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

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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 carries functional tccP and tccP2 alleles. Interestingly, intact tccP2 has been identified in the incomplete genome sequence of the prototype EPEC strain B171 (serotype O111 : H

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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 diarrhoea 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 enterocyte effacement (LEE) pathogenicity island (McDaniel et al., 1995), and the EPEC adherence factor (EAF) plasmid (Baldini et al., 2006), which carries the transcriptional regulator locus per (Mellies et al., 1999), and encodes the type IV bundle-forming pilus (BFP) (Girón et al., 1991). The most common O serogroups of typical EPEC are O55, O111, O119, O127 and O142. Typical EPEC are further 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 Verocytotoxigenic (VT)-producing E. coli (VTEC) that can cause bloody diarrhoea, haemorrhagic colitis and haemolytic-uremic 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 (YbPDEP/DV) (Campellone et al., 2002; Gruenheid et al., 2001). Tyrosine phosphorylation of Tir [Tir(Y-P)] 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 in 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 EspF, 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 in 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 in 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(Y-P)] 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 tccP2 prophage ECs1126, 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 tccP with gene-specific tccP-F and tccP-R primers. Forward, gene-specific primers tirY74-F and tirS478-F were used together with a conserved reverse primer (tir-R) to discriminate between tfrE324868 and tfrS6gene 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 taq 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 gene sequencer. When necessary, internal sequencing primers were used.

Preparation of TccP rabbit antiserum. A PCR fragment encoding a truncated TccP2_1935 derivative comprising the unique N terminus and two PRRs (TccP2_N2R) was cloned into pET28-a as described previously (Ogura et al., 2007); TccP2R-His was purified as described by Hartland et al. (1999), and polyclonal TccP2R-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-denaturing 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 B1711tccP2 mutant was constructed using the λ Red recombinase method (Datsenko & Wanner, 2000). Disruption of tccP2 was performed with a kanamycin-resistance cassette generated with primers B171tccP-F1 and B171tccP-R1 using pKD4 as template. Purified PCR product was electroporated into E. coli B171 (pKD46). Clones were grown on LB medium + kanamycin to select for kanamycin (AprccP2) resistance. pKD46 was cured from the resulting strains by growth at 43 °C. Primers flanking the deleted region and inside the kanamycin-resistance cassette were used in PCR to verify the deletion (primer pairs K1 and tccP2-F, and K2 and tccP2-R).

pLCC364 is a derivative of pCX340 (Charpentier & Oswald, 2000), an 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 pCX-B171tccP-F1 and pCX-B171tccP-R1. The 915 bp PCR product, containing terminal EcoRI and NotI sites, was digested and ligated into pCX340, generating pLCC364.

pLCC365 and pLCC366 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, were digested and ligated into pSA10, generating pLCC365 and pLCC366, 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 Int280f antisera (Adu-Bobie et al., 1998), and EPEC E2348/69 was detected with rabbit polyclonal Int280s antisera (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 phallolidin (Invitrogen) were used at dilutions of 1 : 500, 1 : 100 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

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

<table>
<thead>
<tr>
<th>Serotype</th>
<th>No. of strains</th>
<th>tccP2</th>
<th>Tir type</th>
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<tbody>
<tr>
<td><strong>EPEC 1</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>O55 : H6</td>
<td>10</td>
<td>−</td>
<td>Y-P (5)*, ND (5)</td>
</tr>
<tr>
<td>O86 : H34</td>
<td>4</td>
<td>−</td>
<td>Y-P (3), ND (1)</td>
</tr>
<tr>
<td>O127 : H6</td>
<td>5</td>
<td>−</td>
<td>Y-P (3), ND (2)</td>
</tr>
<tr>
<td>O142 : H6</td>
<td>6</td>
<td>−</td>
<td>Y-P (4), ND (2)</td>
</tr>
<tr>
<td>O142 : H34</td>
<td>3</td>
<td>−</td>
<td>Y-P</td>
</tr>
<tr>
<td><strong>EPEC 2</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>O111 : H1−</td>
<td>8</td>
<td>[1150 bp] [+] (6)*, [700 bp] (1), − (1)</td>
<td>Y-P</td>
</tr>
<tr>
<td>O111 : H2</td>
<td>18</td>
<td>[1150 bp] (14), [1000 bp] (3), [1800 bp] (1)</td>
<td>Y-P</td>
</tr>
<tr>
<td>O114 : H2</td>
<td>1</td>
<td>[1150 bp]</td>
<td>Y-P</td>
</tr>
<tr>
<td><strong>EPEC non-1 non-2</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>O119 : H6‡</td>
<td>6</td>
<td>−</td>
<td>Y-P</td>
</tr>
</tbody>
</table>

