/or insulin receptor tyrosine kinase substrate (IRTKS). Here we used C. rodentium to investigate the different Tir signalling pathways in vivo. Following infection with wild-type C. rodentium IRTKS, but not IRSp53, was recruited to the bacterial attachment sites. Similar results were seen after infection of human ileal explants with EHEC. Mutating Y471 or Y451 in TirCR abolished recruitment of Nck and IRTKS respectively, but did not affect recruitment of N-WASP or A/E lesion formation. This suggests that despite their crucial role in actin polymerization in cultured cells the Tir:Nck and Tir:IRTKS pathways are not essential for N-WASP recruitment or A/E lesion formation in vivo. Importantly, wild-type C. rodentium out-competed the tir tyrosine mutants during mixed infections. These results uncouple the Tir:Nck and Tir:IRTKS pathways from A/E lesion formation in vivo but assign them an important in vivo role.

Introduction

Enteropathogenic Escherichia coli (EPEC) and enterohemorrhagic E. coli (EHEC), particularly serotype O157:H7, are important human pathogens (Nataro and Kaper, 1998). EPEC is an important cause of infantile diarrhoea in developing countries, while EHEC is a major foodborne pathogen in developed countries that can cause bloody diarrhoea, haemorrhagic colitis and haemolytic uraemic syndrome, haemolytic uraemic syndrome being the leading cause of paediatric kidney failure in the USA and UK (Nataro and Kaper, 1998). Citrobacter rodentium is a mouse-specific pathogen, the ethiological agent of transmissible colonic hyperplasia, and a model EPEC and EHEC microorganism (Mundy et al., 2005).

While colonizing the gut mucosa EPEC, EHEC and C. rodentium induce attaching and effacing (A/E) lesions, which are characterized by extensive remodelling of the gut epithelium leading to elongation and effacement of the brush border microvilli, intimate bacterial attachment to the enterocyte apical plasma membrane, accumulation of polymerized actin and formation of elevated pedestal-like structures (Knutton et al., 1987). Adhesion of EPEC, EHEC (reviewed in Frankel et al., 1998; Frankel and Phillips, 2008) and C. rodentium (Girard et al., 2009a) to cultured cells also triggers actin polymerization under attached bacteria. For this reason dissecting the actin signalling pathways in vitro was at the heart of EPEC and EHEC research for over two decades.

The ability to induce A/E lesions and actin polymerization is encoded within the locus of enterocyte effacement (McDaniel et al., 1995). The locus of enterocyte effacement encodes a type III secretion system (Jarvis et al., 1995), the outer membrane adhesin intimin (Jerse et al., 1990), regulators, chaperones and translocator and effector proteins (reviewed in Garmendia et al., 2005), including Tir (Kenny et al., 1997). Following translocation, Tir is integrated into the epithelial cell plasma membrane in a hairpin loop topology (Hartland et al., 1999), exposing an extracellular central domain that functions as an intimin receptor. Binding of intimin induces clustering of Tir,
assembly of signalling complexes and actin polymerization (reviewed in Caron et al., 2006).

Using cultured epithelial cells and the prototype EPEC strain E2348/69 have shown that actin polymerization is predominantly dependent on phosphorylation of the C-terminal Tir tyrosine residue 474 (Y474) (Kenny, 1999). It was recently reported that a short polyproline sequence at the N-terminus of TirEPEC is needed for recruitment of redundant tyrosine kinases that phosphorylate Y474; substitution of the polyprolines with alanines prevented actin polymerization (Bommarius et al., 2007). Phosphorylation of Tir residue Y474 provides a binding site for the mammalian adaptor protein Nck (Gruenheid et al., 2001; Campellone et al., 2002; 2004a). Recruitment of Nck leads to activation of the neural Wiskott–Aldrich syndrome protein (N-WASP), recruitment of the Arp2/3 complex and actin polymerization (reviewed in Caron et al., 2006). In addition, TirEPEC can promote weak actin polymerization, in an Nck-independent manner, involving the C-terminal Tir tyrosine residue Y454 (Campellone and Leong, 2005), which is present in the context of a conserved Asn-Pro-Tyr (NPY) motif (Brady et al., 2007).

In contrast to TirEPEC, TirEHEC contains the NPY motif but lacks the Nck binding site (Brady et al., 2007). Nevertheless, EHEC O157:H7 triggers strong actin polymerization as it additionally translocates TccP/EspF0, which is recruited to Tir though indirectly (Campellone et al., 2004b; Garmendia et al., 2004). Recently, Vingadassalom et al. (2009) have shown that the Tir NPY motif recruits the adaptor protein insulin receptor tyrosine kinase substrate (IRTKS), while Weiss et al. (2009) have shown that rather than IRTKS Tir recruits the insulin receptor substrate protein of 53 kDa (IRSp53). IRTKS and IRSp53 were shown to link Tir and TccP/EspF0, which in turn activates N-WASP (Garmendia et al., 2006; Campellone et al., 2008; Cheng et al., 2008; Sallee et al., 2008), leading to actin polymerization.

Importantly, EPEC strains belonging to lineage 2 and non-O157 EHEC strains can simultaneously trigger actin polymerization by the Tir:Nck and Tir:TccP/TccP2 pathways (Whale et al., 2006; 2007; Ogura et al., 2007). This suggests the existence of selective pressure that maintains the actin polymerization capabilities in EPEC and EHEC strains. Nonetheless, a fundamental question, which was the focus of this investigation, remains unanswered: what is the role of the Tir:Nck and Tir:IRTKS/IRSp53 pathways during infection in vivo?

