Modelling of Infection by Enteropathogenic *Escherichia coli* Strains in Lineages 2 and 4 Ex Vivo and In Vivo by Using *Citrobacter rodentium* Expressing TccP^V

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Received 5 November 2008/Returned for modification 31 December 2008/Accepted 28 January 2009

Enteropathogenic *Escherichia coli* (EPEC) strains colonize the human gut mucosa via attaching-and-effacing (A/E) lesion formation, while in vitro they employ diverse strategies to trigger actin polymerization. Strains belonging to the EPEC-1 lineage trigger strong actin polymerization via tyrosine phosphorylation of the type III secretion system (T3SS) effector Tir, recruitment of Nck, and activation of N-WASP. Strains belonging to EPEC-2 and EPEC-4 can trigger strong actin polymerization by dual mechanisms, since while employing the Tir-Nck pathway they can additionally activate N-WASP via the T3SS effectors TccP2 and TccP, respectively. It is currently not known if the ability to trigger actin polymerization by twin mechanisms increases in vivo virulence or fitness. Since mice are resistant to EPEC infection, in vivo studies are frequently done using the murine model pathogen *Citrobacter rodentium*, which shares with EPEC-1 strains the ability to induce A/E lesions and trigger strong actin polymerization via the Tir:Nck pathway. In order to model infections with EPEC-2 and EPEC-4, we constructed *C. rodentium* strains expressing TccP. Using a mouse intestinal in vitro organ culture model and oral gavage into C57BL/6 mice, we have shown that TccP can cooperate with Tir of *C. rodentium*. The recombinant strains induced typical A/E lesions ex vivo and in vivo. Expression of TccP did not alter *C. rodentium* colonization dynamics or pathology. In competition with the wild-type strain, expression of TccP in *C. rodentium* did not confer a competitive advantage.

Enteropathogenic *Escherichia coli* (EPEC) and enterohemorrhagic *Escherichia coli* (EHEC) are diarrheal pathogens which colonize the gut epithelium via attaching-and-effacing (A/E) lesion formation (reviewed in references 9 and 10). A/E lesions are characterized by effacement of the brush border microvilli and intimate bacterial attachment to the mammalian cell plasma membrane (24). The genes required for A/E lesion formation are carried on the locus of enterocyte effacement (26), which encodes transcriptional regulators (21), the adhesin intimin (20), a type III secretion system (T3SS) (19), chaperones, translocators, and several effector proteins (reviewed in references 10 and 12).

While infecting cultured cells in vitro, EPEC and EHEC trigger strong actin polymerization at the site of bacterial attachment. The principal T3SS effector protein needed for A/E lesion formation on mucosal surfaces and actin polymerization in vitro is Tir (23, 33). Once translocated, Tir is integrated into the plasma membrane of the mammalian cell in a hairpin loop topology (18). The extracellular loop, presented above the plasma membrane, serves as an intimin receptor (reviewed in reference 11). In EPEC, actin polymerization in vitro is initiated once clustering of Tir by intimin (34) leads to phosphorylation of a Tir tyrosine (Y) residue at position 474 in the prototype EPEC strain E2348/69 (22), which is present in the

context of a consensus binding site (Y_pDEP/D/V) for the mammalian adaptor protein Nck (5, 17). Binding of Nck to phosphorylated Tir leads to recruitment and activation of the neuronal Wiskott-Aldrich syndrome protein (N-WASP) and actin polymerization via the actin-related protein 2/3 complex (reviewed in reference 7). Tir from E2348/69 can also trigger weak actin polymerization in the absence of Nck recruitment (6). This Nck-independent pathway is dependent on a universally conserved NPY Tir motif (2).

Studying the interaction of EHEC O157:H7 with cultured cells revealed that activation of the actin polymerization cascade occurs by a mechanism that is distinct from that of E2348/69 (reviewed in reference 9) since in contrast to EPEC Tir, EHEC O157:H7 Tir lacks a Y474 equivalent and hence cannot assemble the Tir-Nck signaling complex. Instead, EHEC O157:H7 employs the T3SS effector TccP (also known as EspF_U) (3, 13), which mimics Nck in terms of linking Tir to the N-WASP actin polymerization machinery. Recruitment of TccP to Tir is dependent on the conserved NPY Tir motif (1, 4).

