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TccP2 of O157:H7 and Non-O157 Enterohemorrhagic *Escherichia coli* (EHEC): Challenging the Dogma of EHEC-Induced Actin Polymerization▽

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Enterohemorrhagic *Escherichia coli* (EHEC) O157:H7 and enteropathogenic *E. coli* (EPEC) trigger actin polymerization at the site of bacterial adhesion by inducing different signaling pathways. Actin assembly by EPEC requires tyrosine phosphorylation of Tir, which subsequently binds the host adaptor protein Nck. In contrast, $Tir_{EHEC\ O157}$ is not tyrosine phosphorylated and instead of Nck utilizes the bacterially encoded Tir-cytoskeleton coupling protein (TccP)/$EspF_U$, which mimics the function of Nck. *tccP* is carried on prophage CP-933U/Sp14 (TccP). Typical isolates of EHEC O157:H7 harbor a pseudo-*tccP* gene that is carried on prophage CP-933 M/Sp4 (*tccP2*). Here we report that atypical, β-glucuronidase-positive and sorbitol-fermenting, strains of EHEC O157 harbor intact *tccP* and *tccP2* genes, both of which are secreted by the LEE-encoded type III secretion system. Non-O157 EHEC strains, including O26, O103, O111, and O145, are typically *tccP* negative and translocate a Tir protein that encompasses an Nck binding site. Unexpectedly, we found that most clinical non-O157 EHEC isolates carry a functional *tccP2* gene that encodes a secreted protein that can complement an EHEC O157:H7 Δ*tccP* mutant. Using discriminatory, allele-specific PCR, we have demonstrated that over 90% of *tccP2*-positive non-O157 EHEC strains contain a Tir protein that can be tyrosine phosphorylated. These results suggest that the TccP pathway can be used by both O157 and non-O157 EHEC and that non-O157 EHEC can also trigger actin polymerization via the Nck pathway.

Enterohemorrhagic *Escherichia coli* (EHEC) is a subgroup of verocytotoxin (VT)-producing *E. coli* (VTEC) that can cause bloody diarrhea, hemorrhagic colitis, and hemolytic-uremic syndrome (reviewed in references 31 and 32). There are two major types of VT (VT1 and VT2), and VT2 can be subdivided into at least five subtypes (26). *E. coli* O157:H7 is the most common and virulent EHEC serotype, and it is implicated worldwide in human disease (31). Typical strains of EHEC O157:H7 do not ferment sorbitol and lack β-D-glucuronidase activity. However, β-D-glucuronidase-positive and sorbitol-fermenting EHEC O157:H− strains have been implicated in diarrhea and hemolytic-uremic syndrome in Germany and in other European countries (22). Recent epidemiological studies show that there is a steady increase in the isolation of non-O157 EHEC from humans (1, 35) and animals (21, 27), particularly of serogroups O26, O111, and O103. A case control study of risk factors associated with EHEC infection in Argentina revealed that while EHEC O157 was responsible for 60% of cases, EHEC O145 and O121 were found in 14.5% and 1% of the cases, respectively (29).

The hallmark of infections with EHEC and enteropathogenic *E. coli* (EPEC), a major cause of infantile diarrhea in developing countries (8), is the ability of the bacteria to modulate, while remaining extracellular, the cytoskeleton of eukaryotic cells (reviewed in reference 6), which respond to infection by producing elongated actin-rich pedestal-like structures under attached bacteria. Central to this bacterial activity is translocation through the type III secretion system (T3SS) (14) of a bridging molecule, the translocated intimin receptor protein (Tir)/EspE (10, 24), which is inserted into the epithelial cell plasma membrane in a hairpin loop topology (19). The extracellular Tir loop serves as a receptor for the bacterial outer membrane adhesin intimin (reviewed in reference 12), while the intracellular amino and carboxy termini recruit and bind several cytoskeletal and focal adhesion molecules (17).

Studies of actin polymerization by EPEC and EHEC have

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focused in recent years on two prototype strains: EPEC O127:H6 (strain E2348/69) and EHEC O157:H7 (strain EDL933). Although Tir is essential for actin polymerization in both strains (reviewed in reference 4), the prevailing dogma has been that they induce strain-specific signaling pathways. Actin assembly by EPEC E2348/69 requires phosphorylation of tyrosine Y474 of Tir (23), which maturates a binding site for the adaptor protein Nck (3, 18). Recruitment of Nck activates the neuronal Wiskott-Aldrich syndrome protein (N-WASP), triggering localized actin polymerization. In contrast, Tir_{EDL933} lacks a Y474 equivalent, is not tyrosine phosphorylated, and instead of Nck utilizes the T3SS effector protein TccP (Tir-cytoskeleton coupling protein) (15)/$EspF_U$ (5), which mimics Nck in terms of recruitment of N-WASP to the EHEC-induced actin-rich pedestal (6). In a recent study, Campellone et al. (2) identified a C-terminal 12-residue peptide in Tir EHEC O157:H7 that is essential for activation of the $EspF_U$ actin polymerization pathway.

tccP (ECs2715/Z3072) is carried on prophage Sp14/CP-933U. TccP consists of a unique 80-amino-acid N-terminal region (not 87 amino acids, as was previously reported [16]) that is involved in protein translocation (13) and several almost identical proline-rich 47-residue repeats (5, 15). The two sequenced O157:H7 isolates, Sakai (20) and EDL933 (28), contain a *tccP* gene that encodes a unique N terminus and five proline-rich repeats and the beginning of a sixth repeat, but in other clinical and environmental O157:H7 strains the number of repeats varies from 2 to 8 (16); we have shown experimentally that a TccP variant containing the N terminus and two proline-rich repeats is biologically active (13). Recently, while searching by PCR (using primers based on $tccP_{EHEC\ O157:H7}$) for the presence of *tccP* in a large collection of non-O157 EHEC strains, we found this gene in a small percentage of strains belonging to serotypes O26, O111, and O172 and nontypeable strains (NT) (16).

