TccP2-mediated subversion of actin dynamics by EPEC 2 – a distinct evolutionary lineage of enteropathogenic Escherichia coli

Andrew D. Whale, Rodrigo T. Hernandes, Tadasuke Ooka, Lothar Beutin, Stephanie Schüller, Junkal Garmendia, Lynette Crowther, Mônica A. M. Vieira, Yoshitoshi Ogura, Gladys Krause, Alan D. Phillips, Tania A. T. Gomes, Tetsuya Hayashi and Gad Frankel

1Division of Cell and Molecular Biology, Imperial College London, London SW7 2AZ, UK
2Departamento de Microbiologia, Imunologia e Parasitologia, Universidade Federal de São Paulo, São Paulo, Brazil
3Division of Bioenvironmental Science, Frontier Science Research Center, University of Miyazaki, 5200 Kiyotake, Miyazaki 889-1692, Japan
4Nationales Referenzlabor für Escherichia coli, Bundesinstitut für Risikobewertung, Diedersdorfer Weg 1, D-12277 Berlin, Germany
5Centre for Paediatric Gastroenterology, Royal Free and University College Medical School, London, UK
6Fundación Caubet-Cimera, Recinto Hospital Joan March, Carretera Soller Km 1207 110 Bunyola, Mallorca, Spain

Enteropathogenic Escherichia coli (EPEC) is a major cause of infantile diarrhoea in developing countries. While colonizing the gut mucosa, EPEC triggers extensive actin-polymerization activity at the site of intimate bacterial attachment, which is mediated by avid interaction between the outer-membrane adhesin intimin and the type III secretion system (T3SS) effector Tir. The prevailing dogma is that actin polymerization by EPEC is achieved following tyrosine phosphorylation of Tir, recruitment of Nck and activation of neuronal Wiskott–Aldrich syndrome protein (N-WASP). In closely related enterohaemorrhagic E. coli (EHEC) O157 : H7, actin polymerization is triggered following recruitment of the T3SS effector TccP/EspFU (instead of Nck) and local activation of N-WASP. In addition to tccP, typical EHEC O157 : H7 harbour a pseudogene (tccP2). However, it has recently been found that atypical, sorbitol-fermenting EHEC O157 : H7 strain is functional. EHEC O157 : H7 contains functional tccP and tccP2 alleles. Interestingly, intact tccP2 has been identified in the incomplete genome sequence of the prototype EPEC strain B171 (serotype O111 : H2), but it is missing from another prototype EPEC strain E2348/69 (O127 : H7). E2348/69 and B171 belong to two distinct evolutionary lineages of EPEC, termed EPEC 1 and EPEC 2, respectively. Here, it is reported that while both EPEC 1 and EPEC 2 triggered actin polymerization via the Nck pathway, tccP2 was found in 26 of 27 (96.2 %) strains belonging to EPEC 2, and in none of the 34 strains belonging to EPEC 1. It was shown that TccP2 was: (i) translocated by the locus of enterocyte effacement-encoded T3SS; (ii) localized at the tip of the EPEC 2-induced actin-rich pedestals in infected HeLa cells and human intestinal in vitro organ cultures ex vivo; and (iii) essential for actin polymerization in infected Nck−/− cells. Therefore, unlike strains belonging to EPEC 1, strains belonging to EPEC 2 can trigger actin polymerization using both Nck and TccP2 actin-polymerization signaling cascades.

Abbreviations: A/E, attaching and effacing; BFP, bundle-forming pilus; EAF, enteropathogenic Escherichia coli adherence factor; EHEC, enterohaemorrhagic Escherichia coli; EPEC, enteropathogenic Escherichia coli; FAS, fluorescent actin staining; HA, haemagglutinin; IVOC, in vitro organ culture; LEE, locus of enterocyte effacement; MEF, mouse embryo fibroblast; PRR, proline-rich repeat; N-WASP, neuronal Wiskott–Aldrich syndrome protein; T3SS, type III secretion system.

The GenBank/EMBL/DDBJ accession numbers for the sequences determined in this paper are AB271153 (O111 : H2 strain CB07077), AB271154 (O111 : H2 strain CB03447) and AB271155 (O111 : H2 strain CB03454).
INTRODUCTION

Enteropathogenic Escherichia coli (EPEC) is the leading cause of childhood diarrhea in developing countries (reviewed by Chen & Frankel, 2005). EPEC strains belong to a large number of O:H serotypes (Trabulsi et al., 2002) and are divided into typical and atypical categories (Kaper, 1996). Typical EPEC are defined by the presence of the locus of enteroocyte effacement (LEE) pathogenicity island (McDaniel et al., 1995), and the EPEC adherence factor (EAF) plasmid (Baldini et al., 1996). Typical EPEC are divided into two distinct evolutionary lineages known as EPEC 1 and EPEC 2 (Orskov et al., 1990). The EPEC 1 branch is characterized by expression of flagella serotypes H6 and H34 (Whittam et al., 1993), the presence of a complete tra region on the EAF plasmid (Brinkley et al., 2006), and a distinctive antigenic type α of the outer-membrane adhesin intimin (Adu-Bobie et al., 1998), while the EPEC 2 branch expresses the flagella serotype H2 (or H−) (Whittam et al., 1993), lacks the tra region (Brinkley et al., 2006), and expresses the intimin subtype β (Adu-Bobie et al., 1998).