Recently, while screening for the presence of *tccP* in clinical EPEC isolates, we unexpectedly found that strains belonging to EPEC-2 (represented by EPEC O111:NM strain B171) and EPEC-4 (represented by EPEC O119:H6 strain ICC199) (25, 36) encode TccP and TccP2, respectively, which are functionally interchangeable (36). In contrast, strains belonging to the EPEC-1 lineage (represented by EPEC O127:H6 strain E2348/69) are *tccP/tccP2* gene negative (25, 36). As such, strains belonging to EPEC-1, EPEC-2, and EPEC-4 are able to trigger strong actin polymerization via the Tir-Nck pathway, while strains belonging to EPEC-2 and EPEC-4 can additionally

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^V Published ahead of print on 2 February 2009.

TABLE 1. Strains, plasmids, and primers used in this study

Strain or plasmid	Description or nucleotide sequence ^a	Reference or source
Strains		
ICC169	Wild-type <i>C. rodentium</i>	37
ICC180	Luminescent ICC169 derivative	37
ICC302	ICC169 containing <i>tccP</i> constitutively expressed from the <i>tet</i> promoter, inserted into the <i>xylE</i> locus	This study
DBS255	<i>eae</i> deletion mutant	32
Plasmids		
pACYC184	Expressing vector	NEB
pET28a	Expressing vector	Novagen
pICC439	pACYC184 expressing <i>tccP</i> under <i>Ptet</i>	This study
pICC440	pACYC184 containing the 5' and 3' regions of <i>xylE</i>	This study
pICC441	pACYC184 containing the 5' and 3' regions of <i>xylE</i> and expressing <i>tccP</i> under <i>Ptet</i>	This study
pICC431	pET28a expressing N-terminal His-tagged EVHI domain of N-WASP	This study
Primers		
EcoRV-rbsTccP-Fw	5'-TTT GATAT CAAGAAGGAGATATACCATGATTAACAATGTTTCTTCACTTTTCC-3'	
BamHI-TccP-Rv	5'-CGCG GATCCT CACGAGCGCTTAGATGTATTAATGC-3'	
AseI-N-XylE-Fw	5'-CCC ATTAATG TTTAAACCTATTGCAGCAACCGCTTTGG-3'	
AseI-N-XylE-Rv	5'-CCC ATTAAT ACGCAGTACACCAGCAGCTG-3'	
XmnI-C-XylE-Fw	5'-CCC GAACTG TTTCCCGTCTTTTCAGCAGTTTGTCG-3'	
XmnI-C-XylE-Rv	5'-CCC GAACTG TTTCGTTTAAACAAGTAGTTTGCCAGCCACTGG-3'	
NdeI-nwasp-Fw	5'-GGAATTCC ATATG AGCTCCGTCCAGCAGCAG-3'	
EcoRI-nwasp-Rv	5'-CCG GAA TTCTCATTTCAGATTTCCTTTGTGTCG-3'	

^a Restriction sites in sequences are shown in bold.

assemble the Tir-TccP/2 actin polymerization complex (35). It is currently not known if the ability to simultaneously trigger strong actin polymerization by the twin Tir-Nck and Tir-TccP pathways changes the course of the disease or provides EPEC-2 and EPEC-4 strains with an in vivo advantage.

A difficulty in studying EPEC infection in vivo is attributed to the fact that after oral gavage into mice, the bacteria behave similarly to commensal *E. coli*, showing continuous population decline to clearance (27). For this reason, the mouse pathogen *Citrobacter rodentium*, which colonizes the mouse colon via A/E lesion formation, has become a popular surrogate model for in vivo studies (reviewed in reference 28). *C. rodentium* is a diarrheal mouse pathogen that while causing colonic hyperplasia shares many virulence factors with EPEC, including the locus of enterocyte effacement and Tir, which can trigger strong actin polymerization in vitro via the Tir:Nck pathway. *C. rodentium* is missing *tccP/tccP2* and as such serves as an ideal model for EPEC-1. The aim of the study was to construct a *C. rodentium* strain that expresses TccP, which would model infection with EPEC-2 and EPEC-4 in vivo. In particular, we wanted to determine if expression of TccP changes the morphology of the A/E lesions, the infection characteristics, or the in vivo fitness.