EHEC strains Sakai and EDL933 also contain a pseudo-*tccP* gene (ECs1126 and Z1385, respectively), which is carried on prophage Sp4/CP-933 M. A single-base-pair deletion at position 28 results in a frameshift mutation (15). The aim of this study was to determine the prevalence of ECs1126 among O157 and the most common non-O157 EHEC serotypes.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains used in this study include EHEC O157:H7 Sakai (20), Sakai Δ*sepL* (SKI1204), and Sakai Δ*sepL*/Δ*escR* (SKI1205) and O157:H7 strain EDL933 and its *tccP* deletion mutant (EDL933 Δ*tccP*). Disruption of *tccP2* in EPEC strain O111 was performed by the λ Red recombinase method (9), with a kanamycin resistance cassette generated with primers tccP-F1 (CACAGCACAAAAGCACACCTAACACGGTAAAAACCA GCTCACCTCTTTCTCGTGTAGGCTGGAGCTGCTTC) and tccP-R1 (GAG GTCTTGATTGTTCATTTTGTACTGGCGGCGTTGGCGGAGGCCAGTT ACATATGAATATCCTCCTTAG) and pKD4 used as the template. In order to express intact TccP2 in O157:H7 Sakai, the *tccP2* gene of EHEC O157 strain 980938 was amplified by PCR using the specific primers 5′-GGAGGATCCAT AAGACTATCCAATAAAGCTC-3′ and 5′-AAGAAGCTTCAGGTCAGAGC TAATATAGG-3′ and cloned into the BamHI-HindIII site of pTB101, a pBR322-derived vector carrying the *tac* promoter, $lacI^q$, and ampicillin and trimethoprim resistance genes (34), generating plasmid pTB101-*tccP2*. To overexpress the transcriptional regulator PchA (33), which induces *ler* and hence LEE-gene expression, a DNA fragment corresponding to the *pchA* gene was amplified by PCR from the chromosomal DNA of O157:H7 Sakai with the specific primers 5′-CACAGGAATATATCCGTACCC-3′ and 5′-AGTATGTG TCACTGGCCTATACGG-3′ and cloned into pGEM-T (Promega), generating pGEM-*pchA*. pWKS-*pchA* was constructed by transferring the *pchA*-containing EcoRI fragment of pGEM-*pchA* to pWKS130, a pSC101-derived expression vector carrying a kanamycin resistance gene (36). For complementation of EDL933 Δ*tccP* and O111 Δ*tccP2*, *tccP2* was also amplified from the chromosomal DNA of EHEC O26:H11 by PCR using the specific primers 5′-CCGGAATTC ATGATAAATAGCATTAATTCTTTT-3′ and 5′- AAAACTGCAGTCAAGC GTAGTCTGGGACGTCGTATGGGTAAGCGTAGTCTGGGACGTCGTAT GGGTACGAGCGCTTAGATGTATTAATGCC-3′, which introduce terminal EcoRI and PstI restriction sites and encode a C-terminal hemagglutinin (HA) tag. Digested product was then cloned into pSA10 (30). Clinical EHEC isolates are listed in Tables 1 to 5. The eight O157 strains isolated in Japan (see Table 1) were described previously (25).

PCR amplification of ECs1126/Z1385, *tccP*, and *tir*. Conventional PCR was used to amplify ECs1126 and *tccP*. Forward, gene-specific primers (tccP2-F, 5′-ATGATAAATAGCATTAATTCTTT-3′; tccP-F, 5′-ATGATTAACAATGT TTCTTCACTT-3′) were used together with a conserved reverse primer (5′-TC ACGAGCGCTTAGATGTATTAAT-3′). Forward, gene-specific primers (tirY474-F, 5′-CATATTTATGATGAGGTCGCTC-3′; tirS478-F, 5′-TCTGTT CAGAATATGGGGAATA-3′) were used together with a conserved reverse primer (tir-R, 5′-TAAAAGTTCAGATCTTGATGACAT-3′) to discriminate between $tir_{E2348/68}$ and tir_{Sakai} gene types.

Locus-specific sequencing. The *tccP* and *tccP2* genes and their 5′ and 3′ flanking regions were amplified with an Ex *Taq* PCR amplification kit (Takara Bio, Japan) and the PCR primer pairs tccP-SF (5′-GATGAGTATTGCATCG AGTGTC-3′) and tccP-SR (5′-CGGTAACTGTCAGGTCAGAGC-3′) for *tccP* of O157 strains, tccP2-SFa (5′-AAACGGATAAATAAGACTATCC-3′) and tccP2-SRa (5′-AATAACCGGTAACTGTCAGGTC-3′) for *tccP2* of O157 and most O26 strains, tccP2-SFb (5′-GGTAGATTTCATGCAAACGG-3′) and tccP2-SRb (5′-TTCCGGGGCGGTTCAGGTC-3′) for *tccP2* of O111 strains, tccP2-SFb and tccP2-SRa for *tccP2* of O103 strains, and *tccP2*-SFb and tccP2-SFa for *tccP2* of one O26 strain (strain ED411). Direct sequencing of the PCR products was done with 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 (forward primer 5′-CATGCCATGGTTAACAATGTTTCTTCACTT-3′ and reverse primer 5′-C CGGAATTCGGCAGACGCTGAGCCACATCAGG-3′) encoding a truncated $TccP_{EDL933}$ derivative comprising the unique N terminus and two proline-rich repeats ($TccP_{N2R}$) was cloned into pET28-a as described previously (13); $TccP_{N2R}$-His was purified as described previously (19), and polyclonal $TccP_{N2R}$-His antiserum was produced in rabbits at CovalAb Lyon (France). We confirmed that the TccP antiserum is not cross-reactive with EspF.

Detection of TccP and TccP2 by Western blotting. EHEC strains were cultured overnight at 37°C in LB medium, and 0.15 ml of each culture was inoculated into 15 ml of Dulbecco minimal essential medium (DMEM) and incubated for 7 h at 37°C with shaking. When necessary, IPTG (isopropyl-β-D-thiogalactopyranoside) (at a final concentration of 5 μM) was added to the culture at an optical density at 600 nm (OD_{600}) of 0.4 to 0.5, and cells were further incubated for 4 h. Bacterial cells and culture supernatants were separated by centrifugation at 3,000 × *g* for 10 min at 4°C. The supernatants were then filtrated through a 0.20-μm-pore-size filter (Sartorius AG, Goettingen, Germany) to remove the cells and cell debris and concentrated up to 200 μl with an Amicon Ultra centrifugal filter device (Millipore Corp., Bedford, MA). Cells and concentrated supernatants were each dissolved in sodium dodecyl sulfate (SDS) sample buffer and subjected to SDS-polyacrylamide gel electrophoresis and Western blotting analyses. TccP was detected as described previously (15) with polyclonal rabbit TccP antiserum and alkaline phosphatase-conjugated anti-rabbit immunoglobulin G (Bio-Rad Laboratories).

Cell culture and infection. HeLa cells (clone HtTA1) and $Nck1^-$ $Nck2^-$ mouse embryo fibroblasts (MEFs) were grown in DMEM supplemented with 10% fetal calf serum (FCS) and 2 mM glutamine at 37°C in 5% CO_2. $Nck1^+$ $Nck2^+$ MEFs were grown in DMEM supplemented with 15% FCS and 2 mM glutamine at 37°C in 5% CO_2. Cells were seeded onto glass coverslips (12 mm in diameter) in 24-well plates at a density of 5×10^4 cells per well at 24 h before infection. Overnight bacterial cultures were diluted 1 in 500 into DMEM and incubated at 37°C in 5% CO_2 overnight prior to infection. Tir tyrosine phosphorylation and fluorescent actin staining were detected by immunofluorescence as described previously (31). A mouse monoclonal anti-HA antibody (HA.11) was used at a dilution of 1 in 200.