Enterohaemorrhagic E. coli (EHEC) is a subgroup of Verocytotoxin (VT)-producing E. coli (VTEC) that can cause bloody diarrhoea, haemorrhagic colitis and haemolytic-uraemic syndrome (HUS). E. coli O157 : H7 is the most common and virulent EHEC serotype that is implicated worldwide in human disease (reviewed by Karch et al., 2005).

EPEC and EHEC colonize the gut mucosa via attaching and effacing (A/E) lesion formation, which is characterized by intimate attachment of the pathogen to the host intestinal epithelium, localized effacement of the brush border microvilli (Knutton et al., 1987), and localized actin polymerization. A/E lesion formation and actin polymerization are dependent on the LEE-encoded type III secretion system (T3SS) (Jarvis et al., 1995), the adhesin intimin (Jerse et al., 1990), and translocation of the effector protein Tir (translocated intimin receptor) (Kenny et al., 1997). Once translocated, Tir is integrated into the host-cell plasma membrane, in which it adopts a hairpin loop topology (Hartland et al., 1999), with the extracellular loop presented above the plasma membrane acting as a receptor for the bacterial adhesin intimin (reviewed by Frankel et al., 2001). Intimin-mediated clustering of Tir (Campellone et al., 2004b) leads to accretion of several cytoskeletal proteins to the intracellular amino- and carboxy-terminal Tir domains, linking the extracellular bacterium to the host-cell cytoskeleton (Goosney et al., 2001) and triggering actin remodelling into pedestal-like structures. Tir of the prototype EPEC O127 : H6 strain E2348/69, which belongs to EPEC 1, harbours a tyrosine residue Y474 that is present in the context of a consensus binding site for the mammalian adaptor protein Nck (Y36PDE/D/V) (Campellone et al., 2002; Gruenheid et al., 2001). Tyrosine phosphorylation of Tir [Tir(S)] by host-cell kinases (Phillips et al., 2004; Swimm et al., 2004) recruits Nck to the site of bacterial attachment, which in turn binds the actin nucleation-promoting factor neuronal Wiskott–Aldrich syndrome protein (N-WASP), initiating actin polymerization via activation of the actin-related protein 2/3 (Arp2/3) complex (Lommel et al., 2001). In contrast, the equivalent position in Tir of EHEC O157 : H7 is occupied by serine [Tir(S)], and as such, Nck is not involved in actin polymerization by EHEC O157 : H7 (Gruenheid et al., 2001). Instead, EHEC O157 : H7 requires a second bacterial T3SS effector protein, TccP (Tir-cytoskeleton coupling protein; also termed EspFU, because it shares 35 % identity with the T3SS effector EspF), which binds, recruits and activates N-WASP to trigger localized actin polymerization (Campellone et al., 2004a; Garmendia et al., 2004). The minimal region of Tir EHEC O157 that is needed for recruitment of TccP and induction of actin polymerization is a 12 aa motif at the C terminus (Campellone et al., 2006; Allen-Vercoe et al., 2006). This 12 aa motif is conserved in Tir of EPEC O127 : H6, and has been implicated in an Nck-independent actin-remodelling pathway during infection with O127 : H6 EPEC (Campellone and Leong, 2005). Importantly, TccP does not bind Tir directly (Campellone et al., 2004a; Garmendia et al., 2004).

tccP is carried on prophage CP-933U/Sp14 (Campellone et al., 2004a; Garmendia et al., 2004) and consists of a unique 80 aa N-terminal region (involved in protein translocation) and several almost identical 47 aa proline-rich repeats (PRRs) (Garmendia et al., 2006). In a recent survey of clinical and environmental strains, tccP was found in 100 % of EHEC O157 : H7 and in a minority of EPEC and non-O157 EHEC strains (Garmendia et al., 2005). Of particular importance is the fact that in tccP-positive EPEC, Tir is tyrosine-phosphorylated [Tir(S)] and simultaneously recruits Nck and TccP under attached bacteria during infection of cultured epithelial cells (Whale et al., 2006).

EHEC O157 : H7 strains Sakai and EDL933 also contain pseudo tccP genes (ECs1126 and Z1385, respectively, which have also been referred to as espFM by Campellone et al., 2004a), which are carried on prophage Sp4/CP-933M. A deletion of a single (T/A) base pair at position 28 introduces a translational frameshift and a premature stop codon. However, we have recently found that β-glucuronidase-positive/sorbitol-fermenting strains of EHEC O157 harbour an intact ECs1126 gene, in addition to tccP (Ogura et al., 2007). In order to discriminate between the tccP alleles, we named ECs1126, which is carried on prophage Sp4/CP-933M, tccP2. The aim of this study was to determine the distribution and function of tccP2 in typical EPEC belonging to serogroups O55, O84, O111, O114, O127 and O142, which we have previously reported to be tccP gene-negative (Garmendia et al., 2005) O119 : H6.
METHODS

PCR amplification of tccP2 and tir and colony blot hybridization. Clinical EPEC strains are listed in Table 1. Conventional PCR was used to amplify tccP2 with gene-specific tccP2-F and tccP2-R primers. Forward, gene-specific primers tirY474-F and tirS478-F were used together with a conserved reverse primer (tir-R) to discriminate between tirY474R68 and tirS478R gene types [that encode Tir(Y,P) and Tir(S), respectively]; primers used in this study are listed in Table 2. Colony blot hybridization was performed using standard protocols and a tccP2 gene probe.