MATERIALS AND METHODS

Strains and plasmids. The strains and plasmids used in this study are listed in Table 1. For mouse intestinal in vitro organ culture (mIVOC) experiments, strains were grown for 8 h in Luria-Bertani (LB) broth and then transferred to sterile Dulbecco's modified Eagle medium (DMEM) containing 4,500 mg liter⁻¹ of glucose and grown overnight (static) at 37°C in 5% CO₂. For in vivo experiments, strains were grown overnight in LB and the pellet was concentrated 10 times in sterile phosphate-buffered saline (PBS) prior to oral gavage. When appropriate, nalidixic acid, chloramphenicol, and kanamycin were used at concentrations of 50 µg ml⁻¹, 34 µg ml⁻¹, and 50 µg ml⁻¹, respectively.

Construction of pACYC-*tccP* expression vector. Full-length *tccP* was amplified using EHEC O157:H7 EDL933 genomic DNA as a template and the primer pair EcoRV-rbsTccP-Fw and BamHI-TccP-Rv (Table 1). The PCR product was digested and ligated into the EcoRV/BamHI sites of pACYC184 to produce the plasmid pICC439, which constitutively expresses *tccP* from the tetracycline promoter (Table 1). The construction was confirmed by DNA sequencing.

Construction of a *xylE-tccP C. rodentium* strain. For expression of *tccP* from the chromosomal *xylE* locus, the 5' and 3' ends of the *C. rodentium xylE* gene were amplified using the primer pairs AseI-N-XylE-Fw and AseI-N-XylE-Rv and XmnI-C-XylE-Fw and XmnI-C-XylE-Rv, respectively (Table 1). The PCR products were digested with AseI and XmnI, respectively, and sequentially cloned into pACYC184 to produce the plasmid pICC440 (Table 1). Full-length *tccP* from EHEC O157:H7 EDL933 was then cloned into the EcoRV/BamHI sites of pICC440 to produce the plasmid pICC441 (Table 1). A fragment of pICC441 consisting of 5' *xylE*, the tetracycline promoter, *tccP*, the chloramphenicol resistance cassette of pACYC184, and 3' *xylE* was amplified by PCR using the primer pair AseI-N-XylE-Fw and XmnI-C-XylE-Rv. The PCR product was electroporated into *C. rodentium* strain ICC169 containing pKD46, which encodes λ Red recombinase (8). Transformants were selected on chloramphenicol plates, and the insertion of *tccP* in the *xylE* locus was confirmed by PCR.

Preparation of polyclonal N-WASP antiserum. The EVHI (Ena/VASP and Homer/Vesl domains) domain of N-WASP was PCR amplified from a transfection vector harboring full-length N-WASP (kindly provided by David Holden, Imperial College London) using primers NdeI-N-WASP-Fw and EcoRI-nwasp-Rv (Table 1). The resulting PCR product was NdeI-EcoRI digested and ligated into pET28a, generating the plasmid pICC431, for expression as an N-terminal His-tagged protein. His-EVHI was then purified from induced BL21-star culture using a nickel column as previously described (30). Rabbit polyclonal N-WASP antiserum was generated commercially at CovaLab United Kingdom.

Cell culture procedures. Swiss 3T3 and embryonic Nck1⁻ Nck2⁻ and Nck1⁺ Nck2⁺ fibroblast cell lines were grown in DMEM supplemented with 10% fetal calf serum and 2 mM glutamine at 37°C in 5% CO₂. Cells were seeded onto glass coverslips in 24-well plates at a density of 5 × 10⁴ cells per well 24 h before infection. *C. rodentium* strains used for in vitro assays were grown for 8 h in LB broth and then transferred into fresh, sterile DMEM containing 1,000 mg liter⁻¹ of glucose and subjected to static incubation at 37°C in 5% CO₂ overnight prior to infection. Each coverslip was infected with 100 µl of the appropriate overnight culture, centrifuged at 1,000 rpm for 5 min at room temperature, and then incubated at 37°C in 5% CO₂ for 6 h. The cell culture medium was renewed

halfway through the infection period. Immunostaining of the coverslips was done as described previously (35).

Mouse in vitro organ cultures. A mIVOC model was developed using conditions similar to those used for bovine intestinal organ culture (14). Briefly, six mice were used in four independent experiments. Fresh segments from the terminal ileum and colon were inoculated with 50 μ l of the appropriate overnight bacterial culture, corresponding to approximately 10^7 CFU, and incubated at 37°C in a 5%-CO₂ atmosphere on a seesaw rocker (18 cycles min⁻¹) for 8 h. Uninfected explants were cultured in each experiment as controls.