*The number of strains is given in parentheses.
†The size of tccP2 amplicons is indicated in brackets.
‡tccP-positive strain.
Table 2. Primers used in this study

<table>
<thead>
<tr>
<th>Primer</th>
<th>Nucleotide sequence (5’–3’)</th>
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<tr>
<td>tccP2-F</td>
<td>ATGATAAATAGCATTAATTCTTT</td>
</tr>
<tr>
<td>tccP2-R</td>
<td>TCACGAGCCTTAGATGTTAAT</td>
</tr>
<tr>
<td>tirY474-F</td>
<td>CATATTATGAGTAGCTGGCTC</td>
</tr>
<tr>
<td>tirS478-F</td>
<td>TCCTTCAGAATTGAGAATA</td>
</tr>
<tr>
<td>tir-R</td>
<td>TAAAAGTCAGATCTTGATGACAT</td>
</tr>
<tr>
<td>tccP2-SFb</td>
<td>GGTAGATTTTCTGCAAAGG</td>
</tr>
<tr>
<td>tccP2-SRb</td>
<td>AATAACCGTAACTGTCAGGGTC</td>
</tr>
<tr>
<td>B171tccP-F1</td>
<td>CACAGCAAAAAAGCAGCCTAAGACGGTAAAAACGCTACCTTCCTTC-</td>
</tr>
<tr>
<td>B171tccP-R1</td>
<td>GAGGGCTTGTATGCTCATTTTTTGTACTGGCGGCGTTGGCGAGGCGCACTTT-</td>
</tr>
<tr>
<td>k1</td>
<td>CAGTCTAGATCCGATAGCC</td>
</tr>
<tr>
<td>k2</td>
<td>GGTTGCCGGCTGAATGACGGC</td>
</tr>
<tr>
<td>pkk-tccP2-F1</td>
<td>CCGGAATTCATGATAATAGCATTTCTTTAATCTCTCTTAG</td>
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<tr>
<td>pkk-tccP2-R1</td>
<td>AAAACTGCAGTCAAGCGTAGTCTGGGACCTGTATGCTTAGAAGGCT-</td>
</tr>
<tr>
<td>pCX-B171tccP-F1</td>
<td>GGTTTCTATAGTAAATAGCATTAAATCTCTTT</td>
</tr>
<tr>
<td>pCX-B171tccP-R1</td>
<td>CCGGAATTCTCGGACCGCTTGAGAATAGTCATTTAGG</td>
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Table 3. E. coli strains and plasmids