Locus-specific sequencing. The tccP2 genes and their 5' and 3' flanking regions were amplified using a blend tag PCR amplification Kit (Toyobo) and the PCR primer pairs tccP2-SFb and tccP2-SRb (Table 2). Direct sequencing of the PCR products was done using the primers used for amplification and an ABI PRISM 3100 automated sequencer. When necessary, internal sequencing primers were used.

Preparation of tccP rabbit antiserum. A PCR fragment encoding a truncated TccP2L23993 derivative comprising the unique N terminus and two PRRs (TccP2N2R) was cloned into pET28-a as described previously (Ogura et al., 2007); TccP2N2R-His was purified as described by Hartland et al. (1999), and polyclonal TccP2N2R-His antiserum was produced in rabbits at Covalab.

Preparation of protein samples for detection of TccP and TccP2 by Western blotting. Protein preparations from whole-cell extracts were dissolved in protein-denaturating buffer before PAGE and Western blotting. TccP was detected using a rabbit polyclonal anti-TccP primary antibody (diluted 1:1000) and porcine anti-rabbit IgG–horseradish peroxidase conjugate secondary antibody (Dako).

Bacterial strains, growth conditions and plasmids. Bacterial strains used for the functional analysis are listed in Table 3. Bacteria were grown at 37 °C, with aeration in Luria–Bertani (LB) medium or Dulbecco’s Modified Eagle’s Medium (DMEM), supplemented with ampicillin (100 μg ml⁻¹) or kanamycin (50 μg ml⁻¹), or both, when necessary. E. coli B171TccP2 mutant was constructed using the λ Red recombinase method (Datsenko & Wanner, 2000). Disruption of tccP2 was performed with a kanamycin-resistance cassette generated by PCR from B171 genomic DNA using primers pkk-tccP2-F1 and pkk-tccP2-R1. The 970 bp (B171 tccP2) and 1252 (ICC215 tccP2) bp PCR products, containing terminal EcoRI and PstI sites, were digested and ligated into pCX340, generating pICC364.

pICCSS6 and pICCSS6 are derivatives of pSA10 (Schlosser-Silverman et al., 2000), a vector containing multiple cloning sites downstream of the tac promoter. An 894 bp fragment containing tccP2 was amplified by PCR from B171 genomic DNA using primers pkk-tccP2-F1 and pkk-tccP2-R1. A 1176 bp fragment containing tccP2 was amplified by PCR from EPEC O111:H2 strain ICC215 genomic DNA using primers pkk-tccP2-F1 and pkk-tccP2-R1. The 970 bp (B171 tccP2) and 1252 (ICC215 tccP2) bp PCR products, containing terminal EcoRI and PstI sites, was digested and ligated into pCSX40, generating pICCSS6 and pICCSS6, respectively.

Antibodies and reagents. Anti-E. coli O157:H7 goat polyclonal antibody (Fitzgerald Industries International) was diluted 1:500. EPEC ICC199 and B171 strains were detected with rabbit polyclonal Int280 antisum (Adu-Bobie et al., 1998), and EPEC E2348/69 was detected with rabbit polyclonal Int280 antisum (Adu-Bobie et al., 1998), both diluted 1:500. Rabbit polyclonal TirEPEC antiserum was diluted 1:500. Phosphotyrosine and Nck were detected using monoclonal mouse anti-phosphotyrosine clone 4G10 (Sigma) and rabbit polyclonal anti-Nck (Upstate) antibodies, diluted 1:250 and 1:150, respectively. Mouse anti-haemagglutinin (HA) mAb HA.11 (Covance) was diluted 1:200. Rhodamine-, Alexa 633- and Oregon Green-conjugated phalloidin (Invitrogen) were used at dilutions of 1:500, 1:500 and 1:100, respectively. Cy5-conjugated donkey anti-goat, rhodamine-conjugated donkey anti-goat, donkey anti-rabbit and Cy2-conjugated donkey anti-mouse antibodies (Jackson Immunoresearch Laboratories) were diluted 1:200. Samples were analysed using either a Zeiss LSM510 confocal laser scanning

| Serotype | No. of strains | tccP2 | Tir type |
|----------|---------------|-------|----------|
| EPEC 1   |               |       |          |
| O55 : H6 | 10            | –     | Y-P      |
| O86 : H34| 4             | –     | Y-P      |
| O127 : H6| 5             | –     | Y-P      |
| O142 : H6| 6             | –     | Y-P      |
| O142 : H34| 3          | –     | Y-P      |
| EPEC 2   |               |       |          |
| O111 : H   | 8             | +[1150 bp]†|  Y-P  |
| O111 : H2  | 18            | +[1150 bp] (14), +[1000 bp] (3), +[1800 bp] (1) | Y-P   |
| O114 : H2  | 1             | +[1150 bp] | Y-P    |
| EPEC non-1 non-2 | 6 | – | Y-P |

*The number of strains is given in parentheses.
†The size of tccP2 amplicons is indicated in brackets.
‡tccP-positive strain.