Explants were gently rinsed with PBS and fixed in 10% buffered formalin for microscopic examination. Formalin-fixed tissues were processed, paraffin embedded, sectioned at 5 μ m, and stained with hematoxylin and eosin (H&E) according to standard techniques. Sections were then examined by light microscopy, and the mucosal epithelium located between adjacent crypts (designated the intercept mucosal epithelium [ICME]) was examined for the presence of intimately adherent bacteria, as described previously (16). The mean percentage of ICME demonstrating intimately adherent bacteria per section was calculated. Additional explants were fixed in 2.5% glutaraldehyde for electron microscopy analysis.

Oral inoculation of mice. Pathogen-free female C57BL/6 mice (18 to 20 g) were purchased from Charles River, Inc. All animals were housed in individually HEPA-filtered cages with sterile bedding and free access to sterilized food and water. All animal experiments were performed in accordance with the Animals Scientific Procedures (Act 1986) and were approved by the local Ethical Review Committee. For in vivo experiments, independent single-infection experiments were performed twice using four to eight mice per group; wild-type *C. rodentium*-infected and uninfected mice were included alongside every experiment as controls.

Mice were inoculated by oral gavage with 200 μ l overnight LB-grown *C. rodentium* suspension in PBS ($\approx 5 \times 10^9$ CFU). The number of viable bacteria used as an inoculum was determined by retrospective plating onto LB agar containing antibiotics. Stool samples were recovered aseptically at various time points after inoculation, and the number of viable bacteria per gram of stool was determined after homogenization in PBS and plating on LB agar containing antibiotics (38).

In mixed-infection experiments, the two overnight LB-grown bacterial cultures were combined in a ratio of 1:1 (approximately 2×10^9 CFU for each strain) in 200 μ l PBS and used to inoculate female C57BL/6 mice by oral gavage. Dilutions of the inoculum were plated on respective antibiotic-containing plates to determine the ratio of the two bacterial strains (test strain/reference strain) in the inoculum. Stool samples were collected at regular intervals, and the competitive index (CI) was calculated by dividing the ratio of test strain CFU and reference strain CFU from the stools by the ratio of test strain CFU to reference strain CFU in the inoculum (29). The CI was analyzed using five animals per group and was determined at days 7, 9, 11, and 15 postinoculum.

Histopathology and indirect immunofluorescence. Tissues were harvested at 8 and 17 days postinoculation. Segments of the terminal colon, collected postmortem, were rinsed of their content, fixed in 10% buffered formalin, processed, paraffin embedded, sectioned at 5 μ m, and stained with H&E according to standard techniques. Sections were examined by light microscopy for the presence of intimately adhering bacteria on intestinal cells. Crypt length, as a measure of crypt hyperplasia, was evaluated from at least six well-oriented crypts. Additional segments were fixed in 2.5% glutaraldehyde or embedded in optimal-cutting-temperature medium (Raymond A Lamb Limited, United Kingdom), snap-frozen in liquid nitrogen, and kept at -80°C for further electron microscopy analysis and cryosectioning, respectively.

Indirect immunofluorescence (IFA) using formalin-fixed and paraffin-embedded sections was used for detection of O152-positive bacteria (corresponding to the *C. rodentium* serogroup), as described previously (14, 15). Cryosections (two serial sections per strain per mouse per experiment), fixed in 3% paraformaldehyde in PBS, were used for detection of *C. rodentium*, TccP, Nck and N-WASP. Sections were examined with an Axio Imager M1 microscope (Carl Zeiss MicroImaging GmbH); images were acquired using an AxioCam MRm monochrome camera and computer processed using the AxioVision (Carl Zeiss MicroImaging GmbH) and Photoshop 5.0 and Illustrator 8.0 (Adobe Systems Incorporated) software programs.