Results

Construction of chromosomal tirCR mutants

We constructed C. rodentium mutants expressing TirFpa (mutated in the N-terminal polyproline sequence), TirY451A (mutated in the IRSp53/IRTKS binding site), TirY471A (mutated in the Nck binding site) and TirY451A/Y471A (Fig. 1A). To this end, we developed a lambda red-based mutagenesis system (Datsenko and Wanner, 2000), involving insertion of a kanamycin cassette in the map-tir or tir-cesT intergenic regions, which allowed us to introduce site-directed tir mutations into the bacterial chromosome (Fig. 1B). Control mock mutants were generated by inserting kanamycin cassettes in the map-tir (N-terminal control – TirFpa) and tir-cesT (C-terminal control – TirC-ctrl) intergenic regions without affecting the tir coding sequence. A nonsense mutant at Tir position 33 (Tir1_33stop) was used as a further control. Growth curves in minimal and rich media confirmed that the mutants and parental wild-type strains had identical growth rates (data not shown).

Testing the TirCR mutants during infection of cultured cells

We have recently shown that C. rodentium can efficiently adhere to and trigger actin polymerization in Swiss 3T3 fibroblast cells (Girard et al., 2009a). Before investigating the effect of the mutagenesis in vivo, we characterized the Tir mutants in vitro. Infection of Swiss 3T3 cells showed recruitment of Tir, Nck and polymerized actin under adherent wild-type C. rodentium and C. rodentium expressing TirFpa, TirC-ctrl, TirFpa and TirY451A (Fig. 2). Focused Tir without detectable Nck or actin polymerization (Fig. 2) was detected under adherent C. rodentium expressing TirY471A and TirY451A/Y471A, while neither focused Tir nor actin polymerization was detected under adherent C. rodentium expressing Tir1_33stop (Fig. 2). These results show that the Tir polyproline sequence is dispensable for Nck recruitment and actin polymerization by C. rodentium both of which are dependent on TirY471.

The role of the polyproline region and Tir residues Y451 and Y471 in vivo

We investigated the impact of Tir mutagenesis on colonization and persistence of C. rodentium in vivo by enumerating colony-forming units per gram of stools (cfu g⁻¹) collected at regular intervals following oral inoculation of C57BL/6 mice. This has shown that inoculation with C. rodentium expressing TirFpa, TirY451A, TirY471A and TirY451A/Y471A (Fig. 3A) resulted in the same colonization dynamics as wild-type C. rodentium or the control strains expressing TirFpa and TirC-ctrl (Fig. 3A); infection with all strains peaked at day 7 and started to clear from day 15 post inoculation. C. rodentium expressing Tir1_33stop was rapidly cleared and failed to initiate an infection. C. rodentium expressing TirFpa, TirY451A, TirY471A and TirY451A/Y471A (Fig. 3B) were as efficient in triggering colonic hyperplasia as wild-type C. rodentium.
The polyproline region (PPR) of Tir EPEC is reported (Bommarius et al., 2007) to recruit a number of protein tyrosine kinases that phosphorylate Y474 on adjacent Tir molecules and to maturate an Nck binding site, leading to activation of N-WASP and strong actin polymerization. Tir EPEC/CR can trigger inefficient actin polymerization via the NPY454/1-IRTKS/IRSp53 pathway.

B. Schematic representation of the tirCR chromosomal mutagenesis strategy. The genetic organization of the map-tir-cesT locus in wild-type C. rodentium is shown in (i). Recombinant pGEMT plasmids, containing tirCR mutations were used as PCR templates and the amplified fragments, together with the lambda red recombinase, were used to introduce the mutations in tandem with an aphT kanamycin-resistance cassette into the endogenous tirCR locus. Distinct constructs were used to introduce 5'-specific mock (TirN-ctrl), TirP5A and Tir1-33stop mutations (ii) or 3'-specific mock (TirC-ctrl), TirY451A, TirY471A and TirY451A/Y471A mutations (iii).

Fig. 1. A. A diagram illustrating the Tir Y454/451 and Y474/471 actin polymerization pathways used by EPEC E2348/69 and C. rodentium. The polyproline region (PPR) of Tir EPEC is reported (Bommarius et al., 2007) to recruit a number of protein tyrosine kinases that phosphorylate Y474 on adjacent Tir molecules and to maturate an Nck binding site, leading to activation of N-WASP and strong actin polymerization. Tir EPEC/CR can trigger inefficient actin polymerization via the NPY454/1-IRTKS/IRSp53 pathway.
as wild-type *C. rodentium* and *C. rodentium* expressing TirN-ctrl and TirC-ctrl (Fig. 3B); crypt length was significantly higher than in mice infected with *C. rodentium* expressing Tir1-33stop or uninfected mice (Fig. 3B).

Recruitment of adaptor and signalling molecules to the site of *C. rodentium* attachment in vivo

We first processed thin colonic sections to determine if Nck is recruited under adherent *C. rodentium in vivo*. Nck was detected in mice infected with wild-type *C. rodentium* and *C. rodentium* expressing TirN-ctrl, TirC-ctrl, TirPSA, TirY451A, TirY471A and TirY451A/Y471A. Nck (B) and polymerized actin (A) were detected under wild-type *C. rodentium* and *C. rodentium* expressing TirN-ctrl, TirC-ctrl, TirPSA and TirY471A. No Nck or polymerized actin was detected under *C. rodentium* expressing TirY471A or TirY451A/Y471A. Tir, Nck or polymerized actin were not detected under *C. rodentium* expressing Tir1-33stop.