Nucleotide sequence accession numbers. All DNA sequences determined in this study have been submitted to the DDBJ/GenBank/EMBL database (accession no. AB253537 to AB253549 for *tccP* of O157 strains, AB253550 to AB253562 for *tccP2* of O157 strains, AB253563 to AB253570 for *tccP2* of O26

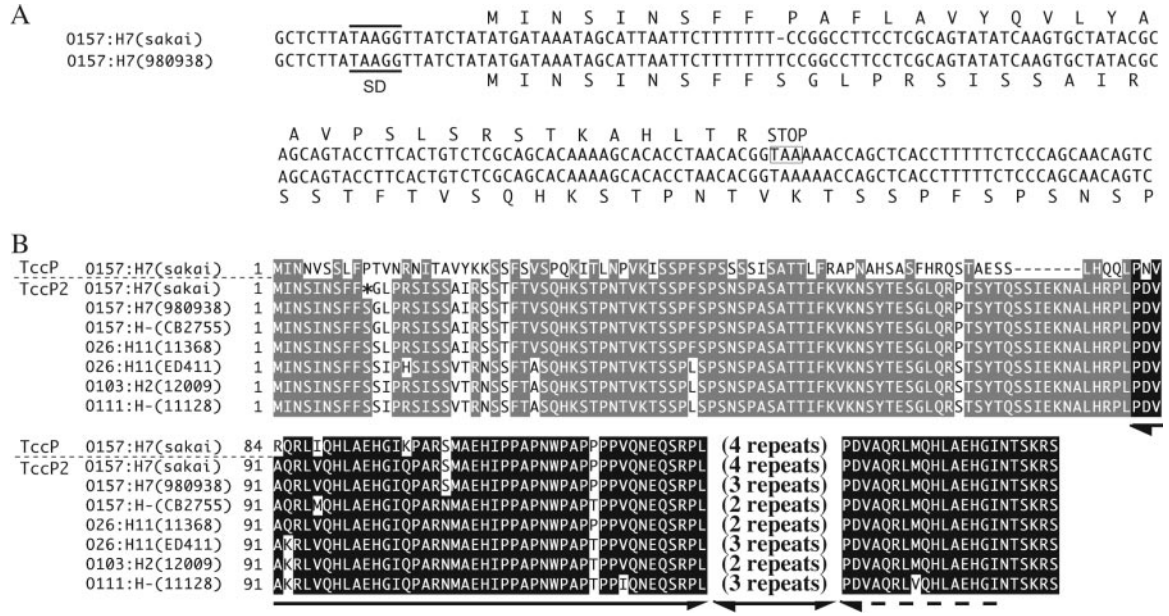

FIG. 1. (A) DNA and protein sequence alignment of the 5′ region/N terminus of *tccP2*/TccP2 from typical O157 (strain Sakai) and atypical O157 (strain 980938). *tccP2* in Sakai has a single-base-pair deletion at position 28 (indicated by a hyphen), resulting in frameshift mutation downstream of codon 10. All of the typical O157 (H7 and H−) strains we examined contain the same deletion in *tccP2.* The Shine-Dalgarno (SD) sequences are indicated by underlining. (B) Multiple-sequence alignment of TccP and TccP2 of representative O157, O26, O111, and O103 EHEC strains. Strain names are indicated in parentheses. The unique N terminus involved in protein translocation is shaded gray, and the proline-rich repeats are shaded black. The proline-rich repeats and the partial repeat in the TccP proteins are indicated by arrows and a dashed arrow, respectively. The position of the frameshift in TccP2 of the O157 Sakai strain, which corresponds to codon 10 of the intact TccP2, is indicated by an asterisk. In silico translation of Sakai *tccP2* from codon 11 revealed that it potentially encodes a TccP2 protein containing five proline-rich repeats.

strains, AB253571 to AB253576 for *tccP2* of O111 strains, and AB253577 to AB253582 for *tccP2* of O103 strains).

RESULTS

Intact ECs1126/Z1385 is found in atypical EHEC O157:H7. ECs1126, which is carried on prophage CP-933 M/Sp4, is a pseudogene in EHEC O157:H7 strains Sakai and EDL933 due to a single-base-pair deletion at position 28 (Fig. 1A). Indeed, it was shown previously that deletion of ECs1126 does not affect the ability of EHEC ΔECs1126 to trigger actin polymerization (5, 15). In silico translation of the gene downstream of the mutation site revealed that if it were not for the apparent single-base deletion at position 28, this gene would encode a protein comprising part of the unique N terminus and five proline-rich repeats (Fig. 1B). We used ECs1126-specific primers to determine whether the pseudogene is conserved in eight randomly selected O157:H7/H− strains (expressing VT2 only or VT2 and VT1) isolated in Japan from human feces (Table 1). *tccP* primers were used as a control. DNA amplification revealed that both genes were present in all tested strains. However, while all of the *tccP* amplicons were of ca. 1,050 bp in length, there was considerable strain-to-strain variation in the sizes of the *ECs1126* amplicons, which ranged from ca. 750 to 1,200 bp (Table 1).

To determine whether ECs1126 is a pseudogene in all eight O157:H7/H− strains, we used locus-specific primers to amplify ECs1126 and its flanking regions. Sequence analysis revealed that while in seven strains ECs1126 was indeed a pseudogene, in one strain, 980938, ECs1126 (894 bp) was intact and comprised a complete open reading frame (ORF) encoding the unique N terminus and four proline-rich repeats (Fig. 1B). Interestingly, this strain was atypical, β-D-glucuronidase-positive EHEC O157:H7. Sequence analysis of the *tccP* locus revealed an intact ORF of 1,014 bp in all eight strains (Table 1). Sequence alignment of TccP and ECs1126 revealed a low level of conservation at the N-terminal domain, which in TccP is involved in protein translocation (13). In contrast, other than differences in their numbers, the proline-rich repeats were almost identical (Fig. 1B).

To determine whether an intact ECs1126 is found in other β-D-glucuronidase-positive EHEC O157 strains, the gene was analyzed in four more β-D-glucuronidase-positive, sorbitol-fermenting O157 EHEC strains (producing VT2) that were isolated from human feces in Germany (Table 1). Sequence analysis of the amplicons (753 bp and 1,317 bp) revealed an intact ECs1126 ORF in all four strains (Table 1). These strains also had an intact *tccP* ORF of 1,155 bp (Table 1). These results show for the first time the existence of a subpopulation of EHEC O157 that harbors two potentially functional *tccP*-like alleles. In order to distinguish between the two alleles, we designated ECs1126/Z1385, which is carried on prophage Sp4/CP-933 M, *tccP2*.