Antibodies and reagents. Sections were immunostained using the following antibodies: rabbit anti-O152 (kindly provided by Lothar Beutin, The National Reference Laboratory for *Escherichia coli*, Federal Institute for Risk Assessment, Berlin, Germany), chicken anti-intimin (kindly provided by John M. Fairbrother, *Escherichia coli* Laboratory, Faculté de Médecine Vétérinaire, Université de Montréal, Montreal, Canada), rabbit anti-TccP (31), rabbit anti-N-WASP (this study), and rabbit anti-Nck (Millipore Upstate, Lake Placid, NY). Cy2-

conjugated donkey antichick and tetramethyl rhodamine isocyanate-conjugated donkey antirabbit (Jackson ImmunoResearch Europe Ltd., Soham, Cambridgeshire, United Kingdom) were used as secondary antibodies. Phalloidin-Alexa Fluor 633 (Invitrogen, United Kingdom) was used to stain F-actin, while DNA was counterstained with Hoechst 33342.

Electron microscopy. Ex vivo- and in vivo-derived tissue samples were processed for electron microscopy, as described previously (14). Samples for scanning electron microscopy (SEM) were examined blindly at an accelerating voltage of 25 kV using a Jeol JSM-5300 scanning electron microscope. Samples for transmission electron microscopy (TEM) were observed using a Phillips 201 transmission electron microscope at an accelerating voltage of 60 kV.

Statistical analysis. Results are presented as a scatter plot with the median (ICME with adherent bacteria and CI), as a line plot with the mean and its standard deviation (colonization), or as a vertical bar chart with the mean and its standard deviation (crypt length). Fisher's exact test (number of explants with adherent bacteria) or a Mann-Whitney test (median percentage of ICME with adherent bacteria, colonization, crypt length, and CI) was performed using commercially available GraphPad InStat v3.06 software (GraphPad Software Inc., San Diego, CA). A *P* value of ≤ 0.05 was considered significant.

RESULTS

Interaction of *C. rodentium* with mouse intestinal epithelium ex vivo. In order to determine if expression of TccP affects the interaction of *C. rodentium* with the mouse gut mucosa, we first developed and tested an ileal and colonic mIVOC infection model. H&E-stained sections revealed that the architecture of uninfected tissue was well preserved following an 8-h ex vivo incubation compared to biopsy specimens collected at the out-set (data not shown). Following infection with wild-type *C. rodentium* strain ICC169 and O-antigen staining of formalin-fixed and paraffin-embedded sections, O152-positive bacteria were found intimately associated with the epithelium of the terminal ileum and the terminal colon (Fig. 1; Table 2). Only a few bacteria, mostly above the epithelium, were observed on explants infected with strain DBS255 (*Deae*; intimin mutant) (Fig. 1; Table 2). No O152-positive bacteria were observed on uninfected explants (Fig. 1). SEM and TEM analysis confirmed the presence of typical A/E lesions on infected ileum and colon explants, although elongation of microvilli was less obvious in the terminal colon (Fig. 1). No A/E lesions were observed on explants infected with strain DBS255 (Fig. 1) or ICC169 Δ tir (data not shown). These results validated the mIVOC model and show that despite being restricted to the colon in vivo, *C. rodentium* can induce typical A/E lesions on ileal mucosal surfaces.

Interaction of *C. rodentium* expressing TccP with fibroblasts and mIVOC. In order to determine if TccP can cooperate with Tir of *C. rodentium*, wild-type *C. rodentium* strain ICC169 was transformed with pICC439, encoding TccP. The wild-type and recombinant strains were used to infect Nck1⁻ Nck2⁻ cells and Nck1⁺ Nck2⁺ cells, which were used as a control.

The wild-type ICC169 *C. rodentium* strain triggered actin polymerization in Nck1⁺ Nck2⁺ cells but not in Nck1⁻ Nck2⁻ cells (Fig. 2A). In contrast, ICC169(pICC439) triggered actin polymerization on both cell lines, and TccP was found at the tip of the F-actin pedestals (Fig. 2B). These results show that Tir *C. rodentium* can cooperate with TccP in triggering actin polymerization in vitro.

We next investigated what effect expression of TccP has on the interaction of *C. rodentium* with mIVOC. No significant differences in adherence levels (Fig. 3A) were seen between explants infected with ICC169 and ICC169(pICC439). More-

FIG. 1. Interaction of *C. rodentium* with mIVOC. *C. rodentium* strain ICC169 (WT, arrows) adhered intimately (IFA panel; O152-positive bacteria are stained in red, and DNA is stained in light blue) and induce typical A/E lesions (arrows and arrowheads in SEM and TEM panels, respectively) on both ileal and colonic explants. *C. rodentium* Δeae was observed above the epithelium (asterisks). Neither bacteria nor A/E lesions were observed on uninfected explants. Bar = 20 (IFA), 5 (SEM), or 2 μ m (TEM).