Table 1. Distribution of tccP2 among clinical EPEC 1 and EPEC 2 isolates

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microscope or a Zeiss Axio Imager fluorescence microscope. Infected cells were fixed and fluorescent actin staining (FAS) used to determine the ability of each strain to trigger the formation of actin-rich pedestals. The efficiency of pedestal formation was quantified by measuring the percentage of adherent bacteria that associated with intense actin accretion. Randomly chosen cells harbouring discrete regions of 2–30 bacteria per cell were examined for each strain, and 300 bacteria, 50 per coverslip, from two or three independent infections carried out in duplicate, were counted in a blinded fashion. Statistical differences between groups were determined by ANOVA. To quantify the adherence of strains B171 and \( B171 \) to HeLa cells, the percentage of cells with at least three adhering bacteria was counted. For each strain, 200 randomly chosen cells from two independent infections carried out in duplicate were examined.

**Cell culture and infection.** HeLa cells (clone HtTA1) and mouse embryo fibroblast (MEF) N-WASP\(^{-/-}\), N-WASP\(^{+/+}\) (a kind gift from S. Snapper, Harvard Medical School, Boston, MA, USA), Nck1–/Nck2– and Nck1\(^{+/+}\)/Nck2\(^{+/+}\) cell lines were grown in DMEM supplemented with 15% fetal calf serum (FCS), respectively, containing 2 mM glutamine at 37 \( ^\circ \)C in 5% CO\(_2\). Cells were seeded onto glass coverslips (12 mm diameter) in 24-well plates at a density of 5 \( \times 10^4 \) cells per well, 24 h before infection. Bacterial cultures grown in LB medium for 8 h were diluted 1 : 500 into DMEM and incubated as static cultures at 37 \( ^\circ \)C in 5% CO\(_2\) overnight, and were used to

### Table 2. Primers used in this study

| Primer | Nucleotide sequence (5’–3’) |
|--------|-----------------------------|
| tccP2-F | ATGATAAAATAGCATAATTCCTTT |
| tccP2-R | TCACGACGCCGCTTAGGTATTAAT |
| tirY474-F | CATATTTATGAGTGACTGCTC |
| tirS478-F | TCTGTTCAGAAATATGGGAATA |
| tir-R | TAAAGTGTACAGTCTGTGATGACAT |
| tccP2-5Fb | GTTAGATTTATGCAAAAGG |
| tccP2-5Rb | AAAACTGCCATATATAGGAATT |
| B171tccP-F1 | CACAGCACAAGACACCTAAGAGGTAAACCAGCTACCTCTTC |
| B171tccP-R1 | GAGGTCCTTATTGTTCATTTATACAGGCAGTAGGAGCGAGCACAGTT-ACATATGAAATATTCCTCTTAG |
| k1 | CAGTCATAGCGAATAGCCT |
| k2 | CCGTGCCCTGTAATGAACTGAC |
| pkk-tccP2-F1 | CCGGAATTCATGATAAATAGCATTAATTCTTTT |
| pkk-tccP2-R1 | AAAACTGCCATATATAGGAATT |
| pCX-B171tccP-F1 | GGTTTCATATGAAATTAGCATAATTCCTTTT |
| pCX-B171tccP-R1 | CCGGAATTCATGATAAATAGCATTAATTCTTTT |

### Table 3. *E. coli* strains and plasmids

| Strain or plasmid | Description | Source |
|-------------------|-------------|--------|
| **Strains** | | |
| EDL933 | EHEC O157 : H7 \( stx^- \) | ATCC* |
| E2348/69 | Wild-type EPEC 1 O127 : H6 | Levine et al. (1978) |
| ICC192 | \( \Delta escN \) : Km in *E. coli* O127 : H6 E2348/69 | Garmentia et al. (2004) |
| ICC199 | Human clinical isolate *E. coli* O119 : H6 \( tccP^+ \) (non-1 non-2 EPEC) | Whale et al. (2006) |
| ICC215 | Human clinical isolate *E. coli* O111 : H2 \( tccP^+ \) (EPEC 2) | This study |
| B171 | Wild-type EPEC 2 O111 : H– \( tccP^+ \) | Riley et al. (1987) |
| ICC216 | \( \Delta tccP \) : Km in *E. coli* O111 : H– strain B171 | This study |
| ICC185 | \( \Delta tccP \) : Km in *E. coli* O115 : H7 EDL933 | Garmentia et al. (2004) |
| **Plasmids** | | |
| pCX340 | pBR322 derivative used to generate \( blaM \) gene fusions | Charpentier & Oswald (2004) |
| pICCC364 | pCX340 derivative encoding TccP2\(_{B171}\) fused to TEM-1 | This study |
| pKD46 | Helper plasmid | Datsenko & Wanner (2000) |
| pSA10 | pKK177-3 derivative containing lac\(^E\) | Schlösser-Silverman et al. (2000) |
| pICCC281 | pSA10 derivative encoding TccP\(_{O157}–\)FLAG fusion protein | Garmentia et al. (2004) |
| pICCC365 | pSA10 derivative encoding TccP2\(_{B171}–\)HA | This study |
| pICCC366 | pSA10 derivative encoding TccP2\(_{ICC215}–\)HA | This study |

*American Type Culture Collection.*
Effectors protein translocation assay. Overnight EPEC cultures were diluted 1:100 into 5 ml DMEM containing 10% FCS and 2 mM glutamine, and incubated at 37 °C in 5% CO2 for 3.5 h (preactivation). HeLa cells were infected with the preactivated bacteria for 30 min before IPTG was added at a final concentration of 1 mM, and the infection was allowed to proceed for an additional 1 h. Cell monolayers were washed three times with PBS, and covered with 100 μl PBS and 25 μl 6× concentrated β-lactamase substrate CCF2/AM (Charpentier and Oswald, 2004). The cells were incubated for 2.5 h at room temperature, washed three times with PBS, covered with coverslides, and live cells were observed under a Nikon Eclipse E600 fluorescence microscope using a UV-2A filter set (330-380 nm excitation). Pictures were taken under a Nikon digital camera DXM1200.