$TccP2_{O157}$ is secreted by the LEE-encoded T3SS. We investigated whether the apparently intact TccP2 expressed by β-D-glucuronidase-positive strains is secreted via the LEE-encoded T3SS by Western blotting. First, we optimized detection con-

TABLE 1. Presence and status of *tccP* and *tccP2* in EHEC O157[a]

Serotype	Strain(s)[b]	Phenotype[c]	No. of strains	VT	Length (bp) (status) of[d]:		Origin
					tccP	*tccP2*	
O157:H7	Sakai*†	GUD^-SOB^-	1	1 + 2	1,014 (Int)	1,034 (PG)	Japan
O157:H7	EDL933[e]	GUD^-SOB^-	1	1 + 2	1,014 (Int)	1,034 (PG)	United States
O157:H7	980706, 981456	GUD^-SOB^-	2	1 + 2	1,014 (Int)	752 (PG)	Japan
O157:H−	982243	GUD^-SOB^-	1	1 + 2	1,014 (Int)	752 (PG)	Japan
O157:H7	981795†	GUD^-SOB^-	1	1 + 2	1,014 (Int)	1,175 (PG)	Japan
O157:H7	980551	GUD^-SOB^-	1	1 + 2	1,014 (Int)	1,034 (PG)	Japan
O157:H7	990570†	GUD^-SOB^-	1	2	1,014 (Int)	1,034 (PG)	Japan
O157:H7	990281†	GUD^-SOB^-	1	2	1,014 (Int)	752 (PG)	Japan
O157:H7	980938*	GUD^+SOB^-	1	1 + 2	1,014 (Int)	894 (Int)	Japan
O157:H−	CB7184	GUD^+SOB^+	1	2	1,155 (Int)	1,317 (Int)	Germany
O157:H−	CB2755*, CB6161, CB7324	GUD^+SOB^+	3	2	1,155 (Int)	753 (Int)	Germany

[a] All strains express intimin type γ1 and a Sakai-like Tir.
[b] *, nucleotide sequences of *tccP2* are shown in Fig. 1; †, results of Western blot analysis of TccP2 are shown in Fig. 2.
[c] GUD, β-glucuronidase; SOB, sorbitol fermentation; +, positive; −, negative.
[d] Int, intact; PG, pseudogene.
[e] We have resequenced the *tccP* gene of EDL933 and found that its sequence is identical to that of Sakai (i.e., 1,014 bp and five and a half [rather than six and a half] proline-rich repeats).

ditions by using the typical EHEC O157:H7 strain Sakai. Application of anti-TccP antiserum to blots containing whole-cell extracts and culture supernatants revealed that the protein is inefficiently expressed and detectable in bacterial culture pellets but not in the supernatant (Fig. 2A). As EHEC Δ*sepL* is a hypersecretor of T3SS effectors (11) and PchA is a transcriptional regulator that enhances LEE gene expression (32), we determined whether TccP is secreted from Sakai Δ*sepL* over-

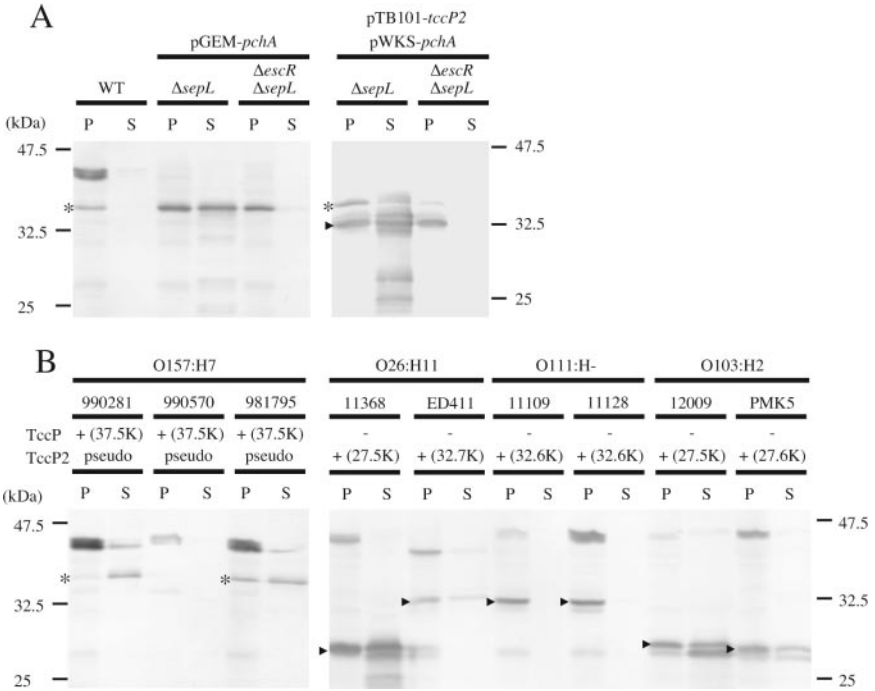

FIG. 2. Expression and secretion of TccP and TccP2 proteins in EHEC strains overexpressing PchA. Whole-cell lysates (P) and culture supernatants (S) were prepared from EHEC strains and analyzed by Western blotting using rabbit TccP antiserum. Positions of TccP and TccP2 proteins are indicated by asterisks and arrowheads, respectively. The amount of sample loaded onto the gel was standardized by bacterial cell concentrations that were estimated from OD_{600} values; each whole-cell lysate corresponds to 1×10^8 cells, and each supernatant is derived from the culture containing 2×10^9 cells. (A) TccP is not detected in culture supernatants of wild-type (WT) Sakai; secretion and detection of TccP is enhanced in Sakai Δ*sep* but abolished in Sakai Δ*sepL*/Δ*escR* (left panel). Overexpression of intact $TccP2_{O157}$ from pTB101-*tccP2* leads to protein secretion from Sakai Δ*sepL* (the endogenous *tccP2* of Sakai is a pseudogene) but not from Sakai Δ*sepL*/Δ*escR* (right panel). (B) Detection of TccP and TccP2 in clinical EHEC isolates: O157:H7 strains 990281, 990570, and 981795; O26:H11 strains 11368 and ED411; O111:H− strains 11109 and 11128; and O103:H2 strains 12009 and PMK5. The presence or absence of *tccP* genes in each strain and the predicted molecular masses of each TccP protein are indicated; strain 990570 appears to be a nonexpressor. The immunoreactive bands at about 42 kDa are likely to be bacterial immunoglobulin-binding proteins, as they reacted in the absence of primary antibody (data not shown).