<table>
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<th>Strain or plasmid</th>
<th>Description</th>
<th>Source</th>
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<tr>
<td>Strains</td>
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<tr>
<td>EDL933</td>
<td>EHEC O157 : H7 stx&lt;sup&gt;*&lt;/sup&gt;</td>
<td>ATCC*</td>
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<tr>
<td>E2348/69</td>
<td>Wild-type EPEC 1 O127 : H6</td>
<td>Levine et al. (1978)</td>
</tr>
<tr>
<td>ICC199</td>
<td>Human clinical isolate E. coli O119 : H6 tccP&lt;sup&gt;+&lt;/sup&gt; (non-1 non-2 EPEC)</td>
<td>Whale et al. (2006)</td>
</tr>
<tr>
<td>ICC215</td>
<td>Human clinical isolate E. coli O111 : H2 tccP&lt;sup&gt;+&lt;/sup&gt; (EPEC 2)</td>
<td>This study</td>
</tr>
<tr>
<td>B171</td>
<td>Wild-type EPEC 2 O111 : H– tccP&lt;sup&gt;–&lt;/sup&gt;</td>
<td>Riley et al. (1987)</td>
</tr>
<tr>
<td>ICC216</td>
<td>ΔtccP2 : Km in E. coli O111 : H– strain B171</td>
<td>This study</td>
</tr>
<tr>
<td>ICC185</td>
<td>ΔtccP : Km in E. coli O157 : H7 EDL933</td>
<td>Garmendia et al. (2004)</td>
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<td>Plasmids</td>
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<tr>
<td>pCX340</td>
<td>pBR322 derivative used to generate blaM gene fusions</td>
<td>Charpentier &amp; Oswald (2004)</td>
</tr>
<tr>
<td>pICC364</td>
<td>pCX340 derivative encoding TccP&lt;sub&gt;2,B171&lt;/sub&gt; fused to TEM-1</td>
<td>This study</td>
</tr>
<tr>
<td>pKD46</td>
<td>Helper plasmid</td>
<td>Datsenko &amp; Wanner (2000)</td>
</tr>
<tr>
<td>pSA10</td>
<td>pKK177-3 derivative containing lacI&lt;sup&gt;*&lt;/sup&gt;</td>
<td>Schlösser-Silverman et al. (2000)</td>
</tr>
<tr>
<td>pICC281</td>
<td>pSA10 derivative encoding TccP&lt;sub&gt;2,0157–FLAG&lt;/sub&gt; fusion protein</td>
<td>Garmendia et al. (2004)</td>
</tr>
<tr>
<td>pICC365</td>
<td>pSA10 derivative encoding TccP&lt;sub&gt;2,B171&lt;/sub&gt;–HA</td>
<td>This study</td>
</tr>
<tr>
<td>pICC366</td>
<td>pSA10 derivative encoding TccP&lt;sub&gt;2,ICC215&lt;/sub&gt;–HA</td>
<td>This study</td>
</tr>
</tbody>
</table>

*American Type Culture Collection.
Mechanism of actin polymerization of EPEC lineage 2

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/TccP (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 TccP2B171 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 TccP2B171 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, i). Cells infected with E2348/69(pIC364) expressing TccP2B171−TEM-1 appeared blue (Fig. 2b, ii), 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 (pIC364) (Fig. 2b, ii).

**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
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 (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). Note that the tccP2 gene is not annotated in the B171 genome sequence.
Mechanism of actin polymerization of EPEC lineage 2

Fig. 2. (a) TccP was detected with TccP antiserum in bacterial whole-cell lysates of EHEC EDL933 and EPEC ICC199, but not EDL933 ΔtccP or EPEC E2348/69. TccP2 was also detected using TccP antiserum in lysates of EPEC O111 : H2 strain ICC215 and EPEC O111 : NM strain B171. (b) TccP2 is a T3SS-translocated effector. Translocation of the EPEC B171 effector protein TccP2 into live HeLa cells using TEM-1 fusion and fluorescence microscopy is shown. HeLa cells were infected with wild-type EPEC E2348/69 carrying pCX340 (negative control) (i), and E2384/69 (ii) and E2384/69 ΔesecN (iii) strains expressing TccP2-B171-TEM fusion protein. β-Lactamase activity in HeLa cells was revealed by the blue fluorescence emitted by the cleaved CCF2 product (cells infected with E2348/69 expressing TccP2-TEM), whereas CCF2 emitted a green fluorescence (cells infected with ΔesecN mutant expressing TccP2-TEM).