IRSp53 was undetectable (using an IRSp53 antiserum made against a 21-amino-acid peptide that is conserved between the human and the mouse IRSp53) (Weiss *et al.*, 2009)) (Fig. 5B). IRTKS was also detected at the attachment sites of *C. rodentium* expressing TirN-ctrl, TirC-ctrl, TirPSA and TirY471A (data not shown) but was not detected under adherent *C. rodentium* expressing TirY451A/Y471A, Tir1-33stop (data not shown), TirY451A or on uninfected tissues (Fig. 5A). These results show that IRTKS is recruited in the mouse gut mucosa beneath adherent *C. rodentium* in a NPY motif-dependent manner, while IRSp53 does not seem to be recruited under adherent bacteria in the *in vivo* mouse model.

In order to support our conclusions using an alternative model, we tested the recruitment of IRSp53 and IRTKS to the site of EHEC O157:H7 (strain TUV 93-0) attachment on human ileal *in vitro* organ culture (IVOC). Similarly to the results of *C. rodentium* infection, IRTKS, but not

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**Fig. 2.** Infection of Swiss 3T3 cells with *C. rodentium*. Tir (A) was detected under adherent wild-type *C. rodentium* and *C. rodentium* expressing TirN-ctrl, TirC-ctrl, TirPSA, TirY451A, TirY471A and TirY451A/Y471A. Nck (B) and polymerized actin (A and B) were detected under wild-type *C. rodentium* and *C. rodentium* expressing TirN-ctrl, TirC-ctrl, TirPSA and TirY471A. No Nck or polymerized actin was detected under *C. rodentium* expressing TirY471A or TirY451A/Y471A.
IRSp53, was detected at the bacterial attachment sites in a TifEHEC Y458-dependent manner (Fig. 5C).

As we were unable to observe IRSp53 recruitment under adherent bacteria either during C. rodentium mouse infection or in EHEC-infected human IVOC, we controlled our ability to detect IRSp53 recruitment to bacterial attachment sites \textit{in vitro}. Consistent with the published data (Weiss \textit{et al.}, 2009) we found that IRSp53 was recruited at the site of \textit{C. rodentium} and EHEC adhesion on Swiss 3T3 cells in a NPY motif-dependent manner (Fig. 5D and E). Recruitment of IRSp53 was also detected by immunofluorescence in infected HeLa cells (data not shown). Therefore, our inability to detect recruitment of IRSp53 to \textit{C. rodentium} and EHEC adhering to mucosal surfaces might be due to the fact that the protein is either not expressed in these tissues or the expression level is below the detection sensitivity.

Finally we investigated recruitment of N-WASP \textit{in vivo} following \textit{C. rodentium} infection. Immunofluorescent staining revealed recruitment to \textit{C. rodentium} attachment sites independently of the polyproline sequence and Tir residues Y451 and Y471 as N-WASP was detected in mice inoculated with any of the site-directed mutants except \textit{C. rodentium} expressing Tir1-33stop (Fig. 4B). These results show that N-WASP is recruited \textit{in vivo} to the bacterial attachment sites by a novel mechanism independent of the Tir:Nck and Tir:IRTKS complexes.

\textbf{Formation of A/E lesions by the different Tir mutants in vivo}

The data presented thus far show that assembly of the Tir:Nck and Tir:IRTKS complexes by \textit{C. rodentium} \textit{in vivo} is dependent on Y471 and Y451 residues respectively, but independent of the polyproline N-terminal sequence. We next investigated the role of these Tir residues in A/E lesion formation. Transmission and scanning electron microscopy revealed typical A/E lesions on colonic mucosa in mice inoculated with wild-type \textit{C. rodentium} and \textit{C. rodentium} expressing TirN-ctrl, TirC-ctrl, TirP5A, TirY451A, TirY471A and TirY451A/Y471A (Fig. 6). No morphological differences or variation in the overall level of electron-dense

\textbf{Fig. 3.} Colonization and clearance dynamics of \textit{C. rodentium} strains after oral inoculation of C57Bl/6 mice. Mice inoculated with \textit{C. rodentium} expressing TirP5A, TirY451A, TirY471A and TirY451A/Y471A exhibit similar colonization dynamics (A) and crypt hyperplasia, measured at day 8 post inoculation (B), as wild-type \textit{C. rodentium} or \textit{C. rodentium} expressing Tir1-33stop; \textit{****}P < 0.0001. The inoculum count is the number of viable bacteria in 200 µl used to inoculate mice by oral gavage. Data are represented as mean ± SD.
material was seen at the site of attachment of any of the C. rodentium strains. No A/E lesions were detected in mice infected with C. rodentium expressing Tir1-33stop. These results show that, while Nck and IRTKS are recruited to C. rodentium attachment sites in mice, their recruitment is not essential for A/E lesion formation.