TABLE 2. Presence of *tccP2* and Tir type among 63 strains of EHEC O26

Serotype[a]	No. of strains	VT	Intimin type	*tccP2* product (bp)	Tir type[b]	Origin
O26:H11	1	1	β1	1,200	E2348/69-like	United Kingdom
O26:H11	3	1	β1	900	E2348/69-like	Germany
O26:H−	3	1	β1	900	E2348/69-like	Germany
O26:H11	5	1	β1	750	E2348/69-like	Germany
O26:H11	2	1	β1		ND	Germany
O26:H11	3	1 + 2	β1	750	E2348/69-like	Germany
O26:H11	6	2	β1	900	E2348/69-like	Germany
O26:H11*†	4	1	β1	750	E2348/69-like	Japan
O26:H−*	3	1	β1	750	E2348/69-like	Japan
O26:H21	1	1	β	900	E2348/69-like	Australia
O26:H11*‡	1	2	β1	750	E2348/69-like	Italy
O26:H−	1	1	β1	1,050	E2348/69-like	Italy
O26:H11	3	1	β1	900	E2348/69-like	Italy
O26:H11	2	2	β1	1,050	E2348/69-like	Italy
O26	3	1	β1	900	E2348/69-like	Italy
O26	1	2	β1	900	NT	Italy
O26	1	2	β1	1,050	E2348/69-like	Italy
O26	12	1, 1 + 2	β1		ND	Australia
O26	3	1	β	900	E2348/69-like	Argentina
O26	1	2	β	1,050	E2348/69-like	Argentina
O26	4	1	β		E2348/69-like	Argentina

[a] *, *tccP2* was sequenced; †, amino acid sequences and expression of TccP2 for a representative isolate (strain 11368) are shown in Fig. 1 and 2, respectively; ‡, amino acid sequences and expression of TccP2 for a representative isolate (strain ED411) are shown in Fig. 1 and 2, respectively.
[b] Tir typing was done by discriminatory PCR primers. ND, not done; NT, not typeable.

TABLE 3. Presence of *tccP2* and Tir type among 13 strains of EHEC O103

Serotype[a]	No. of strains	VT	Intimin type	*tccP2* product (bp)	Tir type[b]	Origin
O103:H2*	3	1	ε	750	E2348/69-like	Japan
O103:H2*	1	1	ε	900	E2348/69-like	Japan
O103:H2*†	1	1 + 2	ε	750	E2348/69-like	Japan
O103:H2*‡	1	1	ε	750	E2348/69-like	France
O103:H2	1	1 + 2	ε	900	E2348/69-like	Argentina
O103:H2	1	1	ε	900	NT	Argentina
O103:H2	2	1	ε		E2348/69-like	Argentina
O103	2	1	ε	900	E2348/69-like	Italy
O103	1	2	ε		ND	Italy

[a] *, *tccP2* was sequenced; †, amino acid sequences and expression of TccP2 of a representative isolate (strain 12009) are shown in Fig. 1 and 2, respectively; ‡, production of TccP by this strain (strain PMK5) is shown in Fig. 2.
[b] Tir typing was done by discriminatory PCR primers. ND, not done; NT, not typeable.

expressing PchA. Western blot analysis revealed that TccP was readily detected in culture supernatants of this strain (Fig. 2A). Secretion was T3SS dependent, as TccP was not detected in culture supernatants of a Sakai Δ*sepL*/Δ*escR* double mutant (Fig. 2A).

Analysis of other clinical O157 isolates by Western blotting revealed strain-to-strain variation in both protein expression and secretion. As in the case of EHEC Sakai, TccP was detected only in the cell extract of some strains, while in other strains TccP was found either exclusively in the supernatant or in both supernatant and whole-cell extracts (Fig. 2B). These results show diversity in expression and secretion of TccP in different EHEC O157 strains.

In order to determine whether $TccP2_{O157}$ is secreted, an intact *tccP2* gene of atypical O157 strain 980938 was expressed in Sakai Δ*sepL* and Sakai Δ*sepL*/Δ*escR* mutants overexpressing PchA. Expression and secretion of $TccP2_{O157}$ was determined with the TccP antiserum which cross-reacts with TccP2, as the proline-rich repeats of TccP and TccP2 are almost identical. Both the endogenous TccP and the recombinant TccP2 were detected in bacterial cell pellets and supernatants of Sakai Δ*sepL* but only in cell pellets of Sakai Δ*sepL*/Δ*escR* (Fig. 2A). These results confirmed that $tccP2_{O157}$ encodes a protein that is secreted by the LEE-encoded T3SS.

Prevalence of *tccP2* in non-O157 EHEC. The realization that some O157 EHEC strains express secreted *tccP2* prompted us to determine whether *tccP2* is present in non-O157 EHEC strains isolated from human feces in different countries (Argentina, Australia, Germany, Italy, Japan, and the United Kingdom). A total of 63 O26, 13 O103, 84 O111, 4 O121, 13 O145, and 5 O NT strains were screened by *tccP2*-specific PCR (Tables 2 to 5). *tccP2* was detected in 100% of the O111, O121, and O145 isolates and in 76.9% of the O103 strains. Among the O26 EHEC strains, *tccP2* was found in 40 (95.2%) of 42 strains isolated in Europe and Japan and in 50% of strains isolated in Argentina, but in only one of 13 strains isolated in Australia (Table 2). The PCR amplicons ranged from 600 to 1,200 bp. *tccP2* was found in strains expressing VT1, VT2, or VT1 and VT2.

Sequence analysis of 20 *tccP2*-positive strains of different serotypes (eight O26, six O111, and six O103) revealed a complete ORF. Amino acid alignment of TccP2 from different serotypes, including $TccP2_{O157}$, revealed that other than dif-