Human intestinal in vitro organ culture (IVOC). Tissue was obtained with fully informed parental consent and local ethical committee approval, using grasp forceps during routine endoscopic investigation of intestinal disorders. Small intestinal mucosal biopsies, which appeared macroscopically normal, were taken for organ culture experiments as described by Hicks et al. (1998). Adherence of strains B171 and B171tccP2 was examined using tissue from two patients (aged 159 and 181 months) for scanning electron microscopy, and two further cases (aged 194 and 195 months) for cryosectioning and immunostaining. In each experiment, a non-infected sample was included to exclude endogenous bacterial adhesion. Samples for scanning electron microscopy were processed as described by Hicks et al. (1998). For immunofluorescence, samples were embedded in OCT compound (Sakura), snap-frozen in liquid nitrogen, and stored at -70 °C until use. Serial sections of 8 μm were cut with an MTE cryostat (SLEE Technik), picked up on poly-L-lysine-coated slides, and air-dried. Tissue sections were fixed in formalin for 10 min, and blocked with 0.5% BSA and 2% normal goat serum in PBS for 20 min at room temperature. Slides were incubated in primary antibody (rabbit anti-Nck, rabbit anti-TccP) for 60 min at room temperature, and washed and incubated in Alexa Fluor 488-conjugated goat anti-rabbit IgG (Molecular Probes) for 30 min. Counterstaining of bacteria and cell nuclei was performed using propidium iodide (Sigma). Epithelial cells were stained with mouse anti-cytokeratin (Dako) and Alexa Fluor 647-conjugated goat antimouse IgG (Molecular Probes). Sections were analysed with a Radiance 2100 confocal laser scanning microscope equipped with an argon–krypton laser and a red diode (Bio-Rad).

**RESULTS**

**tccP2 is absent from EPEC 1 and associated with the EPEC 2 lineage**

We employed conventional PCR to amplify tccP2 (Fig. 1a) using DNA from typical EPEC isolates as a template and gene-specific primers. tccP2 was found in 26 of 27 (96.2%) strains belonging to EPEC 2, but in none of the 34 EPEC 1 isolates (Table 1) and O119 : H6, which is evolutionarily distinct from EPEC 1 and EPEC 2 (Whittam & McGraw, 1996), and which we have previously shown encodes biologically active TccP (Whale et al., 2006). The tccP2 amplicons varied in length from 700 to 1800 bp (Table 1). We further confirmed the absence of tccP or tccP2 in 14 randomly chosen, PCR-negative isolates by colony blot hybridization (data not shown).

Locus-specific PCR was used to amplify and sequence the tccP2 locus of representative strains, confirming the presence of an intact ORF. Amino acid sequence alignment of the TccP2 polypeptides revealed that other than differences in the number of PRRs, which ranged from three to 10, the proteins shared a high level of sequence similarity (Fig. 1b). Moreover, the PRRs of TccP2 overlapped almost exactly with those of TccP of EHEC O157 : H7 and EPEC O119 : H6 (Fig. 1b).

TccP2 is a translocated effector protein

Antiserum raised against TccP is cross-reactive with TccP2 due to the sequence identity of the PRRs. Using the antiserum to analyse whole-cell extracts of tccP-negative/ tccP2-positive EPEC 2 lineage strains B171 (O111 : H−) and ICC215 (O111 : H2), and tccP-positive/tccP2-negative strains EHEC EDL933 (O157 : H7) and EPEC ‘non-1 non-2 lineage’ ICC199 (O119 : H6) as controls, revealed reactive bands of different sizes that correlated with differences in the number of PRRs; no band was detected in the tccP-negative/tccP2-negative EPEC 1 strain E2348/69 (O127 : H6) or in EDL933 ATccP (Fig. 2a). These results show that tccP2 is expressed in EPEC 2 strains.

TccP2 of EPEC 2 strain B171 is 77% identical (87% similar) to TccP of EHEC O157 : H7. However, while the PRRs were nearly identical, the N termini showed only 40% identity (Fig. 1b). Since this region of TccP contains the critical translocation signal (Garmendia et al., 2006), we used the TEM-1 β-lactamase-based translocation assay (Charpentier & Oswald, 2004) to determine if TccP2_B171 was translocated into the host cell. Translocation was detected directly within living host cells by using the fluorescent β-lactamase substrate CCF2/AM. HeLa cells were infected with wild-type and T3SS-deficient ΔescN mutant EPEC strains carrying pICC364, a plasmid that encodes a translational fusion of TccP2_B171 to TEM-1. Expression of the fusion protein in these strains was verified by Western blot (data not shown), and translocation of the protein into infected HeLa cells was analysed (Fig. 2b). Uninfected HeLa cells or cells infected with E2348/69(pCX340) (negative control, empty vector) appeared green, indicating the absence of TEM-1 activity (Fig. 2b). Cells infected with E2348/69(pICC364) expressing TccP2_B171–TEM-1 appeared blue (Fig. 2b), indicating that TEM-1 was translocated into the host cells. Moreover, this translocation was fully dependent on a functional T3SS, given that it was not observed when HeLa cells were infected with E2348/69ΔescN (pICC364) (Fig. 2b,
These results show that TccP2 is an effector protein translocated into host cells by the LEE-encoded T3SS. B171 triggers Nck-independent actin polymerization.