C. rodentium expressing TirY451A, TirY471A and TirY451A/Y471A are out-competed by the wild-type strain during mixed infections

As none of the Tir mutations, but Tir1-33stop, affected bacterial load or A/E lesion formation, we compared their

**Fig. 4.** Recruitment of Nck and N-WASP to the C. rodentium attachment sites in vivo.
A. Nck (arrowheads) is recruited under adherent wild-type C. rodentium (arrows) and C. rodentium expressing TirN-ctrl, TirC-ctrl, TirP5A and TirY451A. Nck was not detected under intimately adherent C. rodentium expressing TirY471A or TirY451A/Y471A.
B. N-WASP (arrowheads) is recruited *in vivo* under intimately adherent wild-type C. rodentium (arrows) and C. rodentium expressing TirN-ctrl, TirC-ctrl, TirP5A, TirY451A, TirY471A and TirY451A/Y471A. Neither intimately adherent bacteria, nor Nck or N-WASP recruitment was observed on sections derived from mice inoculated with C. rodentium expressing Tir1-33stop or uninfected mice. Bacteria were labelled with anti-intimin [l]. Bar = 20 μm.
Fig. 5. Recruitment of IRTKS (A) and IRSp53 (B) to the C. rodentium attachment sites in vivo. IRTKS (arrows) is recruited under adherent wild-type C. rodentium. IRSp53 was not detected under intimately adherent C. rodentium expressing TirY451A or on uninfected tissues. IRSp53 was not detected in either the infected or uninfected tissues. Bar = 20 μm. (C) Immunofluorescence staining of human terminal ileal mucosa infected with wild-type EHEC, EHEC Δtir (pIC422), expressing TirY458A and EHEC Δtir (pIC421), expressing TirTir. IRTKS (green) was detected under adherent wild-type EHEC and EHEC Δtir expressing TirTir but not under adherent EHEC Δtir expressing TirY458A. No IRSp53 staining was observed under attached wild-type EHEC bacteria. Tir was stained in blue (lower panel) to facilitate detection of A/E lesions. Sections were counterstained with anti-cytokeratin (blue, upper panels) and propidium iodide (red) to visualize epithelial cells and cell nuclei/bacteria respectively. Shown are merged images of all fluorescence channels. Infection of Swiss 3T3 cells with C. rodentium expressing TirY451A, TirY471A and TirY451A/Y471A to a reference strain (wild-type C. rodentium). At days 2, 4, 7, 9 and 11 post inoculation the ratio between the two populations (test strain versus reference strain) and the competitive index (CI) were calculated for each group (Fig. 7). In order to neutralize any potential negative effects of the chromosomal kanamycin cassette insertion on the in vivo fitness of the mutants, the CI of the mock mutant strain was directly compared with the CI of the mock mutant control. Generally, a test strain with a CI of less than 0.5 is considered attenuated, whereas a CI equal or higher than 1 indicates that the test strain colonizes at least as well as the reference strain (Mundy et al., 2004).

We conducted pairwise and global statistical analysis of the CI of C. rodentium expressing TirY451A, TirY471A and TirY451A/Y471A compared with the CI of C. rodentium expressing TirTir. We also compared the CI of C. rodentium expressing TirTir, with the CI of C. rodentium expressing TirTir. While C. rodentium expressing TirTir was as competitive as the wild-type strain (data not shown), pairwise analysis showed that from day 9 post inoculation C. rodentium expressing TirY451A and TirY471A were significantly out-competed with C. rodentium expressing TirTir. C. rodentium expressing TirY451A/Y471A showed a greater degree of attenuation, which was seen as early as day 4 post inoculation. Global statistical analysis of the CI of C. rodentium expressing TirY451A, TirY471A and TirY451A/Y471A showed that from day 4 post inoculation, C. rodentium expressing TirY451A/Y471A strain was highly attenuated compared with C. rodentium expressing TirTir, with a CI of 0.0044 compared with 3.5 for the control strain at day 11 (Fig. 7). These results show that although not affecting bacterial load and A/E lesion formation in vivo, Tir residues Y451 and Y471, and by extension Tir:Nck and Tir:IRTKS complexes, provide the bacterium with competitive edge and higher fitness in vivo.

**Discussion**

The Nck binding site and the NPY motif within Tir are conserved among typical EPEC, non-O157 EHEC and C. rodentium (reviewed in Garmentia et al., 2005; Franken and Phillips, 2008). The absence of the Nck binding site in Tir EHEC O157:H7 is compensated by expression of TccP/EspFU (Campellone et al., 2004b; Garmentia et al., 2004), which similarly to Nck, activates N-WASP (Garmentia et al., 2006; Campellone et al., 2008; Cheng et al., 2008; Sallee et al., 2008). This suggests the existence of selective pressure for the preservation of these signalling pathways. However, despite intense investigations, the role of these signalling pathways in vivo is not fully understood.

In this study we investigated the role of Tir tyrosine residues Y451 and Y471 during the entire infection cycle using the C. rodentium model. After confirming that C. rodentium expressing TirY451A, TirY471A and TirY451A/Y471A had the same in vitro phenotypes as their corresponding EPEC Y454 and Y474 Tir mutants (in terms of actin polymerization and Nck recruitment), we investigated their phenotype in vivo. We found that C. rodentium expressing the single TirY451A and TirY471A substitutions and the double TirY451A/Y471A mutation exhibited colonization dynamic, pathogen load, tissue targeting, pathology (i.e. colonic hyperplasia) and A/E lesion formation abilities that were indistinguishable from the parental wild-type strain. Importantly, we found that even though C. rodentium expressing TirY471A lost the ability to recruit Nck, N-WASP was still found recruited under attached bacteria in vivo. C. rodentium expressing TirY451A recruited both Nck and N-WASP to the site of bacterial attachment in vivo. In addition, A/E lesions induced by C. rodentium expressing any of the Tir mutants, but TirY471stop, showed similar accumulation of electron-dense material under adherent bacteria.