TABLE 4. Presence of *tccP2* and Tir type among 84 strains of EHEC O111

Serotype[a]	No. of strains	VT	Intimin type	*tccP2* product (bp)	Tir type[b]	Origin
O111:H8	10	1	γ2	900	E2348/69-like	Germany
O111: H,ND	1	1	γ2	750	E2348/69-like	Germany
O111:H8	1	1	γ2	900	NT	Germany
O111:H8	8	1 + 2	γ2	900	E2348/69-like	Germany
O111:H8	1	1 + 2	γ2	600	E2348/69-like	Germany
O111:H8	3	2	γ2	900	E2348/69-like	Germany
O111:H−*†	3	1	γ2	900	E2348/69-like	Japan
O111:H−*	1	1	γ2	900	E2348/69-like	Italy
O111:H−*‡	2	1 + 2	γ2	900	E2348/69-like	Japan
O111:NM	1	2	θ	1,050	Sakai-like	Argentina
O111:NM	2	1 + 2	θ	1,050	Sakai-like	Argentina
O111:H−	5	1	γ2	900	E2348/69-like	Italy
O111:H−	1	1	γ2	900	Sakai-like	Italy
O111:H−	1	1 + 2	γ2	900	E2348/69-like	Italy
O111	8	1	γ2	900	E2348/69-like	Italy
O111	1	1	γ2	600	E2348/69-like	Italy
O111	1	1 + 2	γ2	750	E2348/69-like	Italy
O111	7	1 + 2	γ2	900	E2348/69-like	Italy
O111	2	1 + 2	γ2	1,050	E2348/69-like	Italy
O111:H−	3	1	γ2	1,050	E2348/69-like	Italy
O111:H−	3	1 + 2	γ2	1,050	E2348/69-like	Italy
O111:H8	2	1 + 2	γ2	900	E2348/69-like	Australia
O111	4	1 + 2	γ2	1,200	E2348/69-like	Australia
O111	6	1 + 2	γ2	900	E2348/69-like	Australia
O111:H−	3	1 + 2	γ2	1,200	E2348/69-like	Australia
O111:H−	1	1 + 2	γ2	1,200	E2348/69-like	England
O111:H−	3	1 + 2	γ2	900	E2348/69-like	Australia

[a] *tccP2* was sequenced; †, production of TccP2 by a representative isolate (strain 11109) is shown in Fig. 2; ‡, amino acid sequences and expression of TccP2 for a representative isolate (strain 11128) are shown in Fig. 1 and 2, respectively.
[b] Tir typing was done by discriminatory PCR primers. NT, not typeable.

TABLE 5. Presence of *tccP2* and Tir type in other EHEC serotypes

Serotype[a]	No. of strains	VT	Intimin type	*tccP2* product (bp)	Tir type[b]	Origin
O121:H19	4	2	ε	900	E2348/69-like	Argentina
O145:H25	3	2	β	900	E2348/69-like	Argentina
O145:H−*	10	2	γ	1,200	NT	Argentina
O171:H−	1	2	ι	1,200	E2348/69-like	Argentina
ONT:H−	1	1	β		E2348/69-like	Argentina
ONT:H−	1	1	ε		E2348/69-like	Argentina
ONT:H−	1	2	γ	1,200	NT	Argentina
ONT:H−*	1	2[c]	ε	900	E2348/69-like	Argentina
ONT:H−	1	1 + 2	γ2	1,050	E2348/69-like	Argentina

[a] *, also *tccP* positive.
[b] Tir typing was done by discriminatory PCR primers. NT, not typeable.
[c] Genotype is *stx*2+*stx*2vh-a.

ferences in the number of proline-rich repeats, the proteins have a high level of sequence similarity (representative strains are shown in Fig. 1B).

Secretion, translocation, and function of non-O157 TccP2. As the proline-rich repeats of TccP2 from non-O157 EHEC strains are also almost identical to TccP, the TccP antiserum can be used to detect both TccP homologues. We analyzed the expression and secretion of TccP2 from representative non-O157 strains. This revealed relatively high levels of expression of TccP2 in O26 strains. The protein was detected in bacterial cell pellets, but the amount secreted by different strains varied (Fig. 2B). TccP2 was readily detected in whole-cell extracts, but not in the culture supernatant, of EHEC O111. In contrast, TccP2 was secreted efficiently by EHEC O103 (Fig. 2B). These results show that *tccP2* encodes a secreted protein and that expression and secretion of TccP2 in non-O157 EHEC follows the same pattern as expression and secretion of TccP in EHEC O157. Using the TEM-1 protein translocation assay (7), we confirmed that TccP2 is translocated by the LEE-encoded T3SS (data not shown).

In order to determine whether TccP2 of non-O157 EHEC is a functional translocated effector, *tccP2* was amplified from EHEC O26:H11, cloned, and expressed as an HA-tagged protein in EDL933 Δ*tccP*, a mutant which is unable to trigger actin polymerization on its own (5, 15). Infection of HeLa cells resulted in restoration of actin polymerization activity and focusing of TccP2 in the host cytoplasm beneath attached bacteria, showing that TccP2 is functional when expressed in typical EHEC O157 (Fig. 3).

Tir typing of *tccP2*-positive non-O157 EHEC. The observation that the majority of the non-O157 EHEC strains appear to express a functional *tccP2* prompted us to determine whether they express an EHEC O157-like (Sakai) Tir (Tir_S) or an EPEC (E2348/69) O127-like Tir that can undergo tyrosine (Y474) phosphorylation (Tir_Y) and utilize the Nck-driven actin polymerization pathway. Use of discriminatory PCR revealed that all but three O111 TccP2-positive strains harbor Tir_Y (Tables 2 to 5).

Sequence alignment of the carboxy-terminal Tir domains of EHEC O26, O103, and O111, which were retrieved from the database, revealed the presence of a conserved sequence that comprises the Nck binding site (including a Y474 equivalent) (Fig. 4A), clustering of which was previously shown to be sufficient for triggering actin assembly (3). Consistent with this, infection of HeLa cells with representative strains expressing TccP2 and Tir_Y revealed Tir tyrosine phosphorylation in association with attached bacteria (Fig. 4B) and recruitment of Nck (data not shown). These results suggest that the *tccP2*-positive EHEC strains possess a redundant mechanism of actin polymerization.

TccP2 mediates actin polymerization during infection of $Nck^{-/-}$ cells. We aimed to determine whether, like TccP, TccP2 can trigger actin polymerization after infection of $Nck^{-/-}$ cells. Unfortunately, we found that all of the non-O157 EHEC strains we tested adhered very poorly to the $Nck^{-/-}$ cells. For this reason, the function of TccP2 was tested with an adherent *tccP2*-positive EPEC O111 strain (which is *tccP* gene negative and encodes Tir containing a putative Nck binding site) and its isogenic O111 Δ*tccP2* mutant, ICC216. Infection with both wild-type and Δ*tccP2* strains resulted in actin polymerization in infected $Nck^{+/+}$ control cells, while only the wild-type strain triggered actin polymerization in the $Nck^{-/-}$ cells. Complementing the Δ*tccP2* mutant with $tccP2_{O26}$ restored actin polymerization activity in infected $Nck^{-/-}$ cells (Fig. 5). These results confirm that TccP2 is a translocated effector protein, capable of triggering Nck-independent actin polymerization within the eukaryotic cell.