Activation of the phospho-Tir [Tir(P)]–Nck actin-remodelling pathway is necessary for induction of actin-rich pedestals during infection with EPEC 1 strain E2348/69 (O127 : H7) (Campellone et al., 2004b). In contrast, Nck is not recruited to the site of bacterial adhesion during infection with EHEC O157 : H7 (strain Sakai) and EPEC O119 : H6 (strain ICO198), and TccP2 of EPEC O111 : H- (strain B171) and EPEC O111 : H2 (strains CB03454, CB03447 and CB07077). The unique N terminus is shaded grey and the PRRs are shaded black. The complete unit of a PRR and a partial C-terminal repeat are indicated by arrows and a dashed arrow, respectively. The TccP sequence of ICC199 was taken from Whale et al. (2006) (accession no. DQ206456), and that for TccP2 of B171 from its unfinished genome sequence (AAJX01000441). Note that the tccP2 gene is not annotated in the B171 genome sequence.

B171 triggers Nck-independent actin polymerization

Activation of the phospho-Tir [Tir(P)]–Nck actin-remodelling pathway is necessary for induction of actin-rich pedestals during infection with EPEC 1 strain E2348/69 (O127 : H7) (Campellone et al., 2004b). In contrast, Nck is not recruited to the site of bacterial adhesion during infection with EHEC O157 : H7 (EDL933) (Gruenheid et al., 2001), at which Tir and TccP are necessary for A/E lesion formation (Campellone et al., 2004a; Garmendia et al., 2004). In order to characterize TccP2-positive EPEC 2, HeLa cells were infected with strains B171 and ICC215. As controls, HeLa cells were infected with strains E2348/69 and EDL933. Immunostaining revealed tyrosine phosphorylation (a signal that previous studies have shown to correspond to Tir(Y-P); Kenny et al., 1997) below adherent E2348/69, B171 and ICC215, but not EDL933 (Fig. 3a). Accordingly, we compared the ability of EPEC 2, EPEC 1 and EHEC O157 : H7 to induce actin-pedestal assembly during infection of Nck1−/Nck2− and Nck1+/Nck2+ fibroblasts.
Phalloidin staining and quantification of the efficiency of pedestal formation revealed that expression of tccP2 in E2348/69 significantly enhanced its ability to trigger actin polymerization in Nck1−/−Nck2−/− cells (Fig. 4a), suggesting that translocated TccP2 is able to promote host-cell actin polymerization under adherent E2348/69 in the absence of a functional Tir(P)−Nck actin-remodelling pathway.

In order to determine the role of tccP2 in B171-induced A/E lesions, a non-polar deletion of tccP2 was generated, producing strain B171ΔtccP2 (ICC216). Infection of HeLa cells revealed that B171ΔtccP2 induced actin polymerization under attached bacteria in a similar manner to wild-type B171 (Fig. 4b), despite exhibiting reduced cell adherence (B171ΔtccP2 adhered to 48±13 % of cells; in comparison, wild-type B171 adhered to 99.5±1 % of cells). However, B171ΔtccP2 was unable to trigger actin polymerization beneath adherent bacteria during infection of Nck1−/−Nck2− fibroblasts (Fig. 4c). Similar to published observations regarding inefficiency of binding and effector translocation into Nck-deficient MEF cell lines (Campellone et al., 2004a), B171ΔtccP2 interacted with Nck-deficient fibroblasts at levels too low to allow strict quantification of pedestal formation. The ability of B171ΔtccP2 to trigger actin accretion was completely restored by introduction of plasmids encoding TccPEDL933 (pICC281) (Fig. 4c) and TccP2−B171 (pICC365) (data not shown). These results suggest that B171, similar to EHEC O157, is able to trigger localized actin polymerization in an Nck-independent and TccP-dependent manner. Significantly though, B171 is different from EHEC O157, as it can also trigger actin polymerization via a TccP-independent mechanism.

In order to determine whether both the Nck pathway and the TccP pathway are activated at the site of bacterial attachment, pICC365 encoding C-terminal HA-tagged TccP2−B171 was introduced into B171. Co-immunostaining with HA and Nck antibodies revealed that Nck and TccP2 were simultaneously concentrated in actin-rich pedestals beneath adherent bacteria (Fig. 5a), indicating that B171 has the ability to simultaneously utilize the Nck- and TccP2-mediated actin-remodelling pathways. To confirm that TccP2 functions upstream of N-WASP in the actin-polymerization cascade, we infected an N-WASP-deficient fibroblast cell line (N-WASP−/− MEFs with
B171ΔtccP2(pICC365) (expressing HA-tagged TccP2). Co-immunostaining of infected cells with TirEPEC antiserum and HA antibodies revealed that both Tir and TccP2-HA were recruited to the site of bacterial attachment in the absence of N-WASP (Fig. 5b). As expected, no TccP-HA was detected beneath B171ΔtccP2 (data not shown), and neither strain was able to trigger formation of actin pedestals on N-WASP+/−/− MEFs. Infection and immunostaining of control N-WASP-proficient cells (N-WASP+/−/− MEFs) revealed that Tir and TccP2 were recruited to sites of adherent B171ΔtccP2 expressing HA-tagged TccP2, but crucially, actin pedestals were triggered. Taken together, these data show that TccP2 is recruited in the absence of N-WASP, and suggest that N-WASP is not required for the indirect interaction between Tir and TccP2, and that N-WASP is a critical factor in the B171-induced actin-polymerization cascade.