TABLE 2. Adherence of *C. rodentium* on mouse explants ex vivo

Strain	No. of explants with intimately adherent bacteria/total (% of positive explants)	
	Terminal ileum	Terminal colon
ICC169	33/40 (83)	17/28 (61)
ICC169(pICC439)	21/31 (68)	11/22 (50)
ICC302	5/7 (71)	5/6 (83)
DBS255	0/13 (0)	0/8 (0)

over, ICC169(pICC439) adhered intimately to, and induced typical A/E lesions on, ileal and colonic mIVOC (Fig. 3B).

Role of TccP during mouse infection. Finally, we aimed to determine if expression of TccP changes the course of *C. rodentium* infection in vivo. However, preliminary studies showed that pICC439 is unstable in vivo since it was lost from ICC169 as early as 3 days post-oral gavage into C57BL mice (data not shown).

In order to overcome the problem of plasmid instability in vivo, we constructed *C. rodentium* strain ICC302, which expresses *tccP* constitutively from the *tet* promoter at the chromosomal *xylE* locus; we selected this site since we have previously shown that disruption of *xylE* by insertion of the *luxCDABE* operon and a kanamycin resistance cassette (*C. rodentium* strain ICC180) did not affect the overall growth and virulence capacity of ICC169 (37).

In vitro characterization showed that expression of TccP in ICC302 enabled the strain to trigger strong actin polymeriza-

tion in both Nck1⁺ Nck2⁺ and Nck1⁻ Nck2⁻ cells (Fig. 2C). When tested ex vivo on mIVOC, ICC302 adhered intimately to ileal and colonic mIVOC (Fig. 3C) and induced typical A/E lesions (Fig. 3D).

Since constitutive expression of *tccP* did not affect the ability of ICC302 to infect mammalian cells in vitro and mucosal surfaces ex vivo, the recombinant strain was tested in vivo. C57BL/6 mice were challenged by oral gavage with ICC302 expressing TccP; wild-type *C. rodentium* strain ICC169 was used as a reference. In comparison to results for wild-type *C. rodentium*, expression of TccP did not affect the colonization dynamics, as reflected by the number of CFU per gram of stools recovered over time (Fig. 4A).

Histopathological examination revealed that the constitutive expression of *tccP* did not influence colonic hyperplasia (as reflected by the crypt length) (Fig. 4B and C) compared to results for wild-type *C. rodentium*. Colonic sections from ICC169 and ICC302 challenge showed similar levels of intimately adherent O152-positive bacteria at the apical surface of the mucosal epithelium (Fig. 4C).

Recruitment of TccP, Nck, and N-WASP and A/E lesion formation in vivo. Examination of infected cryosections revealed that *C. rodentium* strain ICC302 was able to recruit TccP, Nck, and N-WASP underneath intimately adherent bacteria while Nck and N-WASP, in the absence of TccP, were detected under adherent ICC169 (Fig. 5). This suggests that in vivo *C. rodentium* can recruit both TccP and Nck, although in the absence of double staining it is not possible to assess if

FIG. 2. Interaction of *C. rodentium* with Nck1⁺ Nck2⁺ (Nck^{+/+} panel) or Nck1⁻ Nck2⁻ (Nck^{-/-} panel) embryonic fibroblasts. Wild-type *C. rodentium* strain ICC169 (A) adhered to both cell lines (arrowheads) but was able to trigger actin polymerization only in the Nck^{+/+} cells (arrowheads). Expression of *tccP* from pICC439 in *C. rodentium* ICC169 (B) or from the *xylE* locus in ICC302 (C) enabled the strains to trigger strong actin polymerization in both Nck^{+/+} and Nck^{-/-} cells (arrows). TccP (asterisks) was detected in both strains at the tip of the actin-rich pedestals. DNA was stained blue, TccP was stained red, and actin was stained green. Bar = 2 μ m.