Consistent with our data, Deng et al. (2003) have previously shown that C. rodentium expressing TirY471F induced typical A/E lesions in vivo at day 8 post oral challenge. However, as these studies were done using plasmid-encoded Tir, the level of colonization of the tir mutant was greatly lower than that achieved by the wild-type strain. Using the human IVOC model, Schuller et al. (2007) have shown that while Tir is essential for colonization of the human gut mucosa, mutation in Y474 of EPEC E2348/69 Tir abolished Nck recruitment but did not affect the ability of the bacteria to recruit N-WASP and to induce typical A/E lesions. Moreover, and consistent with our
Scanning and transmission electron microscopy of mice colonic epithelium infected with wild-type *C. rodentium* and *C. rodentium* expressing TirN-ctrl, TirC-ctrl, TirP5A, TirY451A, TirY451/Y471A. Local effacement of the brush border microvilli, intimately adherent bacteria (arrow) and accumulation of electron-dense material (arrowheads) were observed following inoculation of mice with any of the *C. rodentium* strains. Intact brush border microvilli were observed following mice infection with *C. rodentium* expressing Tir1-33stop or on uninfected mice. Tissues were collected at day 8 post inoculation. Bar = 5 μm (SEM), 2 μm (TEM) or 500 nm (TEM, insets).
C. rodentium data, EPEC E2348/69 expressing TirY454F/Y474F still recruited N-WASP to the bacterial attachment sites on human IVOC. When considered together these data suggest that actin polymerization on mucosal surfaces is triggered by EPEC and C. rodentium independently of Y454/1 and Y474/1 residues by a yet unidentified mechanism.

Recent reports have shown that Tir recruits IRTKS (Vingadassalom et al., 2009) and/or IRSp53 (Weiss et al., 2009) in an NPY-dependent manner. Therefore, we investigated if IRTKS or IRSp53 are recruited to the C. rodentium attachment sites in vivo. IRTKS was detected under attached bacteria and its recruitment was Y451-dependent. In contrast, no IRSp53 was detected under adherent bacteria in vivo. Similarly we found that IRTKS, but not IRSp53, was recruited under adherent EHEC bacteria using human IVOC.

Adhesion of EHEC O157 to human (Garmendia et al., 2004) and bovine (Girard et al., 2009b) IVOC and bovine ileal loops (Vlisidou et al., 2006) was reported to be Tir-dependent but independent of the Tir-IRTKS-TccP/EspFU complex, which is consistent with our observation that EHEC adhered to human IVOC independently of IRTKS recruitment. In vivo calf studies revealed no measurable differences in colonization levels between wild type and EHEC O157ΔtccP/espFU (Vlisidou et al., 2006). However, in infant rabbits colonization efficiency of the tccP/espFU EHEC O157 mutant was similar to the parent strain in the ileum but was reduced in the large bowel at 7 days post infection (Ritchie et al., 2008). In gnotobiotic piglets, expression of TccP/EspFU was associated with larger-size adherent bacterial aggregates (Ritchie et al., 2008). Together, these results are consistent with the notion that the Tir NPY signalling pathway is not essential for the formation of A/E lesions and for the establishment of colonization, but promotes bacterial expansion from the initial infection sites.

In addition to the Tir Y451 and Y471 pathways, we investigated the role of the Tir N-terminal polyproline sequence as it was reported to play a role in the recruit-
ment of kinases involved in Tir phosphorylation, Nck recruitment and actin polymerization. Contrary to its reported role in EPEC (Bommarius et al., 2007), the polyproline sequence plays no role in recruitment of Nck and actin polymerization in C. rodentium in vitro and in vivo. These data are consistent with a previous report showing that deletion of the entire N-terminus of EPEC and EHEC Tir did not inhibit actin polymerization (Campellone et al., 2004a; 2006).

Taken together, our results show that while Tir Y471 recruits Nck and Y451 recruits IRTKS, these signaling pathways are dispensable for colonization, colonic hyperplasia and A/E lesion formation. This conclusion raises a fundamental question: does the ability to activate these pathways benefit the bacterium? In order to address this experimentally, we performed mixed infection studies. By comparing the CIs of C. rodentium expressing mock or tir mutants, we have shown that in mixed infections of wild type with either of the single Y451 and Y471 Tir mutants the latter strains were similarly out-competed. Importantly, mixed infections of wild type and C. rodentium expressing the double TirY451A/Y471A mutant resulted in rapid decline of the mutant. These results suggest that TirY451A and TirY471A contribute independently to C. rodentium competitiveness in vivo. The fact that C. rodentium expressing TirY451A/Y471A is significantly more attenuated than each of the single mutants alone suggests a cooperative (accumulative) function of the Y451 and Y471 pathways. We conclude that Tir residues Y454/1 and Y474/1 contribute to in vivo competitiveness, probably during mixed infections.

In conclusion, our results show that despite defining EPEC, EHEC and C. rodentium infection, we lack basic knowledge of the mechanisms involved in A/E lesion formation. Moreover, we have shown that recruitment of N-WASP to the site of bacterial attachment in vivo occurs independently of Tir residues Y451 and Y471. While IRTKS is recruited to bacterial adhesion sites ex vivo and in vivo, it is not essential for A/E lesion formation or recruitment of N-WASP. Finally, our results show that although not involved in A/E lesion formation Tir residues Y451 and Y471 play an important role in pathogen host interaction.

### Experimental procedures

**Bacterial strains and growth conditions**

The bacterial strains, plasmids and primers used in this study are listed in Table 1. Bacteria were grown in Luria–Bertani (LB) medium, M9 minimum media (Mundy et al., 2004) or in Dubacco’s modified Eagle’s medium (DMEM) supplemented with kanamycin (50 mg ml⁻¹), ampicillin (100 mg ml⁻¹) and nalidixic acid (50 mg ml⁻¹) as required.