**Recruitment of TccP2 and Nck during infection of human intestinal biopsy samples with B171**

To investigate the role of TccP2 during IVOC, paediatric small intestinal biopsy samples were infected with strain B171 and its isogenic tccP2 mutant (B171ΔtccP2). As shown in Fig. 6, both wild-type and deletion mutant attached intimately to human intestinal mucosa, causing microvillus elongation in between adhering bacteria. Immunofluorescence staining of cryosectioned organ-culture samples showed that TccP2 was translocated into human intestinal epithelium and localized beneath adherent B171 bacteria. The host adaptor protein Nck was recruited by both wild-type and B171ΔtccP2 bacteria; in contrast, and as expected, no TccP2 staining was observed in B171ΔtccP2-infected samples (Fig. 7).

**tccP and tccP2 are functionally interchangeable**

Considering that TccP ICC199 was 77% identical to TccP ICC215 and that TccP EDL933 complemented B171ΔtccP2 (Fig. 4c), we carried out a reciprocal experiment to determine the ability of TccP2 to complement an EDL933ΔtccP mutant strain. To this end, tccP2 from strains B171 (consisting of four PRRs) and ICC215 (consisting of six PRRs), cloned under the control of an IPTG-inducible promoter and tagged with a C-terminal HA epitope (pICC365 and pICC366, respectively), was introduced into...
strain ICC185 (EDL933ΔtccP). The ability to complement the tccP mutation and induce actin-pedestal assembly during infection was analysed by immunofluorescence. Phalloidin staining revealed that tccP<sup>2</sup>_B171 and tccP<sup>2</sup>_ICC215 complemented the ability of EDL933ΔtccP to generate A/E lesions following infection of HeLa cells (Fig. 8). Co-staining of infected HeLa cells with an anti-HA mAb and phalloidin revealed that TccP<sup>2</sup>_B171 was detected beneath ICC185(pICC365) and ICC185(pICC366) bacteria, co-localizing with F-actin at the tip of the pedestals (Fig. 8). In contrast, introduction of a plasmid-borne copy of Z1385 (pseudo tccP<sup>2</sup> allele of EDL933) did not restore the ability of ICC185 to induce actin pedestals (data not shown). These data indicate that tccP<sup>2</sup> encodes a protein that can functionally substitute for TccP<sub>EDL933</sub>, and that both tccP homologues, tccP and tccP<sup>2</sup>, are functionally interchangeable.

**DISCUSSION**

Until recently, the prevalent dogma concerning EPEC- and EHEC-triggered localized actin polymerization, based on studies of two prototypical strains (O127 : H6 EPEC 1 strain E2348/69 and O157 : H7 EHEC strain EDL933), was that formation of actin-rich pedestals is achieved via distinct signal-transduction pathways. A C-terminal 12 aa motif (including phosphorylated Y474) of TirEPEC binds Nck, which in turn recruits and activates N-WASP beneath adherent bacteria (Campellone et al., 2004b). N-WASP then recruits the Arp2/3 complex, leading to the generation of a network of actin filaments under attached bacteria. In contrast, a different C-terminal 12 aa motif of TirEHEC<sub>O157</sub> (encompassing Y458) (Campellone et al., 2006; Allen-Vercoe et al., 2006) clusters TccP, which leads to the formation of actin-rich pedestals by an Nck-independent mechanism (Gruenheid et al., 2001). However, through the analysis of a large number of clinical and environmental non-O157 EHEC and EPEC isolates, we have identified a subset of strains that have the potential to induce actin polymerization in the host eukaryotic cell by simultaneously utilizing the Tir(P)–Nck and Tir–TccP pathways (Garmendia et al., 2005). The predominant EPEC group in this category are strains belonging to EPEC serotype O119 : H6 (Whale et al., 2006), which is situated in the evolutionary tree in between the EPEC 1 and EPEC 2 lineages (Whittam & McGraw, 1996).
EPEC 1 strains are characterized by expression of flagellar antigens H6 or H34 (Whittam et al., 1993), possession of a complete tra region (Brinkley et al., 2006), and intimin α. In this study, we have shown that other characteristics of EPEC 1 strains are expression of Tir(Y-P) and lack of tccP and tccP2. Importantly, strains belonging to O119 : H6 are unique, as they do not belong to EPEC 1 (Whittam & McGraw, 1996), express intimin type β (Adu-Bobie et al., 1998), almost harbour tccP (Garmendia et al., 2005), and may have a complete tra region (Brinkley et al., 2006). These characteristics suggest that the evolution of this serotype followed a distinct path, through which it acquired virulence determinants horizontally. The EPEC 2 lineage is characterized by expression of flagellar antigens H2 or H2 (Whittam et al., 1993), intimin β (Adu-Bobie et al., 1998) and Tir(Y-P). Unexpectedly, we found that with the exception of only one isolate, all of the EPEC 2 strains tested contained intact tccP2. Sequence analysis of TccP2 from different isolates showed that, other than variation in the number of PRRs, the protein sequences were identical.