DISCUSSION

Until the first half of 2004, EPEC- and EHEC-induced actin polymerization was believed to follow distinct signaling cascades. Based on studies with the prototype EPEC strain E2348/69, the prevailing dogma was that phosphorylation of Tir_{EPEC} leads to maturation of an Nck binding site, Nck recruitment, and activation of N-WASP. In contrast, studies with the prototypic EHEC O157:H7 strains (Sakai and EDL933) revealed that Tir_{EHEC} does not use the Nck pathway and employs an

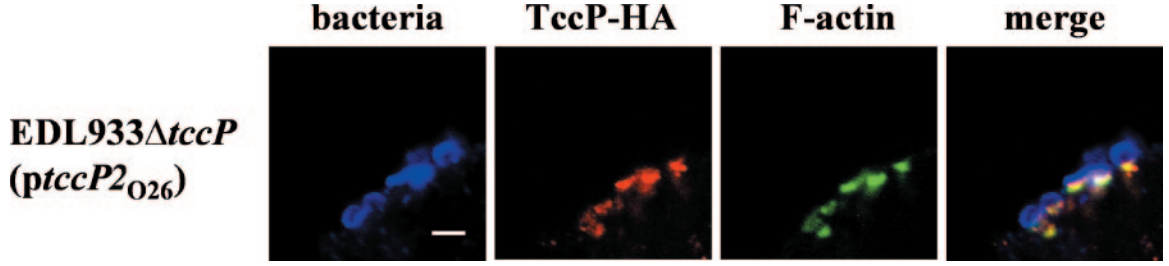

FIG. 3. Expression of $tccP2_{O26}$ in EDL933 Δ*tccP* restores actin polymerization in infected HeLa cells; TccP2-HA is recruited to the site of bacterial attachment. Scale bar, 2 μm.

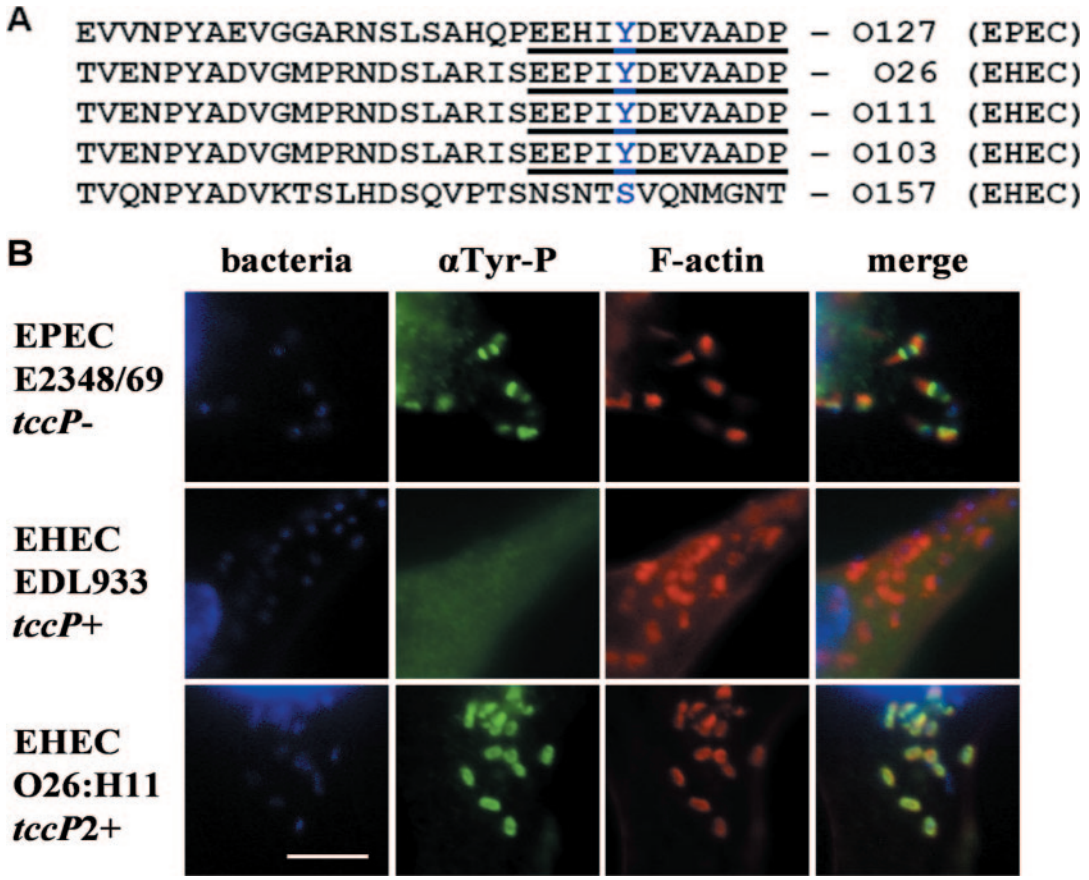

FIG. 4. (A) Sequence alignment of the C-terminal region of Tir from EPEC with the Y474 equivalents from EHEC O26, O111, and O103. The Nck binding site is underlined. (B) Immunofluorescent staining showing Tir tyrosine phosphorylation (αTyr-P) at sites of induced actin polymerization following infection of HeLa cells with E2348/69, an O26 strain expressing E2348/69-like Tir, but not with O157:H7 EDL933. Bar, 5 μm.

alternative, strain-specific adaptor protein to activate the N-WASP signaling cascade, the origin of which (i.e., cellular or bacterial) was not known (4). During the second half of 2004 it became apparent that in addition to Tir, EHEC O157:H7 also translocates an effector protein that, like Nck, is recruited to Tir and activates N-WASP. This effector is carried on prophage Sp14/CP-933U and was originally named $EspF_U$ (5) or TccP (15).

More recently, it emerged that the distinction between the EPEC and EHEC actin polymerization pathways is not absolute, as *tccP* is present in some EPEC and non-O157 EHEC strains (16). Subsequent functional studies showed that in *tccP*-positive EPEC, Tir is tyrosine phosphorylated and both the Nck and TccP signaling pathways are simultaneously functional (37).

EHEC strains Sakai and EDL933 contain a pseudo-*tccP* gene that is carried on prophage Sp4/CP-933 M (*tccP2*). Indeed, deletion of this gene has no effect on EHEC O157:H7 (EDL933)-induced actin polymerization (5, 15). As the selective pressure on pseudogenes is different from that imparted by the action of evolutionary forces on functional genes, we conducted a pilot study to determine the preservation of *tccP2* in eight clinical EHEC O157:H7 strains isolated in Japan. We found that in addition to *tccP*, which was uniform in length and encodes the unique N terminus and five proline-rich repeats, all of the tested strains also harbored *tccP2*. *tccP2* amplicons exhibited considerable variation in size. In seven of the O157 strains, *tccP2* was a pseudogene. Unexpectedly, in one of the strains, *tccP2* was intact and encoded a protein comprising a unique N terminus and four proline-rich repeats. The strain harboring the intact *tccP2* gene was an atypical, β-D-glucuronidase-positive EHEC O157:H7. Analysis of four β-D-glucuronidase-positive and sorbitol-fermenting EHEC O157 strains (22) revealed the presence of intact *tccP* and *tccP2* genes. By expressing $TccP2_{O157}$ in Sakai Δ*sepL* or Sakai Δ*sepL*/Δ*escr*, we have shown that the protein is secreted by the LEE-encoded T3SS. These results suggest that TccP2 in the atypical EHEC O157 strains is biologically active. The presence of intact *tccP* and *tccP2* in this lineage of EHEC O157 is intriguing and raises a number of questions. (i) Is there an advantage in expressing both *tccP* and *tccP2*, and if so, where? (ii) Do the gene products have overlapping functions? Finally, (iii) are the β-D-glucuronidase-positive and sorbitol-fermenting O157 EHEC strains the source of *tccP* and *tccP2*, from which the genes were disseminated to non-O157 bacteria? Large-scale epidemiological and functional studies are needed to address these questions experimentally.