**Introduction of site-directed tirCR mutants into the bacterial chromosome**

The C. rodentium expressing mutated Tir were generated using a lambda red-based mutations system (Datsenko and Wanner, 2000). We introduced site-directed tir mutations into the endogenous tir chromosome, together with a kanamycin cassette, either in the map-tir or the tir-cesT intergenic regions for 5′ and 3′ mutations respectively.

In order to mutate the N-terminal polyproline region we cloned the 3′ end of map (base pairs 377–612) followed by a non-polar aphT cassette (Galan et al., 1992), which confers kanamycin resistance, the map-tir intergenic region and the 5′ end of tirCR (base pairs 1–331) into a pGEMT vector. The 3′ end of map was amplified using primer pair Cmap-Fw and EcoRI-Cmap-Rv and the map-tir intergenic region with the 5′ end of tirCR was amplified using primer pair and EcoRI-[map-tir]-Fw, Ntir-Rv. The two fragments were digested with EcoRI, ligated to each other and then cloned into pGEMT. The non-polar aphT cassette was then inserted into the EcoRI site between the two fragments and the orientation of the kanamycin cassette checked by PCR. The plasmid, named pICCC432, was used to generate the control C. rodentium mutant expressing TirY451A, as described below.

Similarly, in order to mutate the C-terminal tyrosine residues we cloned the 3′ end of tirCR (base pairs 1067–1444), followed by a non-polar aphT cassette, the tir-cesT intergenic region and the 5′ end of cesT (base pairs 1–388) into pGEMT. The 3′ end of tirCR was amplified using primer pair Ctr-Fw and EcoRI-Ctr-Rv and the tir-cesT intergenic region with the 5′ end of cesT was amplified using the primer pair EcoRI-[tir-cesT]-Fw and NcresT-Rv. The two fragments were digested with EcoRI, ligated to each other, cloned into pGEMT and the non-polar aphT cassette was then inserted into the EcoRI site as described above, generating plasmid pICCC433, which was used to generate the control C. rodentium mutant expressing TirP5A.

TirCR mutagenesis was then carried out by inverse-PCR pLCC432 and pICCC433 as templates for 5′ and 3′ site-directed mutagenesis respectively. The primer pair TirP5A-Fw and TirP5A-Rv was used to mutate the five proline residues of tirCR polyproline region into alanines (TirP5A), generating plICCC435. Similarly, the primer pair TirY451A-Fw and TirY451A-Rv and TirY471A-Fw and TirY471A-Rv were used to mutate the tyrosine residues Y451 and Y471 into alanines (TirY451A and TirY471A) generating pICCC436 and pICCC437 respectively. The double tyrosine mutant (TirY451A/Y471A) was generated by superimposing Y471A on the Y451A mutant, generating plICCC438. All pGEMT derivatives were checked by DNA sequencing using an automated DNA sequencer (ABI 377). While sequencing the putative TirP5A mutants we identified a non-specific mutant containing a frameshift that created a stop codon at Tir amino acid position 33 (Tir1-33stop). This clone, named pICCC434, was used as a nonsense mutant control.

To introduce tirF5A tirF33stop and tirP5A site-directed mutants into C. rodentium chromosome, the inserts in pLCC432, pICCC434 and pICCC435 were PCR-amplified using the primer pair Cmap-Fw and Ntir-Rv. Similarly, to introduce tirC355, tirY451A, tirY471A and tirY451A/Y471A site-directed mutants into C. rodentium chromosome, the inserts in pICCC433, pICCC436,
Table 1. Strains, plasmids and primers used in this study.

| Strains          | Description                                      | Reference                  |
|------------------|--------------------------------------------------|----------------------------|
| ICC169           | Wild type *C. rodentium* (serogroup O152); Naι   | Wiles *et al.* (2004)      |
| ICC293           | *C. rodentium* expressing TirFv-ctrl              | This study                 |
| ICC294           | *C. rodentium* expressing TirC-ctrl               | This study                 |
| ICC295           | *C. rodentium* expressing TirF1-33stop            | This study                 |
| ICC296           | *C. rodentium* expressing TirPSA                  | This study                 |
| ICC297           | *C. rodentium* expressing TirY451A                | This study                 |
| ICC298           | *C. rodentium* expressing TirY471A                | This study                 |
| ICC301           | *C. rodentium* expressing TirY451A/Y471A          | This study                 |
| TUV 93-0         | EDL933, EHEC O157:H7, stx1 and stx2 mutant       | Riley *et al.* (1983)      |
| KC5              | TUV 93-0 tir deletion mutant                      | Murphy and Campellone (2003) |