FIG. 3. Interaction of *C. rodentium* expressing TccP with mIVOC. (A) In comparison to results for wild-type (WT columns) *C. rodentium*, expression of *tccP* in ICC169 in *trans* (*ptccP* columns) did not significantly influence the median percentage of intercrypt mucosal epithelium with adherent bacteria (ICME+) on either ileal or colonic explants ex vivo. (B) Intimately adherent, O152-positive *C. rodentium* ICC169(pICC439) (DNA was stained in light blue, and bacteria were stained red; arrows) were observed on both ileal and colonic explants; typical A/E lesions were confirmed by SEM (arrows) and TEM (arrowheads). (C) Adhesion of *C. rodentium* ICC302 constitutively expressing TccP from the *xylE* locus to the ileal and colonic mIVOC (DNA was stained in light blue, and bacteria were stained red; arrows). (D) *C. rodentium* ICC302 triggers typical A/E lesions on ileal and colonic mIVOC. Bar = 20 (IFA), 5 (SEM), or 1 μ m (TEM) (bar in inset = 500 nm).

TccP and Nck are colocalized. SEM (Fig. 6A) and TEM (Fig. 6B) examination revealed that *C. rodentium* strains ICC169 and ICC302 exhibited indistinguishable colonization patterns and A/E lesions.

Expression of TccP does not change in vivo fitness of ICC302. Finally, we determined if expression of TccP provides the bacterium with a competitive advantage. To this end we determined the competitive index of ICC302 during mixed

FIG. 4. Colonization dynamic, hyperplasia, and CI of *C. rodentium* ICC302. ICC302 (*xylE-tccP*) exhibits colonization dynamics (A) and crypt hyperplasia (B and C) similar to those of wild-type *C. rodentium*, which was significantly different from results for uninfected mice; ***, $P < 0.001$. Both strains intimately adhered to the colonic mucosa (C) (O152-positive bacteria are stained in red, and DNA is stained in light blue). No adherent bacteria were observed on colonic sections derived from uninfected mice (Uninfected). Bar = 100 (bar in inset = 20) (C). (D) The in vivo fitness of ICC302 was investigated in mixed-infection experiments with the ICC169 (WT) strain. The CI was determined on days 7, 9, 11, and 15 postinoculation for each of the five mice per group, and individual CI values (open circles) are represented as log_{10} CI values. The median CI of each group is indicated on the graph by a horizontal line, with the corresponding numerical values shown beneath the line. No statistical differences were observed between the CI of ICC302 and the CI of ICC180, used as a control, indicating that none of the strains had a competitive advantage compared to ICC169.

FIG. 5. Detection of TccP, Nck, and N-WASP following infection of mice with *C. rodentium* strain ICC169 (WT) or ICC302 (expressing TccP from the *xylE* locus). While TccP, Nck, and N-WASP (arrowheads) were detected under adherent ICC302 (stained with anti-intimin; arrows), only Nck and N-WASP (arrowheads) were detected under ICC169 (arrows). Bar = 20 μ m.

FIG. 6. Typical A/E lesions were observed by SEM (arrows) and TEM (arrowheads) following infection with *C. rodentium* ICC169 and ICC302. Neither adherent bacteria nor A/E lesions were observed on colonic sections derived from uninfected mice (Uninfected). Bar = 5 μm (A) or 2 μm (bar in insets = 500 nm) (B).

infection with ICC169. In order to neutralize any potential effects of a chromosomal insertion into the *xylE* locus on in vivo fitness, the CI of ICC302 was directly compared to the CI of ICC180, which harbors a chromosomal insertion in the same locus (37). This showed that expression of TccP did not affect the in vivo fitness of ICC302 in comparison to that of ICC180 at any time point after mixed infection (Fig. 4D). This result suggests that EPEC-1 (modeled by ICC169) and EPEC-2 and EPEC-4 (modeled by ICC302) are as infectious and that the ability to trigger strong actin polymerization by twin mechanisms in vitro does not increase the virulence potential or fitness in vivo.

DISCUSSION

The ability to polymerize actin is considered central to EPEC infection. While EPEC-1 strains can only assemble the Tir-Nck actin signaling complex, EPEC-2 and EPEC-4 strains are equipped with the twin Tir-Nck and Tir-TccP mechanisms (reviewed in reference 9). However, it was not known if the ability to assemble more than one actin polymerization complex increases virulence or fitness.