An investigation of the distribution of *tccP2* in the most common non-O157 EHEC serotypes also revealed unexpected results, as *tccP2* was found in most of the 185 strains tested

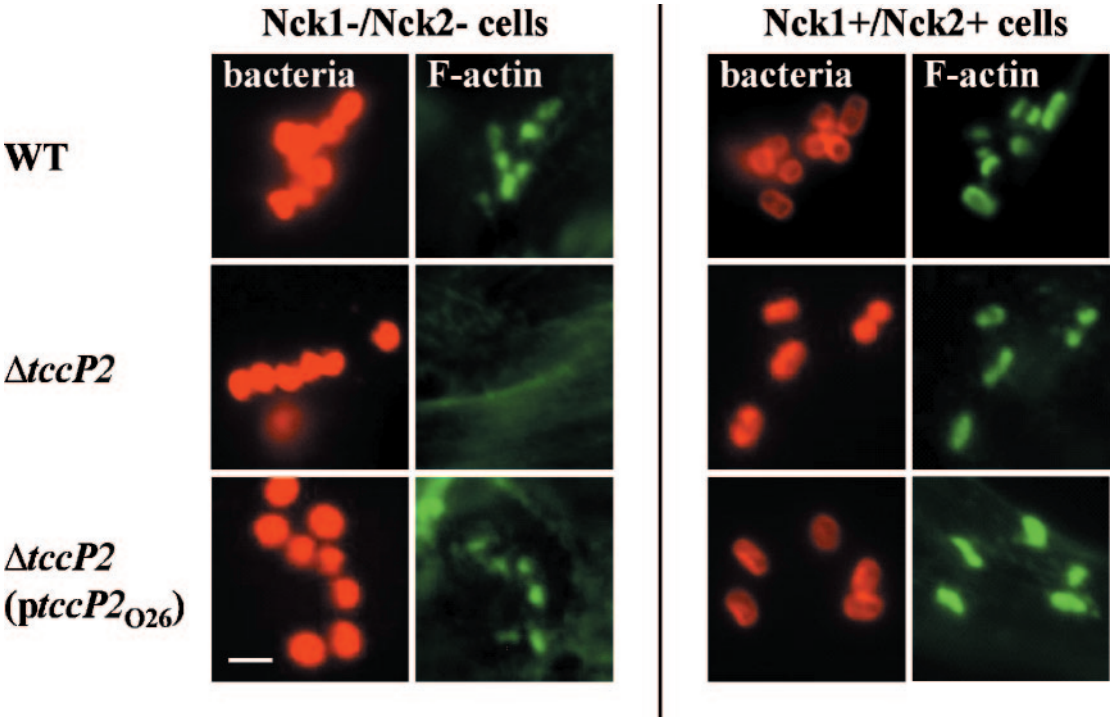

FIG. 5. Deletion of *tccP2* in O111 EPEC results in a loss in the ability to trigger actin pedestal formation in Nck-deficient cells. The wild-type (WT) phenotype is restored by expression of $tccP2_{O26}$. All strains tested were capable of initiating the formation of actin pedestals on Nck-proficient cells. Bar, 2 μm.

(88%). Only a few sporadic isolates within the serotypes and a group of 12 EHEC O26 strains from Australia, possibly belonging to a particular clone, did not possess *tccP2*. Interestingly, two non-O157 EHEC strains (O145:NM and ONT:HNM) isolated in Argentina were positive for both *tccP* and *tccP2*, although we do not know whether the strains can utilize the Nck-binding Y474 peptide, TccP, and TccP2 simultaneously.

Examination of representative non-O157 EHEC strains revealed that although there is considerable strain-to-strain variation, TccP2 is expressed and secreted into culture supernatants. A similar expression/secretion pattern of TccP was observed in EHEC O157 strains. These results are suggestive of strain-specific *tccP*/*tccP2* gene regulation and TccP/TccP2 protein secretion. The significance of these differences for infection and virulence is not known.

Use of the TEM-1 protein translocation assay revealed that TccP2 is injected into host cells by the LEE-encoded T3SS. Expression of $TccP2_{O26}$ in EDL933 Δ*tccP* restored actin polymerization activity in infected HeLa cells and recruitment of $TccP2_{O26}$ to the site of bacterial attachment. These results suggest that the function of TccP2 in non-O157 EHEC is equivalent to that of TccP. Mutagenesis of *tccP2* resulted in loss of actin polymerization activity in infected Nck-deficient cells, which was restored by expression of $TccP2_{O26}$. Using discriminatory allele-specific PCR, we found that over 90% of the *tccP2*-positive strains express Tir_Y (containing a Y474 equivalent). Infections of HeLa cells with representative *tccP2*-positive strains revealed that Tir is tyrosine phosphorylated and colocalized with Nck.

Collectively, these results show that (i) typical EHEC O157 strains trigger actin polymerization by the specialized, Nck-independent TccP signaling pathway; (ii) atypical, β-D-glucuronidase-positive and sorbitol-fermenting EHEC O157 strains also trigger actin polymerization in an Nck-independent mechanism, although these strains have the potential to use either or both of the TccP and TccP2 pathways; and (iii) most non-O157 EHEC strains express TccP2 proteins capable of Nck-independent actin polymerization activity while translocating Tir proteins that can mediate actin polymerization by the Nck pathway.

The findings presented in this report break the prevailing dogma that actin polymerization by EHEC is mediated by either the Nck or TccP pathway and highlight the existence of a previously unrecognized category of non-O157 EHEC strains that express a unique combination of virulence determinants (intimin, $Tir_{E2348/69}$-like protein, and functional TccP2). As *tccP2*-positive non-O157 EHEC strains appear to comprise the majority of strains and to have redundant mechanisms of actin polymerization, it is possible that expression of TccP2 confers a competitive advantage on these bacteria. Whether this advantage is manifest during interactions with specific hosts or cell types or is involved in pedestal formation or other signaling cascades (e.g., interaction with phagocytes) are intriguing questions for further studies.

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