| Plasmids         | Description                                      | Reference                  |
|------------------|--------------------------------------------------|----------------------------|
| pSA10            | pKK177-3 containing lacI gene                    | Schlosser-Silverman *et al.* (2000) |
| pIC421           | pSA10 derivative encoding EHEC Tir               | Mousnier *et al.* (2008)    |
| pIC422           | pIC421 derivative encoding EHEC TirY451A        | Mousnier *et al.* (2008)    |
| pET28a           | Expressing vector                                | Novagen                    |
| pGEMT            | Cloning vector                                   | Promega                    |
| pKD46            | Coding the lambda Red recombinase                 | Datsenko and Wanner (2000) |
| pSB315           | A plasmid coding for the kanamycin resistance aphT cassette | Galan *et al.* (1992)      |
| pIC431           | pET28a expressing N-terminal His-tagged EVH1 domain of N-WASP | Girard *et al.* (2009a)    |
| pIC432           | pGEMT vector containing the 3’ end of map (bp 377–612), the aphT cassette, \( \text{map-tir} \) intergenic region and the 5’ end of \( \text{tir} \) (bp 1–331) | This study              |
| pIC433           | pGEMT vector containing the 3’ end of \( \text{tir} \) (bp 1067–1644), the \( \text{aphT} \) cassette, \( \text{tir-cesT} \) intergenic region and the 5’ end of \( \text{cesT} \) (bp 1–388) | This study              |
| pIC434           | pIC432 containing a stop codon at amino acid position 33 of \( \text{tir} \) CR | This study              |
| pIC435           | pIC432 containing the \( \text{tir} \) CR \( \text{PSA} \) mutation | This study              |
| pIC436           | pIC432 containing the \( \text{tir} \) CR \( \text{Y451A} \) mutation | This study              |
| pIC437           | pIC432 containing the \( \text{tir} \) CR \( \text{Y471A} \) mutation | This study              |
| pIC438           | pIC432 containing the \( \text{tir} \) CR \( \text{Y451A/Y471A} \) mutation | This study              |
| Primer name      | Nucleotide sequence (restriction site in bold)    |                            |
| C map-Fw         | 5’-ggcagaactatcaatacgagc-3’                       |                            |
| EcoRI-Cmap-Rv    | 5’-ccggaattctcagctggcttgcgt-3’                    |                            |
| EcoRI-[map-tr]-Fw| 5’-ccggaattcaggatctggcaaagtt-3’                   |                            |
| Nts-Rv           | 5’-ggaattctcagctagaacaggaacaggc-3’                |                            |
| Ctr-Fw           | 5’-caagcacgctccgacctg-3’                          |                            |
| EcoRI-Ctr-Rv     | 5’-ccggaattctcagctagaacaggaacaggc-3’              |                            |
| EcoRI-[tir-cesT]-Fw| 5’-ccggaattctcagctagaacaggaacaggc-3’              |                            |
| NcesT-Rv         | 5’-ggttgagaccagatgacagc-3’                        |                            |
| TirPSA-Fw        | 5’-gcagccttcagctagaacaggaacaggc-3’                |                            |
| TirPSA-Rv        | 5’-gcatctgcaatcattgaataatggtagtcc-3’              |                            |
| TirY451A-Fw      | 5’-gctgatggtaggttgct-3’                           |                            |
| TirY471A-Fw      | 5’-gagagctgctgcgtgatgtcc-3’                       |                            |
| pIC437 and pIC438 were amplified using the primer pair Ctr-Fw and NcesT-Rv. The PCR products were electroporated into wild-type *C. rodentium* (ICC169) containing pKD46 encoding the lambda Red recombinase (Datsenko and Wanner, 2000). Transformants were selected on kanamycin plates and the insertion of site-directed \( \text{tir} \) CR mutants into *C. rodentium* chromosome was confirmed by PCR and DNA sequencing.  

**Cell culture**

Swiss 3T3 cell line was grown in DMEM containing 4500 mg l\(^{-1}\) glucose and supplemented with 10% fetal calf serum and 2 mM glutamine at 37°C in 5% CO\(_2\). Cells were seeded onto glass coverslips in 24-well plates at a density of 5 \( \times 10^4 \) cells per well, 48 h before infection. EHEC (Mousnier *et al.*, 2008) or *C. rodentium* (Girard *et al.*, 2009a) strains used for *in vitro* assays were grown for 8 h in LB broth, then transferred into fresh, sterile DMEM containing 1000 mg l\(^{-1}\) glucose and incubated static at 37°C in 5% CO\(_2\) overnight prior to infection. Each coverslip was infected with 25 μl of the appropriate overnight culture, centrifuged at 1000 r.p.m. for 5 min at room temperature, and then incubated at 37°C in 5% CO\(_2\) for 5 h. The cell culture medium was renewed half way through the infection period. After washes with phosphate-buffered saline (PBS), infected cells were fixed for 20 min in 4% paraformaldehyde, permeabilized with 0.1% Triton for 5 min and labelled by indirect immunofluorescence, using rabbit anti-TirEHEC (1:500) (Batchelor *et al.*, 2004; Girard *et al.*, 2007), rabbit anti-Nck (1:300) (Millipore (Upstate), Lake Placid, NY, USA) or rabbit anti-IRSp53 (1:50) (Weiss *et al.*, 2009).
studies as described below. Tissues were collected for histopathological and microscopic examination and fixed in 10% buffered formalin for microscopic examination. Formalin-fixed tissues were then processed, paraffin-embedded, sectioned at 5 μm, and stained with haematoxylin and eosin (H&E) according to standard techniques. Formalin-fixed, paraffin-embedded sections (FFPE) were examined by light microscopy for the presence of intimately adhering bacteria on intestinal cells, as previously described (Girard et al., 2005b). Crypts length was also evaluated and the length of at least six well-oriented crypts has been measured on each section.

Additional colonic segments were fixed in 2.5% glutaraldehyde for further electron microscopy analysis, while some were embedded in optimal cutting temperature medium (Raymond A Lamb Limited, UK), then snap-frozen in liquid nitrogen for further cryosectioning.

Mice
Pathogen-free female 18–20 g C57Bl/6 mice were purchased from Charles River. All animals were housed in individually HEPA-filtered cages with sterile bedding and free access to sterilized food and water. All animal experiments were performed in accordance with the Animals Scientific Procedures Act (1986) and were approved by the local Ethical Review Committee. Independent single infection experiments were performed twice using four to eight mice per group. Mice inoculated with mock mutant and nonsense mutant strains were included in every experiment. Mice inoculated with wild-type strain and uninfected mice were included in parallel with mutant strains.