Phalloidin staining and quantification of the efficiency of pedestal formation (see Methods) revealed that EDL933 was able to induce actin accretion during infection of an Nck1−/Nck2− fibroblast cell line, at a similar efficiency to infection of a control Nck1−/Nck2− fibroblast cell line (Fig. 3b). In agreement with Campellone & Leong, 2005, strain E2348/69 induced less intense actin accretion at significantly reduced frequency in Nck1−/Nck2− fibroblast cells in comparison to Nck1−/Nck2+ cells (P<0.05), when preactivated by static growth in tissue-culture medium and 5% CO2 (Fig. 3b). The residual and inefficient ability of EPEC E2348/69 to trigger actin polymerization is likely to be due to a recently identified Nck-independent pathway mediated by unknown cellular or bacterial factors (Campellone & Leong, 2005). In contrast, in a similar manner to TccP-expressing EPEC O119 : H6 strain ICC199 and EHEC O157 : H7 strain EDL933, TccP2-expressing B171 was able to trigger actin polymerization during infection of an Nck1−/Nck2− fibroblast cell line, at a similar efficiency as during infection of an Nck1−/Nck2+ fibroblast cell line (Fig. 3b). Thus, it appears that B171 is able to utilize an Nck-independent pathway to efficiently induce actin polymerization upon infection.

Functional analysis of TccP2

In order to elucidate the function of TccP2, we carried out infections of Nck1−/Nck2− fibroblast cell lines with EPEC 1 E2348/69 expressing tccP2_B171 (pICC365). Phalloidin staining and quantification of 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 B171 ΔtccP2 (ICC216). Infection of HeLa cells revealed that B171 ΔtccP2 induced actin polymerization under attached bacteria in a similar manner to wild-type B171 (Fig. 4b), despite exhibiting reduced cell adherence (B171 ΔtccP2 adhered to 48±13% of cells; in comparison, wild-type B171 adhered to 99.5±1% of cells). However, B171 ΔtccP2 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), B171 ΔtccP2 interacted with Nck-deficient fibroblasts at levels too low to allow strict quantification of pedestal formation. The ability of B171 ΔtccP2 to trigger actin accretion was completely restored by introduction of plasmids encoding TccP EDL933 (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, but 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+/+) 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 B171 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 (EDL933 ΔtccP). The ability to complement the tccP mutation and induce actin-pedestal assembly during infection was analysed by immunofluorescence. Phalloidin staining revealed that tccP2_B171 and tccP2_ICC215 complemented the ability of EDL933 ΔtccP 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 TccP2–HA 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 tccP2 allele of EDL933) did not restore the ability of ICC185 to induce actin pedestals (data not shown). These data indicate that tccP2 encodes a protein that can functionally substitute for TccP_EDL933, and that both tccP homologues, tccP and tccP2, 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 E2348/69(pICC365-tccP2_B171)), 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_O157 (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 B171 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, TIRB171 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 TirEHEC (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.

Fig. 7. Nck was recruited to the site of IVOC adhesion of strains B171 and B171ΔtccP2, while TccP2 was found only under adherent B171. Terminal ileal mucosa was infected with B171 and B171ΔtccP2 for 8 h, and cryosections were processed for immunofluorescence. Staining was performed for TccP2 (green in merged image, upper two panels) or Nck (green, lower two panels). Bacteria and cell nuclei were visualized by propidium iodide (PI) stain (red). Epithelial cells were counterstained with anti-cytokeratin (labelled blue). Separate monochrome images of the red and green fluorescence channels (left and middle column respectively) are shown, as well as merged images of all channels (right column).

Fig. 8. FAS and recruitment of TccP2 to the site of bacterial adhesion. HeLa cells were infected with strain EDL933ΔtccP for 5 h. Following fixation and permeabilization, bacteria were labelled in blue. Actin was detected by rhodamine-phalloidin, and TccP2–HA was labelled in green. Strain ICC185, unable to form actin-rich pedestals during infection, was complemented by both TccP2_B171 (pIC365) and TccP2 ICC215 (pIC366). TccP2 was detected beneath adherent ICC185(pIC365) and ICC185(pIC366), but not ICC185, and co-localized with polymerized actin at the tip of the triggered actin pedestal. Bar, 2 μm.
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