In this study we compared the infectious properties of wild-type *C. rodentium* (ICC169), which can trigger strong actin polymerization in vitro exclusively by the Tir-Nck pathway, with a *C. rodentium* strain that can additionally express TccP (ICC302). First, we demonstrated that TccP can be translocated from *C. rodentium* and cooperate with its Tir protein during infection of Nck-deficient cells. In order to determine if

expression of TccP affects the ability of ICC169 to interact with mucosal surfaces, we developed an mIVOC infection model. As far as we know, this is the first time such a model has been described; we believe the mIVOC model provides an important tool for studying enteric bacterial infection; moreover, it would contribute to the reduction, replacement, and refinement (three Rs) of animal experimentation. Interestingly, although following inoculation by the oral route *C. rodentium* is found attached only to the cecum and colon, ex vivo *C. rodentium* can efficiently induce A/E lesions on the ileum. Importantly, in comparison with infection of mIVOC with ICC169, expressing TccP from a plasmid did not enhance the ability of *C. rodentium* to attach and induce A/E lesions on either the ileal or the colonic mucosa.

We next aimed to determine if expression of TccP influences colonization dynamics, pathology, recruitment of signaling molecules to the site of bacterial attachment, and competitiveness in vivo. Since the *tccP*-encoding plasmid was unstable in vivo, we constructed a recombinant *C. rodentium* strain that constitutively expresses *tccP* from the *tet* promoter. We chose to express TccP from the *xylE* locus, since we have shown before that it can tolerate gene insertions without virulence being affected (37). Comparison of infection profiles of ICC169 and ICC302 has shown that both strains exhibited the same colonization dynamics; infection with both strains peaked at 7 days postchallenge and started to clear 15 days later. Expression of TccP did not exacerbate the overall disease/pathology since similar levels of colonic hyperplasia were seen

following infection with ICC302 and the control ICC169 *C. rodentium* strains.

Immunofluorescence analysis of frozen sections derived from infected colons has shown that TccP is accumulated under adherent ICC302; no signal was seen under the wild-type ICC169 control. This shows that TccP is translocated from *C. rodentium* in vivo and recruited to Tir. Importantly, TccP does not bind Tir directly but via an adaptor protein encoded by the host (3, 13). Our results imply that the adaptor linking Tir and TccP is also expressed in the murine gut. Importantly, qualitative data suggest that TccP competitively excludes Nck, since fewer Nck-positive bacterial foci were seen under adherent ICC302 (data not shown). This suggests that although in vitro Nck and TccP are seen together under adherent bacteria (35), in vivo either Nck or TccP appears to be dominant. It is not currently known if the difference between the in vivo and in vitro data is due to Tir density or to special constraints at the site of bacterial attachment. Nonetheless, both Nck and TccP are capable of efficient recruitment of N-WASP. Finally, we investigated if the ability to activate N-WASP by both Nck and TccP confers a competitive advantage in vivo. Comparison of the competitive indexes of ICC302 and ICC180 in competition with ICC169 has shown that expression of TccP does not increase in vivo competitiveness.

Infections with *C. rodentium* strains ICC169 and ICC302 model those of EPEC-1 and of EPEC-2 and EPEC-4, respectively. Our data show that although EPEC-2 and EPEC-4 can assemble two actin-signaling complexes (compared to one in EPEC-1), this did not have a measurable impact during infection in vivo. These finds are consistent with epidemiological and clinical data, which did not reveal any significant differences between EPEC-1 and EPEC-2. However, the fact that the ability to polymerize actin by the Tir-Nck and Tir-TccP actin polymerization pathways is conserved in EPEC-2 and EPEC-4 suggests that they are under selective pressure. At present it is not known if the selection is at the level of the human host, the animal reservoir, or the environment.

ACKNOWLEDGMENTS

We thank Anthony J. FitzGerald for technical help and Alan Phillips (UCL) for making his SEM available for this project. We thank Lothar Beutin from the Nationales Referenzlabor für *Escherichia coli* (NRL-E. coli) in Berlin for the O152 antiserum.

Francis Girard was supported by a postdoctoral fellowship from the Natural Sciences and Engineering Research Council (NSERC) of Canada and by a Canada-United Kingdom Millennium Research Award, awarded by the NSERC and the Royal Society of London, United Kingdom. This work was supported by the BBSRC and the Wellcome Trust.

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Editor: A. J. Bäuml