Oral infection of mice
For single infection experiments, mice were inoculated by oral gavage with 200 μl of overnight LB-grown C. rodentium suspension in PBS (≈ 5 × 10⁹ cfu). The number of viable bacteria used as inoculum was determined by retrospective plating onto LB agar containing antibiotics. Stool samples were recovered aseptically at various time points after inoculation and the number of viable bacteria per gram of stool was determined by plating onto LB agar (Wiles et al., 2005). At day 8 post inoculum, the mice were culled and the colonic tissues were collected for histopathological and microscopic studies as described below.

For mixed infection experiments, the two overnight LB-grown bacterial strains to be competed against each other in vivo were combined in a ratio of 1:1 (approximately 2 × 10⁹ cfu for each strain) in 200 μl PBS and used to infect mice by oral gavage. Dilutions of the inoculum were plated on respective antibiotic-containing plates to determine the precise ratio of the two bacterial strains (test strain/reference strain). The ratio of viable bacteria determined in our inoculum was of approximately 2:1 for all CI experiments. Stool samples were collected at regular intervals and the CI was calculated by dividing the ratio of test strain cfu and reference strain cfu from the stools by the ratio of test strain cfu to reference strain cfu in the inoculum (Mundy et al., 2003). The CI experiments were carried out using five animals per group and the CI was determined at days 2, 4, 7, 9 and 11 post inoculation.

Harvesting, collection of samples and histopathology
Segments of the terminal colon of each mouse were collected post mortem at day 8 post inoculation, rinsed of their content and fixed in 10% buffered formalin for microscopic examination.

IVOC and immunofluorescence staining of cryosections
Human IVOC, cryosectioning and immunofluorescence staining were performed as described previously (Schuller et al., 2007) with ethical approval and informed consent. Biopsies from the terminal ileum were infected with wild-type EHEC (TUV 93-0), EHEC Δtir (KC5) strain expressing EHEC TirWT (pIC421) or TirF152 (pIC422), for 8 h. Experiments were performed using tissue from four patients (aged between 41 and 181 months). Cryosections were incubated with rabbit anti-IRTKS (1:200) (Millard et al., 2007), rabbit anti-TirEHEC (1:500) (Batchelor et al., 2004; Girard et al., 2007), rabbit anti-IRSp53 (1:50) (Weiss et al., 2009) or mouse anticytokeratin (1:50, Dako) for 60 min at room temperature, washed and incubated in Alexa Fluor 488 or Alexa Fluor 647-conjugated secondary antibody (Molecular Probes) for 30 min. Counterstaining of bacteria and cell nuclei was performed using propidium iodide (Sigma). Sections were analysed with a Zeiss LSM 510 Meta confocal laser scanning microscope.

Indirect immunofluorescence assay on mice colon sections
An indirect immunofluorescence assay using FFPE sections and cryosections fixed in 3% paraformaldehyde in PBS was used as previously described (Girard et al., 2007; 2008). Appropriate sections were immunostained using the following antibodies: rabbit anti-O152 (kindly provided by Dr Lothar Beutin, The National Reference Laboratory for E. coli, Federal Institute for Risk Assessment, Berlin, Germany) was used to visualize C. rodentium on FFPE sections, chicken anti-intimin (kindly provided by Professor John M. Fairbrother, E. coli Laboratory, Faculté de médecine vétérinaire, Université de Montréal, Canada) was used to visualize C. rodentium in multi labelling procedures on cryosections (Girard et al., 2005a), rabbit anti-Tir EHEC (Batchelor et al., 2004; Girard et al., 2007), rabbit anti-N-WASP (Girard et al., 2009a), rabbit anti-Nck [Millipore (Upstate), Lake Placid, NY (USA)], rabbit anti-IRSp53 (Weiss et al., 2009) and rabbit anti-IRTKS (Millard et al., 2007) were used for multilabelling procedure on cryosections. Carbocyanine-2-conjugated donkey anti-chicken IgY, Phalloidin-Tetramethyl Rhodamine Iso-Thiocyanate-conjugated donkey anti-rabbit IgG and Rhodamine RedX-conjugated donkey anti-mouse IgG
(Jackson ImmunoResearch Europe, Soham, Cambridgeshire, UK) secondary antibodies were used in respect of the primary antibodies. Phalloidin-Alexa Fluor 633 (Invitrogen, UK) was used to stain F-actin, while DNA of both bacteria and epithelial cells was counterstained with Hoechst 33342. Sections were examined with an Axio Imager M1 microscope (Carl Zeiss Microlmaging GmbH, Germany), images were acquired using an AxioCam MRm monochrome camera, and computer-processed using AxioVision (Carl Zeiss Microlmaging GmbH, Germany).

**Electron microscopy**

Additional explants/tissue samples were processed for electron microscopy, as previously described (Girard et al., 2007). Samples for scanning electron microscopy were examined without knowledge of the strain used, at an accelerating voltage of 25 kV using a JEOL JSM-5300 scanning electron microscope [JEOL (UK), Herts, UK]. Samples for transmission electron microscopy were observed using a Phillips 201 transmission electron microscope at an accelerating voltage of 60 kV (Phillips, UK).

**Statistical analysis**

Results are presented as a line plot (colonization) or a vertical bar chart (crypt length) with the mean and its standard deviation, or as a scatter plot with the median (CI). The non-parametric Mann–Whitney test and the non-parametric Kruskal–Wallis test with Bonferroni’s corrected *a posteriori* comparisons were used to pairwise and global statistical analysis, respectively, using commercially available GraphPad InStat v3.06 software (GraphPad Software, San Diego, CA, USA). A *P* = 0.05 was considered significant.

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