Fig. 5. (a) Nck and TccP2 were simultaneously recruited and co-localized at the site of strain B171(pICC365-tccP2_B171)-induced actin assembly beneath adherent bacteria during infection of HeLa cells. Nck was labelled in green using an anti-Nck antibody, TccP2–HA was labelled in far red with an anti-HA mAb, and actin was labelled in red, using rhodamine-conjugated phalloidin. Bacteria and cell nuclei were visualized with Hoechst stain (blue). Separate monochrome images of the UV, far-red, red and green fluorescence channels are shown, as well as merged images of all channels (right column). Bar, 10 µm. (b) Tir and TccP2 co-localized at the site of B171ΔtccP2(pICC365-tccP2_B171) attachment during infection of N-WASP+/− and N-WASP+−/− fibroblasts. However, induced actin assembly was only detected beneath adherent bacteria during infection of N-WASP+/− fibroblasts. Tir was labelled in red, TccP2–HA was labelled in green, and actin was labelled in far red (shown in blue). Bacteria were visualized with Hoechst stain (shown in monochrome).

Fig. 6. Both wild-type and ΔtccP2 B171 strains induced A/E lesions in intestinal IVOC. Scanning electron micrographs of duodenal mucosa infected with B171 and B171ΔtccP2 are shown. A non-infected sample was included as a negative control. Bars, 5 µm.
The identification of TccP2 in prototypic EPEC strain B171 highlights the fortuitous nature of studying pathogenesis in prototypical strains, as the commonly used E2348/69, which is tccP- and tccP2-negative, allowed the identification of the role of Nck in A/E lesion formation.

Using B171 as a representative of EPEC 2 tccP2-expressing strains, we have shown that TccP2 is a T3SS-translocated effector involved in triggering actin remodelling during infection. In a similar manner to tccP-positive EPEC and EHEC strains, but in contrast to prototypical EPEC 1 strains, B171 was able to efficiently trigger Nck-independent actin polymerization, an activity dependent on tccP2. Due to the high level of sequence conservation between TccP and TccP2, we observed functional redundancy between the two TccP homologues: TccP2 was able to restore actin-polymerization ability to EDL933 during infection of HeLa cells, and TccP was able to complement B171ΔtccP2 for triggering actin remodelling during infection of Nck1−/Nck2− fibroblasts. Nevertheless, due to the difference between the N termini of TccP and TccP2, we cannot exclude the possibility of subtle functional differences.

In a similar way to TccP-positive EPEC strains, TccP2 is localized at the tip of the pedestal and co-localizes with Nck during B171 infection of epithelial cells. Of note, TifB171 harbours both a Y474 equivalent in the context of a consensus Nck binding site, and a second tyrosine residue within a region that shares 75% amino acid identity with a motif responsible for TccP/EspFU recruitment in TifEHEC (Campellone et al. 2006). Moreover, both TccP2 and Nck are recruited to the site of B171 adhesion to human intestinal IVOC. In the absence of TccP2 (i.e. during infection with B171ΔtccP2), Nck was still recruited,
suggested that recruitment of Nck, bacterial adhesion and A/E lesion formation are not dependent on TccP2. A model describing the actin-polymerization cascades induced by EPEC 1, EPEC 2 and ‘non-1 non-2’ EPEC O119 : H6 is shown in Fig. 9. The ability of strains belonging to EPEC 2 to trigger actin polymerization using apparently redundant mechanisms raises intriguing questions regarding the possible spatial and temporal specificities of their function.

We have shown that possession of \textit{tccP2} and \textit{Tir(Y-P)} is a characteristic of EPEC 2. This observation raises some interesting questions, including whether or not the ability to use both the Nck and TccP2 pathways confers an advantage upon EPEC 2, and if so, in which environments EPEC 2 strains are more virulent than EPEC 1 strains. Finally, strain E2348/69 (O127 : H7), which has been used worldwide as a prototype EPEC strain, harbours \textit{Tir(Y-P)} but is \textit{tccP} negative. As such, when studying actin-pedestal formation, E2348/69 should no longer be used as a general representative of typical EPEC; it should now only be considered representative of EPEC 1, while B171 should be used as a prototype strain representing the EPEC 2 lineage.

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**Fig. 9.** Summary of actin-polymerization cascades induced by EPEC 1, EPEC 2 and EPEC O119 : H6. All EPEC lineages translocate Tir that once inserted into the host-cell membrane serves as a receptor for the bacterial adhesin intimin. Intimin-mediated clustering of Tir triggers phosphorylation of tyrosine residue Y474 and concurrent recruitment of Nck. Nck recruits and activates N-WASP, leading to Arp2/3-dependent actin polymerization and pedestal formation. EPEC 2 and ‘non-1 non-2 lineage’ O119 : H6 EPEC, but not EPEC 1 bacteria, also translocate TccP2 and TccP, respectively, and are capable of recruiting N-WASP directly and triggering Nck-independent actin-pedestal formation. Note that TccP2 does not bind Tir directly, but via an unidentified host-encoded adaptor (indicated by a question mark).
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